

## Article

# VE-Albumin Core-Shell Nanoparticles for Paclitaxel Delivery to Treat MDR Breast Cancer

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**Abstract:** Multi-drug resistance (MDR) presents a serious problem in cancer chemotherapy. In this study, Vitamin E (VE)-Albumin core-shell nanoparticles were developed for paclitaxel (PTX) delivery to improve the chemotherapy efficacy in an MDR breast cancer model. The PTX-loaded VE-Albumin core-shell nanoparticles (PTX-VE NPs) had small particle sizes (about 100 nm), high drug entrapment efficiency (95.7%) and loading capacity (12.5%), and showed sustained release profiles, *in vitro*. Docking studies indicated that the hydrophobic interaction and hydrogen bonds play a significant role in the formation of the PTX-VE NPs. The results of confocal laser scanning microscopy analysis demonstrated that the cell uptake of PTX was significantly increased by the PTX-VE NPs, compared with the NPs without VE (PTX NPs). The PTX-VE NPs also exhibited stronger cytotoxicity, compared with PTX NPs with an increased accumulation of PTX in the MCF-7/ADR cells. Importantly, the PTX-VE NPs showed a higher anti-cancer efficacy in MCF-7/ADR tumor xenograft model than the PTX NPs and the PTX solutions. Overall, the VE-Albumin core-shell nanoparticles could be a promising nanocarrier for PTX delivery to improve the chemotherapeutic efficacy of MDR cancer.

**Keywords:** Vitamin E; albumin; core-shell nanoparticles; paclitaxel; multi-drug resistance; breast cancer

## 1. Introduction

Multi-drug resistance (MDR) is a large obstacle to the success of cancer chemotherapy and is crucial to cancer metastasis and recovery [1]. The well-known P-glycoprotein (P-gp), an ATP-binding cassette transporter, which is over expressed on the surface membrane of cancer cells, is one of the major reasons for the cancer MDR [2]. Many P-gp substrates, such as paclitaxel, were expelled out of the cancer cells, resulting in the reduction of intracellular drug accumulation, thereby leading to the treatment failure [3]. Therefore, it is urgent to explore a more effective strategy for overcoming the cancer MDR.

Paclitaxel (PTX), a water-insoluble compound, is used widely as a first-line drug in clinical treatment against variety of cancers [4]. PTX is commonly formulated as Taxol<sup>®</sup>, which uses Cremophor EL and dehydrated ethanol (50:50, V/V) as delivery vehicles to enhance its solubility. But this formulation often causes side effects, such as hypersensitivity, neuropathy, and neurotoxicity, which associate highly with Cremophor EL [5]. To mitigate these side effects, Abraxane<sup>®</sup>, a new formulation, was developed by using the high affinity between paclitaxel and serum albumin to prepare paclitaxel/albumin nanocomplex [5]. This formulation was approved by Food and Drug Administration in 2005. However, its anticancer effect is still greatly affected by cancer MDR.

P-gp inhibitors have been studied for over P-gp mediated drug efflux, such as verapamil, dexverapamil, and tariquidar [6]. These P-gp inhibitors have been evaluated in clinic, but have

not exhibited a good improvement in the therapeutic efficiency. These failures were mainly ascribed to the undesired toxicities, which have urged us to seek for new, more effective compounds with low toxicity and fewer side effects. Vitamin E (VE) is a lipid-soluble antioxidant, which protects lipids and membranes from oxidative damage [7]. It was reported that VE were not only able to overcome MDR by inhibition of ATPase activity, but also did not consider the toxicity [8,9]. In addition, water insoluble anticancer drugs, like PTX, can be loaded well in the VE-based emulsion for parental delivery [10]. Therefore, the use of VE as a P-gp inhibitor will be an attractive candidate to overcome the cancer MDR. However, VE is water-insoluble, which affects its administration in clinic.

Nano-drug delivery systems have been extensively investigated for anti-cancer drug delivery [11–21]. The nanocarriers could not only increase the water solubility of drugs but circumvent the P-gp efflux pump with entering the cancer cells by an endocytosis process [22]. Moreover, the nanoparticles provided a promising strategy for co-delivery of multiple drugs, in a single carrier, to improve the therapeutic efficiency of cancers [23–28]. Various nanocarriers have been developed for the co-delivery of anti-cancer drugs and P-gp inhibitors, such as the co-delivery of paclitaxel and borneol in lipid-albumin nanocomplex [29–31], docetaxel and verapamil in polymeric micelles [32], and paclitaxel and curcumin in lipid-albumin hybrid nanoparticles [33]. Additionally, VE is a lipid-soluble oil, which could be well encapsulated by the albumin to fabricate the VE-Albumin core-shell nanoparticles, through a hydrophobic interaction; VE and albumin could also make interactions with PTX. These interactions are beneficial for increasing the PTX-loading efficiency. Therefore, we speculate that co-delivery of the PTX and the VE with the VE-Albumin core-shell nanoparticles could improve the therapeutic efficiency of PTX against MDR cancers.

In this study, bovine serum albumin was used as a carrier to fabricate the VE-albumin core-shell nanoparticles co-delivery of the PTX and the VE. VE as the oil core of nanoparticles, not only increases the PTX-loading efficiency but also overcomes the P-gp-mediated drug efflux. The physicochemical properties and in vitro release were characterized. The cytotoxicity and cellular uptake were also investigated. Moreover, the anti-cancer effect was evaluated in breast cancer xenografts, in mice. It was speculated that the VE-albumin core-shell nanoparticles would be a suitable drug delivery system for anticancer drug delivery to overcome MDR, in cancer.

## 2. Materials and Methods

### 2.1. Materials and Animals

Paclitaxel was obtained from the Tianfeng Bioengineering Technology Co., Ltd. (Shenyang, China). Vitamin E and bovine serum albumin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cremophor EL was obtained from BASF Corporation (Ludwigshafen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Sigma (St. Louis, MO, USA). RPMI-1640 and fetal bovine serum were obtained from Gibco (BRL, Gaithersburg, MD, USA). All other chemicals and solvents were of analytical or chromatographic grade and were used without further purification.

The PTX solution was prepared according to the clinical formulation. In brief, PTX (0.012 g) was dissolved in anhydrous ethanol (1 mL) and Cremophor EL (1 mL), under magnetic stirring. PTX solution was diluted with saline, before the test.

Balb/c mice (16–18 g) were obtained from the Experimental Animal Center (Nantong University, China). All animal experiments were approved by Nantong University Ethics Committee (20180512-001) and conformed to the Guidelines for the Use of Laboratory Animals.

### 2.2. Preparation of PTX NPs and PTX-VE NPs

The PTX NPs and PTX-VE NPs were fabricated by a desolvation-ultrasonication technique, as described in our previous report, with some modifications [29]. In brief, BSA (0.20 g) was dissolved in 5 mL deionized water, with magnetic stirring. VE (0.01 g) and PTX (0.01 g) were dissolved in

0.3 mL anhydrous ethanol. Then, the VE and PTX mixed solution were added dropwise to the BSA solution, with magnetic stirring. The mixtures were dispersed by probe ultrasonication (JY92-II, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) at 400 W, for 4 min, in an ice bath with a 3 s pulse-on period and a 1 s pulse-off period. After sonication, anhydrous ethanol was evaporated by a rotator RE-2000 (Ya Rong Biochemical Instrument Factory, Shanghai, China). Subsequently, the samples were centrifuged at 3000 rpm, for 10 min, to remove the unloaded drug and impurities, and passed through a 0.45 µm filter membrane for removing the larger particles. The obtained suspensions were kept at 4 °C.

### 2.3. Characterization of PTX-VE NPs

#### 2.3.1. Size Distribution and Morphology

The particle size and polydispersity index (P.I.) of the NPs was determined by dynamic light scattering (PSS NICOMP 380, Santa Barbara, CA, USA). The morphology of NPs was evaluated by transmission electron microscopy (TEM) (JEOL, Tokyo, Japan). In brief, the samples were diluted with distilled water and dropped onto a copper grid. The excess sample was removed with filter paper. Then, 2% phosphotungstic acid staining solution was dropped onto the grid. Finally, the sample was air-dried and assessed with TEM.

#### 2.3.2. Determination of Entrapment Efficiency (EE) and Loading Capacity (LC)

The EE and LC were determined by the method described in our previous research [29]. To separate PTX from the PTX-VE NPs, acetonitrile was added to precipitate BSA, via sonication, for 5 min. After centrifugation at 12,000 rpm for 10 min, the PTX concentration in the supernatant was determined by HPLC. The drug encapsulation efficiency (EE) and loading capacity (LC) were calculated as follows:

$$EE(\%) = \frac{W_{drug\ in\ NPs}}{W_{total\ drug}} \times 100 \quad (1)$$

$$LC(\%) = \frac{W_{drug\ in\ NPs}}{W_{excipients\ and\ drug}} \times 100 \quad (2)$$

### 2.4. Docking Studies

Geometry of VE was optimized in gas phase. The calculation was conducted using the GAMESS suit of codes with the hybrid functional B3LYP. The 6-31G (dp) basis set was used for all the elements.

Docking studies were executed using Autodock Vina.9 [27]. BSA crystalline protein structure was obtained from protein databank (Bovine serum albumin: 4OR0, <http://www.rcsb.org>). The structure of VE used for docking studies was optimized with DFT calculations. Protein structures were altered to include polar hydrogen atoms. During docking studies, the protein structure was kept rigid. Rotation in the VE complex and the PTX complex was permitted for all single bonds.

### 2.5. In Vitro Drug Release

The release behavior of PTX from the PTX-VE NPs was evaluated by a dialysis method. The phosphate buffered saline (PBS, pH 7.4) containing 0.5% *w/v* Tween 80 was used as the dissolution medium. In brief, the samples were suspended in a flask and then immersed in the dissolution medium at 37 °C under at 120 rpm. The amount of PTX released was determined by HPLC.

### 2.6. Cytotoxicity of PTX-VE NPs

The MCF-7 and MCF-7/ADR cell line were purchased from Nanjing Kaiji Biotech. Ltd. Co. (Nanjing, China). The cells were cultured in RPMI medium, supplemented with 10% FBS and 1% penicillin-streptomycin, at 37 °C, with 5% CO<sub>2</sub> and 95% relative humidity. The Cells were seeded

in 96-well plates, at a density of  $5.0 \times 10^3$  cells/well. After 48 h incubation, the growth medium was replaced with 200  $\mu$ L medium containing VE, PTX solution, and PTX-VE NPs with different concentrations, respectively. Then, each well was added with 20  $\mu$ L MTT (5 mg/mL) solution and was incubated for an additional 4 h. The culture medium was removed and 200  $\mu$ L DMSO was added to each well to dissolve the formazan. The absorbance at 492 nm was measured in a microplate reader (Model 500, San Francisco, CA, USA). The results were expressed as % cell viability (OD of treated group/OD of control group  $\times 100$ ).

### 2.7. Rhodamine 6G Accumulation in MCF-7/ADR Cells

MCF-7/Adr cells were seeded onto the cover glasses at  $1 \times 10^5$  cells in 6-well plate, for 24 h. The cells were washed and incubated with Rhodamine 6G solution (Rho solution), Rho NPs, and Rho-VE NPs, at 37 °C, for 2 h. Then the cells were washed twice with 4 °C PBS and fixed with 4% paraformaldehyde, for 20 min. The nuclei were counterstained by 4',6-diamidino-2-phenylindole (DAPI). The Rho 6G fluorescence was visualized by confocal laser scanning microscope (CLSM, TCS SP2/AOBS, LEICA, Bensheim, Germany).

### 2.8. Therapeutic Efficacy in Resistant Breast Cancer Xenografts Mice

The anti-tumor effect was evaluated in MCF-7/ADR tumor bearing Balb/c mice model. MCF-7/ADR cells were injected subcutaneously, at  $2 \times 10^7$  cells, in the armpit of the right anterior limb. When the tumor sizes reached about 100 mm<sup>3</sup>, the mice were randomly divided into four groups (n = 6), and the formulations of PTX solution, PTX NPs, and PTX-VE NPs were intravenously administrated at a dose of 10 mg/kg, at two-day intervals, for five times, with physiological saline as control. Tumor volumes and Body weights were measured with a caliper every other day. Tumor volume (V) was determined by the following formula:

$$V = \frac{\pi ab^2}{6} \quad (3)$$

where a and b represent the long and short axis of tumor, respectively.

### 2.9. Statistical Analysis

Analysis of statistical significance was performed with the SPSS statistics software 16.0. The data are presented as the mean  $\pm$  SD. Student's *t*-test was used to analyze the differences. The differences were considered significant at *p* < 0.05.

## 3. Results and Discussion

### 3.1. Preparation and Characterization of PTX-VE NPs

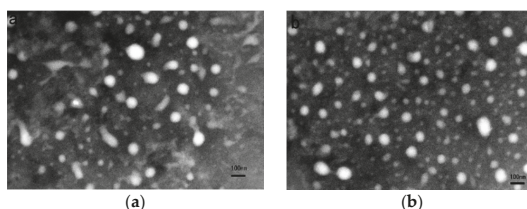
The PTX-VE NPs were fabricated by the desolvation-ultrasonication method. The PTX and VE mixed ethanol solution was added to the BSA solution, and the mixture was subjected to probe sonication. Subsequently, the PTX-VE NPs was formed by the interaction among the PTX, VE, and BSA. Both PTX and VE have high affinity with albumin [34,35], the PTX and VE can bound tightly to BSA via hydrophobic interactions and hydrogen bond, which were in favor of the formation of PTX-VE NPs.

The average size of the PTX NPs and PTX-VE NPs was approximately 100 nm (Table 1), which was considered effective for the accumulation of nanoparticles, in tumor tissue, via passive targeting. The PTX encapsulation in the PTX-VE NPs was increased significantly with a loading capacity > 12%, which was five-fold more than that of PTX NPs, owing mainly to the solubility of PTX in the VE oil. The NPs also have a higher drug encapsulation efficiency (>90%). The morphology of the NPs was evaluated by TEM, which showed a uniform and spherical shape (Figure 1).



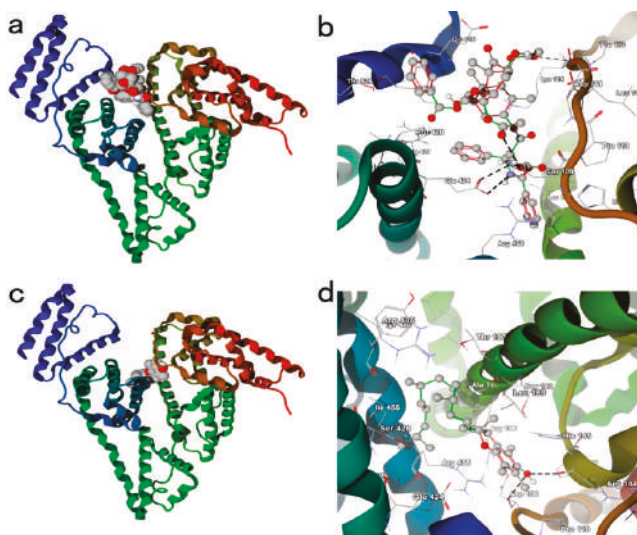
**Table 1.** Physicochemical characteristics of PTX NPs and PTX-VE NPs (n = 3).

Formulation	Size (nm)	P.I.	Zeta Potential (mV)	EE (%)	LC (%)
PTX NPs	101.2 ± 2.8	0.167 ± 0.03	−2.15 ± 0.6	91.2 ± 3.0	2.5 ± 0.08
PTX-VE NPs	106.9 ± 3.2	0.172 ± 0.02	−20.64 ± 0.8	95.7 ± 2.1	12.5 ± 0.15

**Figure 1.** TEM image of PTX NPs (a) and PTX-VE NPs (b).

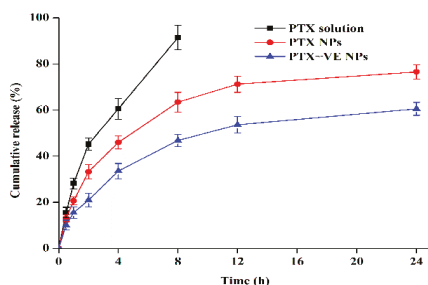
### 3.2. Docking Studies

Docking studies were performed to investigate the interaction between PTX and BSA, VE and BSA, respectively (Figure 2). The crystalline structure of BSA was obtained from the protein databank. In thermodynamics, a negative binding affinity demonstrates a favorable interaction system. The calculated binding affinity of PTX and VE with BSA were  $-8.3$  kcal/mol and  $-7.5$  kcal/mol, respectively, indicating that PTX and VE have strong interaction with BSA. As shown in Figure 2, there were hydrogen bonds between the Glu 424, Ser 109, Lys 114 of BSA and the nitrogen atom, oxygen atom, oxygen atom of PTX, respectively. There also existed hydrogen bonds between the Arg144, Asp108 and the hydroxy of the VE, respectively. Moreover, hydrophobic interactions were another important interaction between the PTX/VE and the BSA. The hydrophobic interactions were established between the Ile 522, Leu 189, Val 423, Ala 193, and PTX; these interactions also occurred between the Ile 455, Val 425, Leu189, Ala 193, and VE. In addition, the hydrophobic interactions and hydrogen bonds between the VE and the PTX cannot be ignored. In summary, the intermolecular force, such as hydrophobic interactions and hydrogen bonds played significantly important role in the formation of the PTX-VE NPs.

**Figure 2.** The molecular docking BSA with PTX (a,b) and VE (c,d).

### 3.3. In Vitro Drug Release

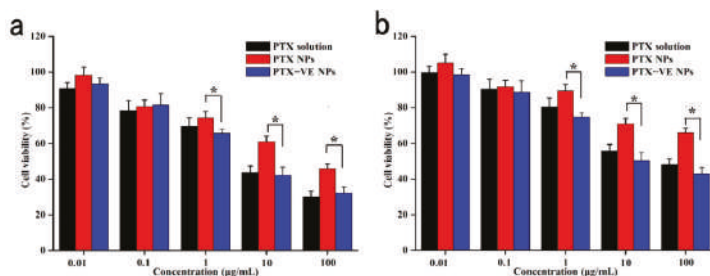
The profiles of the PTX release from the NPs are showed in Figure 3. Nearly 90% of the PTX was released from the PTX solution, in 8 h. Both, PTX NPs and PTX-VE NPs exhibited a sustained release profile, as compared with the PTX solution. After 24 h, the PTX release was about 76% and 60% for the PTX NPs and PTX-VE NPs, respectively. The PTX release from the PTX-VE NPs was slower than that of the PTX NPs. The release of PTX from the NPs might be affected by interactions among PTX, VE, and albumin, such as Hydrogen bonds,  $\pi$ - $\pi$  stacking, and hydrophobic interactions. In addition, the relative rapid release of PTX from NPs, in the early stage, was likely due to the diffusion of drugs absorbed at the outer shell, while the sustained release in the late stage was probably related to the gradual diffusion of drugs in the inner core of NPs.



**Figure 3.** In vitro release profile of PTX from PTX solution, PTX NPs, and PTX-VE NPs, in phosphate buffered saline (0.5% of Tween 80 in PBS, pH 7.4), at  $37 \pm 0.5$  °C (n = 3).

### 3.4. Cytotoxicity of PTX-VE NPs

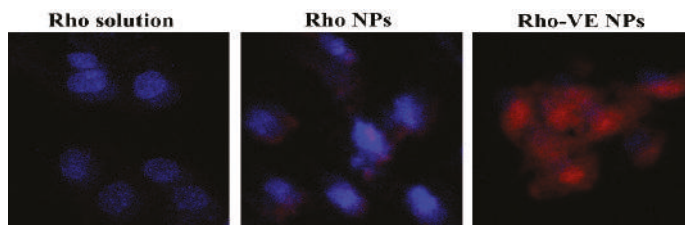
The cytotoxicity study of the PTX solution, PTX NPs, and the PTX-VE NPs was evaluated using the MTT assay and the results are shown in Figure 4. It is clear that cell viabilities for all the formulations in MCF-7 cells were lower than that at concentration 1–100  $\mu$ g/mL in MCF-7/ADR cells, due to P-gp-mediated efflux, which could reduce the drug accumulation in the cells. The PTX-VE NPs exhibited better cytotoxic effect than the PTX NPs, indicating that the PTX-VE NPs could exert stronger MDR-overcoming effects on the MCF-7/ADR cells. In addition, the cytotoxicity of the PTX-VE NPs showed no significant difference, as compared with the PTX solution. The reason was deduced that the cytotoxicity of the PTX solution was partially attributed to the use of Cremophor EL/ethanol mixture, which had 42.1% cytotoxicity on the MCF-7 cells [36]. However, in the case of the PTX-VE NPs, the nanocarriers are biocompatible, and the cytotoxic effect was induced mainly by the PTX incorporated in the nanoparticles [37].



**Figure 4.** In vitro cytotoxicity of PTX solution, PTX NPs, and PTX-VE NPs, against MCF cells (a) and MCF-7/ADR cells (b). Data represented the mean  $\pm$  S.D. (n = 3). \*  $p < 0.05$ , significant difference.

### 3.5. Rhodamine 6G Accumulation in MCF-7/ADR Cells

As a substrate of the P-gp efflux pump, the Rhodamine 6G (Rho) was often used as a fluorescent probe to evaluate the whether the improved cytotoxicity of the PTX-VE NPs was due to the increased intracellular drug delivery in MCF-7/ADR cells. Figure 5 shows the fluorescence images in the MCF-7/ADR cells, after 1 h, following the different Rho-labeled formulation treatments. The cells treated with Rho solution exhibited the least amounts of fluorescence signals, which could be attributed to the uptake inhibition of Rho by the P-gp efflux transporter, overexpressed in the MCF-7/ADR cells. Cells treated with the Rho NPs showed increased fluorescence signals, in comparison with the Rho solution. This was consistent with the research that the nanocarriers were able to circumvent the P-gp efflux pump by entering the cells through an endocytic process. However, the increased cell uptake of the Rho NPs was still restricted. The reason inferred was that the rapid release of encapsulated drugs in the cytoplasm was still probably shuttled out of cells, by the P-gp, which is overexpressed on the MCF-7/ADR cell membranes. Furthermore, the strongest fluorescence signals were observed in the cells treated with the Rho-VE NPs. This could be ascribed to the synergic combination of the nanocarrier and the VE. The nanocarrier increased the intracellular drug delivery by circumventing the P-gp efflux pump and the P-gp ATPase activity was probably inhibited by the released VE from the nanoparticles in cells.

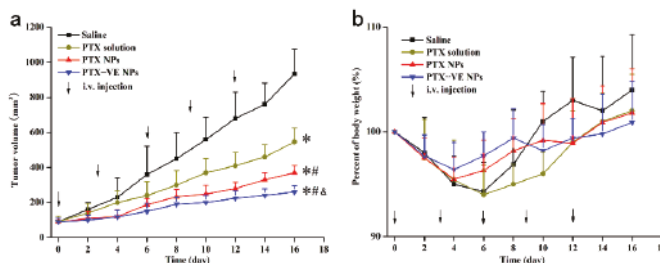


**Figure 5.** Confocal laser scanning microscope images of various Rhodamine 6G (Rho) labeled formulations in the MCF-7/ADR cells. Original magnification:  $10 \times 20$ .

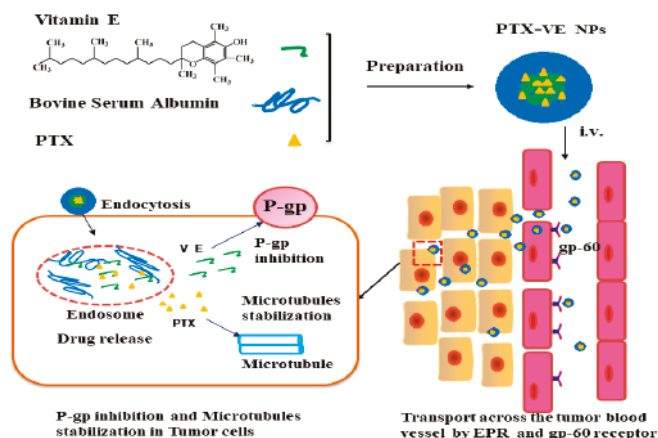
### 3.6. Anti-Tumor Effect and Safety in the Xenograft Model

The anti-tumor effect of the PTX-VE NPs was studied in the Balb/c mice bearing the MCF-7/ADR tumor xenograft model. The changes of tumor value and body weight were shown in Figure 6a, the tumor growth rates of different formulations, in ascending order, was PTX-VE NPs, PTX NPs, PTX solution and Saline, respectively. The results indicated that the PTX-VE NPs were the most effective formulation to treat MDR breast cancer. During the treatment, the relative body weight change of mice was also measured, as shown in Figure 6b. No significant body weight loss was observed for the PTX-VE NPs, PTX NPs, PTX solution and Saline, throughout the experiment, demonstrating good safety and low systemic toxicity of these formulations.

Accordingly, PTX-VE NPs was a safe and efficient drug delivery system to overcome MDR. The reasons could be deduced to the following aspects, as shown in Figure 7. First, the PTX-VE NPs with particle size less than 200nm, which was able to avoid the rapid reticuloendothelial system uptake and achieved a high accumulation in the tumor site by an enhanced permeability and retention (EPR) effect [35]. Second, the NPs could utilize the albumin receptor (gp60) mediated endocytosis through the blood vessel endothelial cells in the angiogenic tumor vasculature and an increased intratumoral accumulation [10,26]. Finally, the combination of the PTX and the VE in the NPs, would significantly enhance the intracellular drug uptake efficiency in MDR tumor cells and improve the therapeutic efficiency of resistant cancers.



**Figure 6.** Anti-tumor effect of PTX formulations on the Balb/c mice implanted with MCF-7/ADR cells. Mice were injected through tail vein with Saline, PTX solution, PTX NPs and PTX-VE NPs, at a dosage of 10 mg/kg. The changes of tumor volume (a) and body weight (b) during 16-day treatments. (n = 6). \*  $p < 0.05$ , significant difference when compared with Saline, #  $p < 0.05$ , significant difference when compared with PTX solution. &  $p < 0.05$ , significant difference when compared with PTX NPs.



**Figure 7.** Schematic illustration of the PTX-VE NPs to reversal of MDR.

#### 4. Conclusions

The present study fabricated the PTX loaded VE-Albumin core-shell nanoparticles to treat MDR breast cancer. The fabricated nanoparticles showed satisfying particle size, drug loading, and sustained-release profile, in vitro. Compared with PTX NPs, PTX-VE NPs exhibited higher cellular uptake and stronger cytotoxic effect in MDR breast cancer cells. Therefore, the PTX loaded VE-Albumin core-shell nanoparticles could be a potential strategy for the treatment of MDR breast cancer.

**Author Contributions:** Conceptualization, B.T. and G.C.; methodology, G.H.F.; software, Y.G.; validation, G.H.F.; formal analysis, G.H.F. and B.T.; investigation, G.H.F. and Y.Q.; data curation, Y.Q.; writing—original draft preparation, B.T.; writing—review and editing, G.H.F.; funding acquisition, B.T.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## Article

# Ultrasensitive (Co)polymers Based on Poly(methacrylamide) Structure with Fining-Tunable pH Responsive Value

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**Abstract:** Novel pH responsive copolymers with tertiary amine groups were prepared by free radical polymerization with 2-(dialkylamino)ethyl methacrylate monomers. These polymers were pH sensitive with the ability to be responsively fine-tuned in aqueous solution, which was proven through titration, transmittance measurements, and proton nuclear magnetic resonance spectroscopy. The polymers were soluble in water at low pH values, induced by electrostatic repulsion between amine groups, and aggregated above their  $pK_a$  value due to the hydrophobic effect of the alkyls. The pH responsive values were precisely tuned from 7.4 to 4.8 by increasing the hydrophobic monomer ratio. Our work provides a novel approach for the development of ultrasensitive pH-responsive polymers for application in biomedical materials.

**Keywords:** fine-tuning; pH responsive; poly(methacrylamide)s; phase transition

## 1. Introduction

The development of tumor-targeted drug delivery systems have attracted increasing interest given their potential to address therapeutic issues in clinical practice [1–7]. Many studies have focused on designing and improving specific transport and activation of intelligent materials that have stimuli responsive properties [8–13]. An interesting aspect of the physiology of tumors is their lower extra-cellular pH (6.5–7.2) compared to the surrounding normal tissues and blood, thus pH can be considered as an ideal trigger for tumor tissues and tumor cells [14]. To target the acidic bio-compartments, several smart materials with pH-responsive properties were designed to increase target efficacy. For instance, the Kobayashi group developed a small molecule system to target the tissue of the mice. This pH of this system can transition across two values of pH value, which means a 200-fold proton concentration change [15–17]. Despite these remarkable advances, selective targeting by pH-sensitive materials of different endocytic components is challenging owing to the small pH differences in these compartments, such as early endosomes (5.9–6.2) versus lysosomes (5.0–5.5) [18–20].

Various pH-responsive polymer systems have been studied, such as liposomes [21], micelles [22,23], nanoparticles [24], and nanogels [25–27]. The pH-responsive polymers can undergo phase transition at a specific pH values and direct spontaneous assemblies [28–31]. Polyelectrolytes, which contain weak acidic or basic groups, have been exploited as pH responsive polymers, inspiring advances in polymer research. Polybases, like poly(2-(diethylamino)ethyl methacrylate) (PDEAEMA),

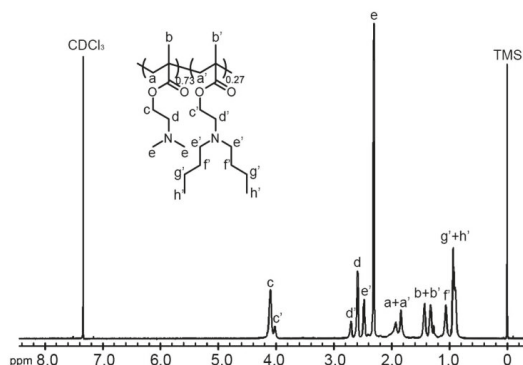
poly(4 or 2-vinylpyridine) (PVP), and poly(vinyl imidazole), were developed for biomedical applications in gene transfer, bio-imaging, and controlled drug release, due to their excellent physicochemical properties [32–34]. The amine group, pyridine, and imidazole group in polymer chains can accept protons under acidic condition and release them in basic environments to induce phase transition [35,36]. For instance, PDEAEMA dissolves in acidic aqueous solution due to the electrostatic repulsions between the protonated amine groups, but undergoes a precipitation above pH 7.5, caused by the hydrophobic effect of substituted ethyl group. However, a challenging issue faced by these widely used polybases is that their phase transition values from the solution state to the precipitation state are usually fixed. Therefore, they are less accurate when targeting cell organelles with different pH microenvironments. The broad pH transition range of these polybases also limits the increase in their responsive property. To develop responsive polymers with narrow transition pH ranges, Armes et al. [37] reported a novel polybase system, poly(2-(diisopropylamino)ethyl methacrylate) (PDPAEMA), with a phase transition at pH 6.3 and a sharp pH transition, providing a method by which to prepare ultra-sensitive pH-responsive polymers.

In this work, we developed a series of novel reversible pH-responsive (co)polymers with a fine-tunable pH transition value. These polymers were synthesized using a free radical polymerization method with tertiary amine-based monomers, 2-(dibutylamino)ethyl methacrylate and 2-(dimethylamino)ethyl methacrylate. At low pH, the (co)polymers were in the unimer state due to the electrostatic interaction and hydrophilicity of the protonated tertiary amine units. At high pH, the stronger hydrophobicity of the alkyl groups on the deprotonated amines led to (co)polymer aggregation. All the (co)polymers showed rapid phase separation at their  $pK_a$ , and the pH transition range can be controlled within a very narrowly defined value.

## 2. Results

### 2.1. Synthesis of the (Co)polymers

A series of random copolymers poly(2-(dimethylamino)ethyl methacrylate)-*co*-2-(dibutylamino)ethyl methacrylate (P(DMAEMA-*co*-DBAEMA)) were obtained by conventional free radical polymerization with varying feed ratios of the monomers containing different tertiary amine groups. Comparing the signal integrals of the proton peaks at 2.3 ppm and at 2.5 ppm (Figure 1, Figures S1–S4), the compositions of the copolymers could be determined, as summarized in Table 1. The molar ratios of the monomer units in the copolymers were relatively close to the feed ratios. Due to the methacrylic acid ester structure of the two monomers, random sequences of two monomer units were expected in the copolymer chains.



**Figure 1.** Proton nuclear magnetic resonance ( $^1\text{H}$ -NMR) spectrum of poly(2-(dimethylamino)ethyl methacrylate)-*co*-2-(dibutylamino)ethyl methacrylate (P(DMAEMA<sub>0.73</sub>-*co*-DBAEMA<sub>0.27</sub>)) in  $\text{CDCl}_3$ .

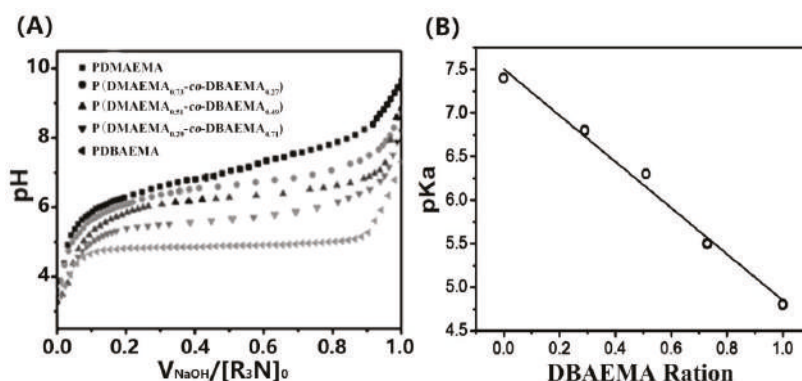
**Table 1.** Characterization of (co)polymers.

Copolymer	DMA Content		Yield (%)	$M_n^b$ ( $\times 10^4$ )	$M_w^b$ ( $\times 10^4$ )	PDI	$pK_a^c$
	In Feed	In Polymer <sup>a</sup>					
PDMAEMA	100	100	73	1.21	1.85	1.53	7.4
P(DMAEMA <sub>0.73</sub> -co-DBAEMA <sub>0.27</sub> )	75	73	67	1.06	1.47	1.39	6.8
P(DMAEMA <sub>0.51</sub> -co-DBAEMA <sub>0.49</sub> )	50	51	62	1.01	1.84	1.82	6.3
P(DMAEMA <sub>0.29</sub> -co-DBAEMA <sub>0.71</sub> )	25	29	65	1.13	2.03	1.80	5.5
PDBAEMA	0	0	56	1.40	2.59	1.85	4.8

<sup>a</sup> Percent molar ratio determined by hydrogen nuclear magnetic resonance (<sup>1</sup>H-NMR); <sup>b</sup> Determined by gel permeation chromatography (GPC) in tetrahydrofuran (THF) with polystyrene standards; <sup>c</sup> Determined by titration during the heating process.

## 2.2. pH Titration of Different Copolymers and Measurement of $pK_a$

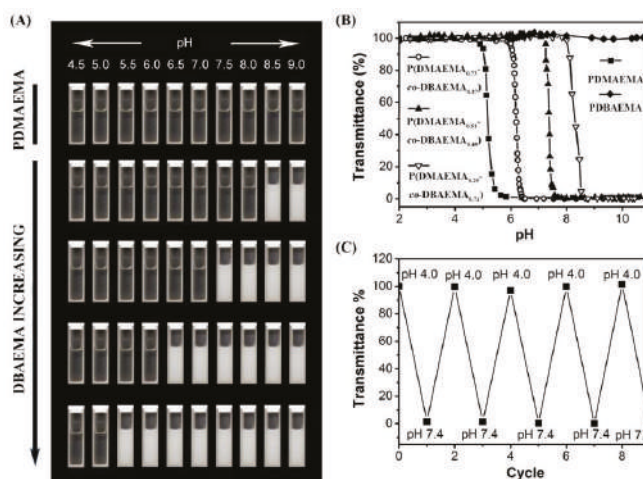
For pH-responsive polymers, the  $pK_a$  value is a critical parameter that can be evaluated through titration experiments. Figure 2A shows the potentiometric titration curves for 0.5 mg/mL (co)polymer solutions. NaOH solution was added to neutralize the protons. Potentiometric titration curves were produced for (co)polymers by plotting the solution pH against the volume of added NaOH. Starting from pH ~3, the addition of small aliquots of NaOH would neutralize the free protons and increase the solution pH. The pH value of PDMAEMA increased continuously with higher NaOH content, whereas for the other (co)polymers, especially PDBAEMA, titration curves were obtained with a plateau at the polymer buffering region. This indicates that the added NaOH was consumed during the deprotonation of the tertiary amine groups on the polymer pendent chain, which corresponds to the phase transition of the polymer. With the completion of the process, further addition of base increased the solution pH rapidly. For each sample, the  $pK_a$  values were calculated as the pH halfway between the two equivalence points in the titration curve. With increasing DBAEMA monomer amounts from 0% to 100%, as shown in Figure 2B, the average  $pK_a$  values of the polymers decreased from 7.4 to 4.8, which was attributed to the hydrophobic property of butyl on amine. An approximately linear relationship was observed between the  $pK_a$  values and DBAEMA monomer molar ratios of the (co)polymers, which indicates that the  $pK_a$  of the pH sensitive polycations can be controlled easily and precisely by adjusting the hydrophobic properties of the pendent group.



**Figure 2.** (A) pH titration curves of (co)polymers. The volumes of NaOH ( $V_{NaOH}$ ) were normalized to the initial amount of amine ( $[R_3N]_0$  in mmol); (B)  $pK_a$  values of the tertiary amine-based polymers as a function of DBAEMA ratio.

### 2.3. Phase Transition Behaviors of the Fine-Tuning pH Responsive Polymers

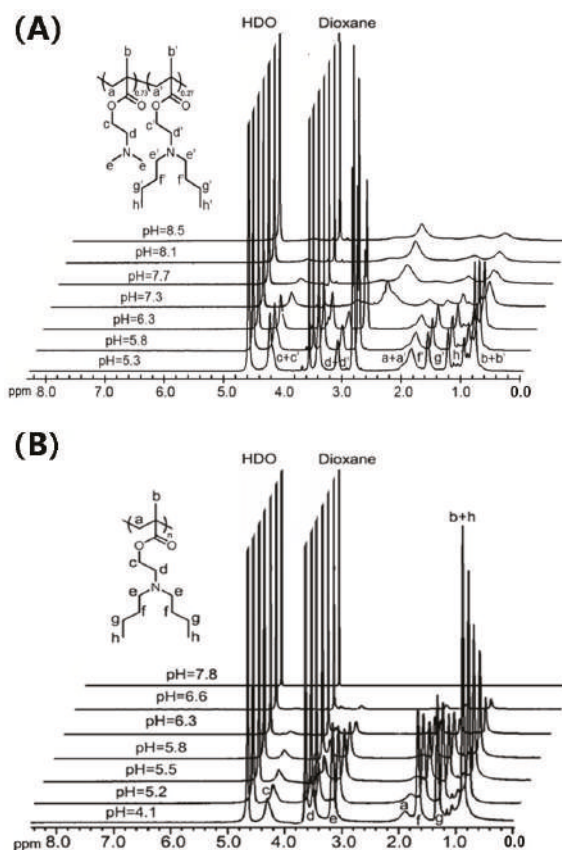
The tenability of the (co)polymers' pH-responsive properties were evaluated by transmittance experiments. Figure 3A,B show the optical images and transmittance changes of the polymer solutions as NaOH was added from initial acid solutions, where all of the polymers were completely dissolved. Over the entire pH range in the measurements, the PDMAEMA solution remained clear with a transmittance around 100%, which was likely due to the hydrophilicity of the dimethyl amino groups, in good agreement with the titration experiment. However, with the incorporation of DBAEMA, the turbidity of the polymer solutions increased sharply at critical pH values due to the aggregation of the polymer chains. The phase transition pH range decreased with the increasing ratio of DBAEMA. Compared to conventional pH responsive materials, the DBAEMA-based polymers displayed a much sharper pH-dependent phase transition, where the transmittance from 100% to 0% was limited to 0.3 pH, indicating that only double the proton concentration could lead to complete turbidity of the copolymers in aqueous solutions. Furthermore, the reversibility of the pH-responsive tertiary amine-based polymers was confirmed by the PDBAEMA transmittance measurement. We also analyzed the polymer solution during cyclic tests between pH 4.0 and pH 7.4. As shown in Figure 3C, the dissolution and aggregation of the polymer solutions are fully reversible for at least five cycles, which indicates the novel pH sensitive materials can be used repeatedly.



**Figure 3.** (A) Optical images of (co)polymer solutions at different pH. (B) The transmittance curves of (co)polymer solutions with increasing pH values. (C) The pH reversibility study of PDBAEMA solution with cycles between pH 4.0 and pH 7.4.

Proton nuclear magnetic resonance spectroscopy ( $^1\text{H-NMR}$ ) was also employed to investigate the effect of pH on the phase transition behaviors of the polymers in aqueous solution. PDBAEMA and  $\text{P(DMAEMA}_{0.73}\text{-co-DBAEMA}_{0.27})$  were investigated as a function of pH in order to compare the aggregation behavior of the polymers with different tertiary amine groups. 1,4-Dioxane, with a single peak at 3.7 ppm, was used as the internal standard, as its intensity remained almost the same before and after the polymer underwent a phase transition. The spectra of the PDBAEMA and  $\text{P(DMAEMA}_{0.73}\text{-co-DBAEMA}_{0.27})$  were recorded in the pH range of 4.1 to 8.5, and Figure 4 shows the  $^1\text{H-NMR}$  spectra of the polymers in  $\text{D}_2\text{O}$  with the tertiary amines in different ionization states. At pH 4.1 and 5.3, the amine groups were protonated; both PDBAEMA and  $\text{P(DMAEMA}_{0.73}\text{-co-DBAEMA}_{0.27})$  were dissolved in the deuterated solution and proton resonance peaks for the segments of each polymer were easily visualized. However, as pH increased from 5.5 to 6.6, the signal intensities

of the PDBAEMA dramatically decreased and disappeared at pH 7.8, when PDBAEMA became completely deprotonated. The suppressed resonances of the polymer were due to the limitation of the motion of the polymer chain, which can be attributed to the phase transition behavior driven by the hydrophobic effect. For P(DMAEMA<sub>0.73</sub>-co-DBAEMA<sub>0.27</sub>), although the signal integrations of the polymer signals decreased gradually when the pH increased above 6.3, the peaks of DMAEMA segments were still observable even at pH 8.5. This drastic difference between PDBAEMA and P(DMAEMA<sub>0.73</sub>-co-DBAEMA<sub>0.27</sub>) spectra can be attributed to the dehydration behaviors of the polymers upon neutralization. The hydrophobic effect involving the butyl groups on the PDBAEMA pendent segments enabled the polymer chains to pack densely in aqueous solution, thus leading to more efficient dehydration and a sharper phase transition.



**Figure 4.** <sup>1</sup>H-NMR spectra of (A) P(DMAEMA<sub>0.73</sub>-co-DBAEMA<sub>0.27</sub>) and (B) PDBAEMA at different pH in D<sub>2</sub>O.

### 3. Materials and Methods

#### 3.1. Materials

2-(dibutylamino) ethanol was purchased from Alfa Aesar Company (Haverhill, MA, USA) and 2-(dimethylamino)ethyl methacrylate (DMAEMA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). CDCl<sub>3</sub>, D<sub>2</sub>O, and 1,4-Dioxane were purchased from Acros Co. (Beijing, China) and used as received. Tetrahydrofuran (THF) was freshly purified by distillation over sodium prior to use.

2,2-Azoisobutyronitrile (AIBN) was recrystallized three times from methanol. Other solvents and reagents were purchased from Beijing Chemical Reagent Co. (Beijing, China) and used as received.

### 3.2. Synthesis of the DBA Monomer

The tertiary amine-based methacrylate monomer 2-(Dibutylamino)ethyl methacrylate (DBAEMA) was synthesized by following a previously reported method [38]. 2-(dibutylamino)ethyl ethanol (17.3 g, 0.1 mol), triethylamine (10.1 g, 0.1 mol), and inhibitor hydroquinone (0.11 g, 0.001 mol) were dissolved in 100 mL THF and placed in a three-neck flask. To this solution, methacryloyl chloride (10.4 g, 0.1 mol) was added dropwise with constant stirring. The resulting solution was refluxed in THF for 2 h and then filtered to remove the precipitated white triethylamine-HCl salts with completion of the reaction. After removing THF solvent by rotary evaporator, the resulting residue was distilled in vacuo (83–87 °C at 0.05 mm Hg) as a colorless liquid. The obtained DBAEMA monomer was characterized by <sup>1</sup>H-NMR. <sup>1</sup>H-NMR (TMS, CDCl<sub>3</sub>, ppm): 6.09 (br, 1H, CHH=C(CH<sub>3</sub>)-), 5.55 (br, 1H, CHH=C(CH<sub>3</sub>)-), 4.19 (t, *J* = 6.3 Hz, 2H, -OCH<sub>2</sub>CH<sub>2</sub>N-), 2.73 (t, *J* = 6.3 Hz, 2H, -OCH<sub>2</sub>CH<sub>2</sub>N-), 2.46 (t, *J* = 7.6 Hz, 2H, -N(CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 1.93 (s, 3H, CH<sub>2</sub>=C(CH<sub>3</sub>)-), 1.41 (m, 4H, -N(CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 1.29 (m, 4H, -N(CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), 0.89 (t, *J* = 7.3 Hz, 6H, -N(CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>)<sub>2</sub>), Yield: 56%.

### 3.3. Synthesis of the (Co)polymers

The P(DMAEMA-*co*-DBAEMA) copolymers, PDMAEMA, and PDBAEMA were prepared by free radical polymerization in THF with varying DBAEMA to DMAEMA molar feed ratios of 0:4, 1:3, 2:2, 3:1, and 4:0, respectively. Typical procedures employed for the polymerization were as follows. Monomers were dissolved in THF at a total concentration of 0.10 g/mL, to which azodiisobutyronitrile (AIBN) (1.0 mol% relative to monomers) was added as a free radical initiator. After three cycles of freeze-thaw to thoroughly remove oxygen, the tube was sealed under reduced pressure and the polymerization was performed at 60 °C for 24 h. The polymers were purified by precipitation from diethyl ether twice, collected by filtration, and dried under vacuum to obtain white powders. The molecular weights and the composition of the (co)polymers were determined by gel permeation chromatography (GPC) and <sup>1</sup>H-NMR, respectively.

### 3.4. Characterization of the (Co)polymers

<sup>1</sup>H-NMR spectra of the monomers and (co)polymers were recorded in CDCl<sub>3</sub> on a Varian-600 MHz spectrometer with tetramethylsilane (TMS) as the internal reference. The molecular weight and molecular weight distributions of the (co)polymers were measured with gel permeation chromatography (GPC) equipment consisting of WGE3010 pump, 3010 refractive index detector, and WGE Styra gel columns. The temperature of the columns was 35 °C and THF was used as an eluent at a flow rate of 1 mL/min. A series of narrowly dispersed polystyrene samples were used as standards and Millennium 32 software was used to calculate the molecular weight and polydispersity.

### 3.5. pH Titration

The pK<sub>a</sub> values of different (co)polymers were detected by pH titration. When the prepared polymer was dissolved in 0.1 N HCl to reach a final concentration of 5–10 mg/mL, the pH titration experiment was performed by adding small volumes (50–100 µL increments) of 0.1 N NaOH solution under stirring. The pH values of the solution were measured continuously using a Sartorius (Germany) pH meter with a microelectrode.

### 3.6. Turbidity Measurements by UV/Vis Spectroscopy

The pH-dependent phase transition of the polymers was determined by the turbidity of the polymer solutions as a function of pH. The transmittance of the 1 mg/mL (co)polymer solutions in 0.1 N HCl were measured at 500 nm through a 1 cm quartz cell on a Shimadzu 2550 ultraviolet



(UV)-vis spectrometer. To adjust the pH, 0.2 N NaOH was added, and the final volumes of the polymer solutions increased about 10% compared with initial volumes. The pH values were monitored with a digital internal pH meter. Polymer-free deionized (DI) water was used as a reference.

### 3.7. <sup>1</sup>H-NMR Measurements of pH-Dependent Phase Transition

P(DMAEMA<sub>0.73</sub>-co-DBAEMA<sub>0.27</sub>) and PDBA were first dissolved in the deuterated buffers at a concentration of 10 mg/mL, and 1,4-dioxane was used as an internal standard. To adjust the pH value, 0.1 N NaOD deuterated solution was added and the Varian Mercury Plu600 MHz NMR spectrometer was used to record the <sup>1</sup>H-NMR spectra at different pH.

## 4. Conclusions

A series of novel pH-responsive polymers based on tertiary amine functional groups were developed by easy free radical copolymerization. The pK<sub>a</sub> values of the polymers were precisely tuned by adjusting the feed ratio between the two monomers, DMAEMA and DBAEMA. The phase transition of polymers occurred in narrow pH ranges, which was demonstrated by transmittance and <sup>1</sup>H-NMR detection. Below pK<sub>a</sub>, the positive charges of the protonated amine groups maintained the solubility of the polymers in aqueous solution, whereas when pH was greater than pK<sub>a</sub>, the hydrophobic butyl groups on neutralized PDBAEMA segments rapidly induced the aggregation and precipitation of the polymers. Compared to widely used PDMAEMA and PDEAEMA, the developed polymers with PDBAEMA segments displayed much sharper pH transition ranges. The finely tunable transition pH values mean the polymers are a promising platform for drug delivery and biomedicine applications, where the encapsulated drugs at physiological pH would be triggered to release in acidic microenvironments.

**Supplementary Materials:** The following are available online.

**Author Contributions:** H.F., P.L., and X.H. conceived and designed the experiments; P.L. and W.L. performed the experiments; H.F. and X.H. analyzed the data; H.L. contributed reagents/materials/analysis tools; X.H. wrote the paper.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## Article

# Effect of Hydrophobic Polypeptide Length on Performances of Thermo-Sensitive Hydrogels

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**Abstract:** Thermosensitive gels are commonly used as drug carriers in medical fields, mainly due to their convenient processing and easy functionalization. However, their overall performance has been severely affected by their unsatisfying biocompatibility and biodegradability. To this end, we synthesized poly(L-alanine) (PLA<sub>l</sub>)-based thermosensitive hydrogels with different degrees of polymerization by ring-opening polymerization. The obtained mPEG<sub>45</sub>–PLA<sub>l</sub> copolymers showed distinct transition temperatures and degradation abilities. It was found that slight changes in the length of hydrophobic side groups had a decisive effect on the gelation behavior of the polypeptide hydrogel. Longer hydrophobic ends led to a lower gelation temperature of gel at the same concentration, which implied better gelation capability. The hydrogels showed rapid gelling, enhanced biocompatibility, and better degradability. Therefore, this thermosensitive hydrogel is a promising material for biomedical application.

**Keywords:** amphiphilicity; phase change; polyamino acids; degradability

## 1. Introduction

The application of hydrogels in the biomedical field has rapidly increased over the past decade, including use in three-dimensional (3D) cell culture [1–3], drug delivery [4–6], and tissue engineering [7–9]. Polymer hydrogels can trap a large amount of moisture inside for easier cell membrane penetration and drug transmission. They enjoy excellent physical properties and exhibit a controllable degradation process. On account of the incomparable convenience, the in situ gelation of biodegradable hydrogels has aroused great interest in many researchers. Among numerous biodegradable hydrogels, thermosensitive hydrogels have specific advantages. On the one hand, they are rather safe for clinical utilization because the common thermosensitive gel preparation process does not involve the use of organic solvents. On the other hand, the gelling conditions of heat-sensitive hydrogels are easy to control, making them applicable in the field of biomedicine [10–12].

Thermosensitive hydrogels can be generated from block copolymers consisting of hydrophilic poly(ethylene glycol) (PEG) and hydrophobic moieties such as poly(lactic acid) (PLA) [13,14], poly(lactic-co-glycolic acid) (PLGA) [15–17], poly( $\epsilon$ -caprolactone) (PCL) [18–20], polyamidoamine (PAMAM) [21], and so on. Thermosensitive hydrogels are soluble at low temperatures, which facilitates the introduction of chemotherapeutic drugs [22–24], functional proteins [25], and cells. Due to the amorphous form of the hydrogel at low temperatures, it can be seamlessly filled into injury sites [26,27]. At body temperature, the soluble hydrogels turn into a gel state and provide a depot for

the encapsulated medical agents to achieve sustained release and long-term therapeutic effect [28]. For example, growth factors can be slowly and continuously released locally for the efficient repair of bone and nerve tissues [29–31]. The hydrogels can also function as 3D scaffolds and as a nutrient resource for cell growth in tissue engineering [32], aiming to help cells grow in a more uniform manner and promote cell proliferation [33].

In 2012, Qian's team prepared a bionic hydrogel composed of three components (i.e., triblock PEG–PCL–PEG copolymer (PECE), nano-hydroxyapatite (n-HA), and collagen), which showed satisfactory efficacy in skull repair [34]. In 2015, Chen's team engineered a thermosensitive PLGA–PEG–PLGA hydrogel loaded with 5-fluorouracil for the prevention of postoperative tendon adhesion, and acquired a good histological score [35]. However, despite the wide application of thermosensitive hydrogels in various diseases in clinical practice, they are not free from flaws. Some hydrogels are difficult to gelatinize, and the degradation time is uncontrollable. Moreover, hydrogels with poor biocompatibility always lead to severe tissue damages. Therefore, there is an urgent need to develop thermosensitive polypeptide polymers with good biocompatibility and biodegradability to avoid the above problems.

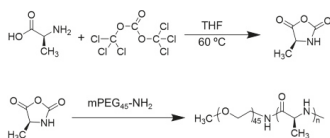
Towards this aim, peptide systems have been developed to avoid evoking the formation of an acidic microenvironment during degradation, and thus reduce the damages to surrounding tissues and minimize the adverse effects toward the bioactivity of the loaded protein or cells. Apart from this, the gelation performance of the polypeptide copolymer can be tuned by copolymerization with various hydrophobic or hydrophilic amino acid monomers, which widens the clinical applications. In 2011, Jeong's team reported the synthesis of poly(alanine-*co*-leucine)–poloxamer–poly(alanine-*co*-leucine) (PAL–PLX–PAL) hydrogels, which exhibited a sustained drug release pattern without producing evident inflammatory effects [22]. In 2017, methoxy poly(ethylene glycol)-*block*-poly(L-alanine-*co*-L-phenylalanine) mPEG-*b*-PLAF hydrogel was synthesized by the ring-opening polymerization (ROP) reaction of L-alanine *N*-carboxyanhydrides (L-Ala NCA) and L-phenylalanine NCA, with amino-terminated mPEG (mPEG-NH<sub>2</sub>) as a macroinitiator. Encapsulated with combretastatin A4 (CA4) and doxorubicin (DOX), mPEG-*b*-PLAF hydrogel had an excellent inhibitory effect on the proliferation of B16F10 melanoma cells [36].

In this work, mPEG–poly(L-alanine) (mPEG–PLAla) polymers with different degrees of polymerization (DPs) were synthesized and named mPEG<sub>45</sub>–PLAla<sub>30</sub>, mPEG<sub>45</sub>–PLAla<sub>22</sub>, and mPEG<sub>45</sub>–PLAla<sub>14</sub>. The influence of different DPs on the gelation properties of hydrogels was studied. It was found that hydrogels with higher DP showed better gelation ability than those with lower DP. In addition, the gelation behavior, tissue safety, and cytotoxicity of these polymers *in vitro* and *in vivo* were also tested and evaluated. The study revealed that these hydrogels had notable potential for employment in the biomedical field.

## 2. Results and Discussion

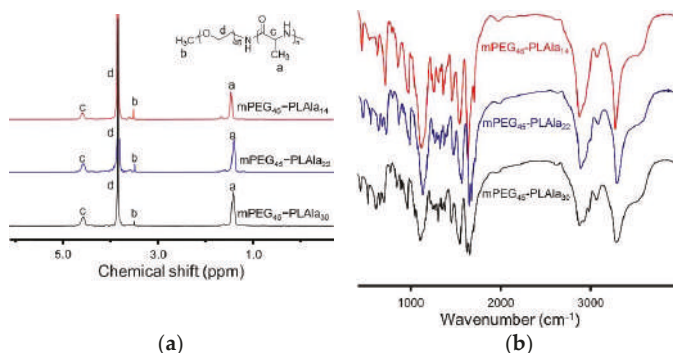
### 2.1. Material Synthesis and Structure Characterization

Triphosgene and L-alanine were synthesized into L-Ala NCA in dried tetrahydrofuran (THF) solvent, as shown in Scheme 1. The polypeptide hydrogels were produced via the ROP of L-Ala NCA initiated by mPEG<sub>45</sub>-NH<sub>2</sub>. The mPEG<sub>45</sub>–PLAla copolymers with different DPs were synthesized by altering the feed amount. The DPs of PLAla were determined by contrasting the integral of the methyl peaks of side chains (–CH<sub>3</sub>) with the methylene peak of PEG (–CH<sub>2</sub>CH<sub>2</sub>O–). The DPs of mPEG<sub>45</sub>–PLAla<sub>30</sub>, mPEG<sub>45</sub>–PLAla<sub>22</sub>, and mPEG<sub>45</sub>–PLAla<sub>14</sub> were 30, 22, and 14, respectively.



**Scheme 1.** Synthetic routes of L-alanine N-carboxyanhydrides (L-Ala NCA) and methoxy poly(ethylene glycol)–poly(L-alanine) (mPEG<sub>45</sub>–PLAla).

The typical proton nuclear magnetic resonance (<sup>1</sup>H NMR) spectrum of block copolymer is shown in Figure 1a, and all peaks could be accurately assigned. The peaks at 1.47, 3.40, 3.50–3.80, and 4.64 ppm stood for the protons of alanine methyl, mPEG terminal methoxy, mPEG backbone, and polymer methine, respectively, indicating the successful synthesis of the three polymers.



**Figure 1.** Structure characterization of mPEG<sub>45</sub>–PLAla. (a) <sup>1</sup>H NMR spectra of mPEG<sub>45</sub>–PLAla<sub>30</sub>, mPEG<sub>45</sub>–PLAla<sub>22</sub>, and mPEG<sub>45</sub>–PLAla<sub>14</sub>, where the subscripts indicate the degree of polymerization (DP); (b) Fourier-transform infrared (FT-IR) spectra of mPEG<sub>45</sub>–PLAla<sub>30</sub>, mPEG<sub>45</sub>–PLAla<sub>22</sub>, and mPEG<sub>45</sub>–PLAla<sub>14</sub>.

The secondary structure of the copolymer was studied by FT-IR. In Figure 1b, characteristic peaks of amide bond at 1627 cm<sup>-1</sup> and 1544 cm<sup>-1</sup> were observed, indicating that all copolymers went through the main β-sheet conformation.

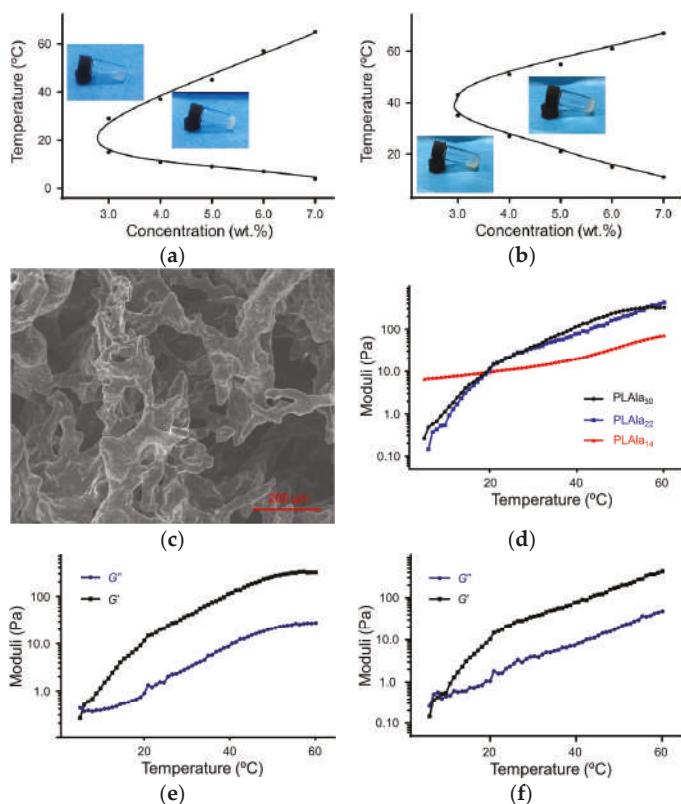
## 2.2. Gelation Ability and Internal Appearance

The tube inversion method was used to determine the transition temperatures. Polypeptides were solubilized in a phosphate-buffered saline (PBS) solution, and went through a solution–gel transition with the change in temperature. The samples were defined as gel if they stayed still when the vial was inverted for as long as 30 s. The phase diagrams of mPEG<sub>45</sub>–PLAla<sub>30</sub> and mPEG<sub>45</sub>–PLAla<sub>22</sub> copolymers are shown in Figure 2a,b. Only copolymers with a DP of no less than 22 could undergo solution–gel transition at the concentration of 3.0–8.0 wt. %. It was noticeable that mPEG<sub>45</sub>–PLAla<sub>22</sub> showed a higher gel transition temperature than mPEG<sub>45</sub>–PLAla<sub>30</sub>. The critical gelation temperatures (CGTs) of mPEG<sub>45</sub>–PLAla<sub>30</sub> and mPEG<sub>45</sub>–PLAla<sub>22</sub> were 5 °C and 15 °C, respectively, at the concentration of 5.0 wt. %. The lower CGT of mPEG<sub>45</sub>–PLAla<sub>30</sub> could be explained by longer hydrophobic end than that of mPEG<sub>45</sub>–PLAla<sub>22</sub> [37].

Solution phase behaviors of the three copolymers at different temperatures were investigated. The images in Figure 2a,b showed the mPEG<sub>45</sub>–PLAla<sub>30</sub> and mPEG<sub>45</sub>–PLAla<sub>22</sub> solutions in PBS (5.0 wt. %) at 4 °C and 37 °C. Although all copolymers were dispersed in PBS at 4 °C, the mPEG<sub>45</sub>–PLAla<sub>14</sub> formed a clear solution while mPEG<sub>45</sub>–PLAla<sub>30</sub> and mPEG<sub>45</sub>–PLAla<sub>22</sub> showed a turbid state. When temperature rose to 37 °C, mPEG<sub>45</sub>–PLAla<sub>30</sub> and mPEG<sub>45</sub>–PLAla<sub>22</sub> were



observed to be stably gelled within 5 min, while mPEG<sub>45</sub>–PLA<sub>14</sub> remained in a viscous flow state. This was because mPEG<sub>45</sub>–PLA<sub>14</sub> had better water solubility. Further, the morphology of the mPEG<sub>45</sub>–PLA<sub>30</sub> hydrogel (5.0 wt. %) was observed by scanning electron microscope (SEM). As can be seen in Figure 2c, it was found that there were numerous tiny pores in the mPEG<sub>45</sub>–PLA<sub>30</sub> gel, which was due to the 3D network structure of the gel. These pores showed a size of about 100  $\mu\text{m}$  and were evenly distributed within the hydrogel, making them suitable as a depot for therapeutic agents. These findings prove that mPEG<sub>45</sub>–PLA<sub>30</sub> and mPEG<sub>45</sub>–PLA<sub>22</sub> hydrogels feature good stability at body temperature and are suitable for biomedical applications.



**Figure 2.** Gelatinization characteristics of mPEG<sub>45</sub>–PLA. (a) Solution–gel phase diagrams of the mPEG<sub>45</sub>–PLA<sub>30</sub> and (b) mPEG<sub>45</sub>–PLA<sub>22</sub> copolymer solutions; (c) Scanning electron microscope (SEM) image of mPEG<sub>45</sub>–PLA<sub>30</sub> hydrogels formed at 40 °C; (d)  $G'$  of the mPEG<sub>45</sub>–PLA<sub>30</sub> (PLA<sub>30</sub>), mPEG<sub>45</sub>–PLA<sub>22</sub> (PLA<sub>22</sub>), and mPEG<sub>45</sub>–PLA<sub>14</sub> (PLA<sub>14</sub>) at the concentration of 5.0 wt. % in phosphate-buffered saline (PBS) solution; (e) Changes of  $G'$  and  $G''$  of mPEG<sub>45</sub>–PLA<sub>30</sub> and (f) mPEG<sub>45</sub>–PLA<sub>22</sub> in PBS solutions (5 wt. %).

### 2.3. Mechanical Performance Test

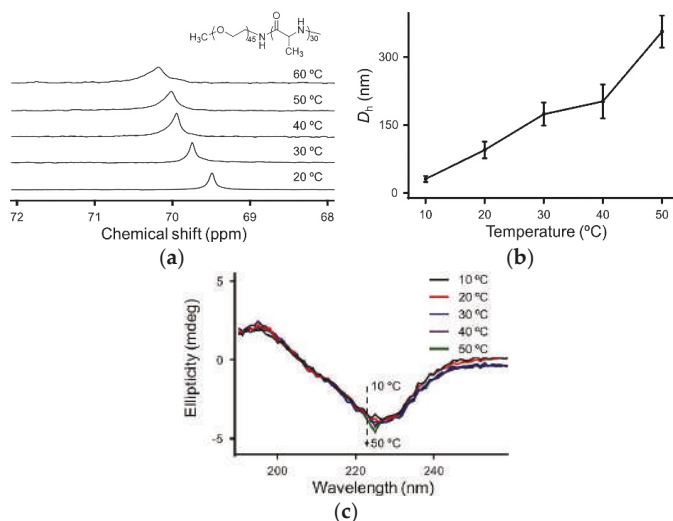
Thermally induced storage modulus ( $G'$ ) and loss modulus ( $G''$ ) changes of the three copolymers were obtained by dynamic mechanical analysis.  $G'$  represents the systematic gel-like behavior of the elastic component of the complex modulus, and  $G''$  is an index of the viscous component of the complex modulus and a measure of the sol-like behavior. The intersection of  $G'$  and  $G''$  reflects the sol–gel transition. As shown in Figure 2d, the  $G'$  of mPEG<sub>45</sub>–PLA<sub>30</sub> and mPEG<sub>45</sub>–PLA<sub>22</sub> obviously increased along with rising temperatures. However, only a minor increment in  $G'$  was

seen in mPEG<sub>45</sub>–PLAla<sub>14</sub>, and no significant change in  $G'$  was detected, even when the temperature rose to about 50 °C, implying that there was no sol–gel modification of mPEG<sub>45</sub>–PLAla<sub>14</sub> within the experimental temperature range. The reason was that hydrophilic mPEG was longer than the hydrophobic end made up of polypeptides. Therefore, with a longer hydrophilic segment, mPEG<sub>45</sub>–PLAla<sub>14</sub> showed better water solubility than mPEG<sub>45</sub>–PLAla<sub>30</sub> and mPEG<sub>45</sub>–PLAla<sub>22</sub>, thus featuring less obvious sol–gel change. Notably, the length of the hydrophobic side chain had a remarkable effect on the gelation function of the mPEG–peptide block copolymer.

In Figure 2e,f,  $G'$  was smaller than  $G''$  when the temperature was lower than CGTs (5 °C in mPEG<sub>45</sub>–PLAla<sub>30</sub> and 12 °C in mPEG<sub>45</sub>–PLAla<sub>22</sub>), which reflected the viscous state of hydrogels. As the temperature increased above CGTs,  $G'$  was sharply elevated and surpassed  $G''$ , confirming the gel formation. This result was in accordance with the findings in Figure 2a,b.

#### 2.4. Mechanism of Gelatinization

In order to study the mechanism of sol–gel transition, the nuclear magnetic peak changes, diameter changes, and conformation evolution of polypeptides in response to temperature were tested by carbon nuclear magnetic resonance (<sup>13</sup>C-NMR), dynamic light scattering (DLS), and circular dichroism (CD). As shown in Figure 3a, when temperature rose from 20 to 60 °C, the characteristic peak of PEG gradually moved from 69.7 to 70.3 ppm, which revealed continuous dehydration of the PEG block during the heat-induced sol–gel transition process. Two main reasons accounted for the gelation of polymers. First, the interaction between hydrophobic blocks became stronger as temperature increased. Secondly, dehydration of PEG segments facilitated the aggregation of the hydrogels [38].



**Figure 3.** Gelation mechanism of the hydrogels. (a) <sup>13</sup>C-NMR spectra (in D<sub>2</sub>O) of 5.0 wt. % mPEG<sub>45</sub>–PLAla<sub>30</sub> solution as a function of temperature; (b) Average hydrodynamic diameter ( $D_h$ ) of micelles of mPEG<sub>45</sub>–PLAla<sub>30</sub> as a function of temperature in water (5.0  $\mu\text{g mL}^{-1}$ ); (c) Circular dichroism (CD) spectra of mPEG<sub>45</sub>–PLAla<sub>30</sub> (0.05  $\text{mg mL}^{-1}$ ) in aqueous solution as a function of temperature.

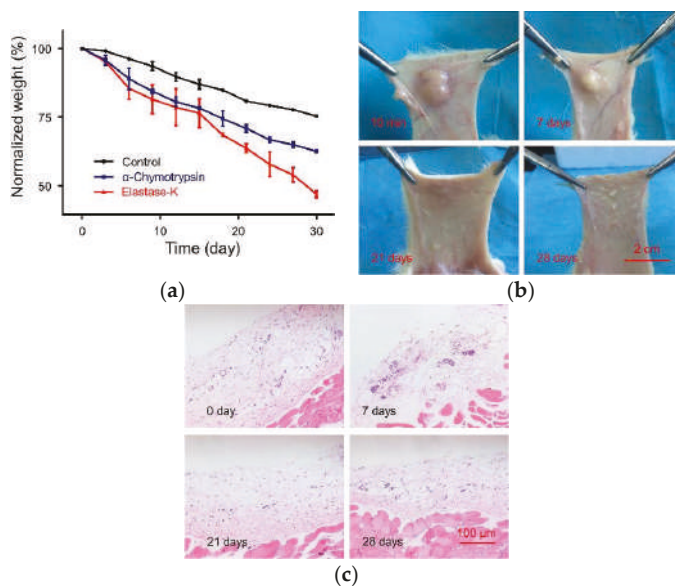
As seen in Figure 3b, the diameter changes of mPEG<sub>45</sub>–PLAla<sub>30</sub> were determined at the concentration of 5.0  $\mu\text{g mL}^{-1}$ . At 10 °C, the average hydrodynamic diameter ( $D_h$ ) of mPEG<sub>45</sub>–PLAla<sub>30</sub> was about 31.4 nm. When the temperature rose to 20 °C and 50 °C, the number swiftly increased to 95.9 nm and 356.2 nm, respectively. The dramatic changes in particle size could be explained by the

interaction between the shell of PEG and core of polypeptides caused by the dehydration process of PEG [38].

In Figure 3c, the CD spectrum illustrated the alteration of the secondary structure of the aqueous mPEG<sub>45</sub>–PLAAla<sub>30</sub> during the sol–gel transition process. The two typical bands corresponding to the  $\beta$ -sheet conformation, a positive Cotton band at 195 nm and a negative Cotton band at 226 nm, were clearly shown. The above results revealed that the synthesized thermosensitive hydrogel had good physicochemical properties and a clear solution–gel transformation mechanism.

## 2.5. Degradability Test and Pathological Analysis

Since hydrogels are often used in the biomedical field, biodegradability is a decisive factor for clinical application. If the degradation rate of the hydrogel is too high, it will lead to a rapid release of the payload. In contrast, if the hydrogel requires a long time to degrade, then it will probably remain at the injection site even after all drugs are released, which is inconvenient for further treatment. The *in vitro* degradation of mPEG<sub>45</sub>–PLAAla<sub>30</sub> hydrogel (5.0 wt. %) was evaluated in PBS and PBS with elastase K or  $\alpha$ -chymotrypsin. As shown in Figure 4a, there was a mass loss of over 70% and 67% hydrogel in the elastase K and  $\alpha$ -chymotrypsin groups on day 15, respectively—considerably higher than 20% in PBS. The results could be explained by the absence of elastase K or  $\alpha$ -chymotrypsin, where the mass loss of gels was merely attributed to the surface erosion of the hydrogels. However, with elastase K or  $\alpha$ -chymotrypsin, the polypeptide chains of the hydrogels were also fast degrading, which sped up the loss of gels.



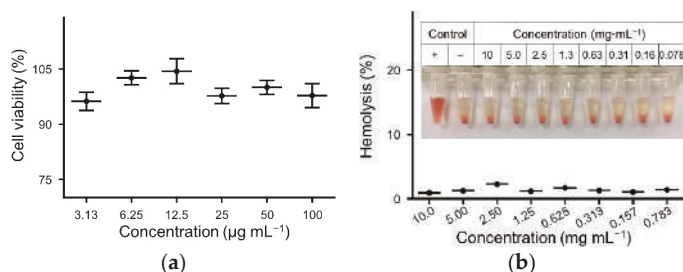
**Figure 4.** Degradation of hydrogel *in vitro* and *in vivo*, and histological analysis of skin. (a) Mass loss curves of *in vitro* degradation of hydrogels in PBS, and PBS with elastase-K or  $\alpha$ -chymotrypsin ( $0.2 \text{ mg mL}^{-1}$ ) groups; (b) Images of *in vivo* gel maintenance at 10 min, 7, 14, 21, and 28 days after the injection of 5.0 wt. % mPEG<sub>45</sub>–PLAAla<sub>30</sub> hydrogels; (c) Hematoxylin and eosin (H&E) images of the skin tissue near the hydrogels on day 7, 14, 21, and 28, respectively.

The degradation process of hydrogels *in vivo* was monitored for 28 days, and images were taken at 10 min, 7, 14, 21, and 28 days after hydrogel treatment. At  $4^\circ\text{C}$ ,  $500.0 \mu\text{L}$  of mPEG<sub>45</sub>–PLAAla<sub>30</sub> solution (5.0 wt. %) was subcutaneously injected into Sprague–Dawley (SD) rats through 21-gauge

syringe needles. In Figure 4b, the solution rapidly turned into gel in 10 min after injection. Seven days later, only a small portion of the hydrogel was degraded. On day 14, the size of the hydrogel shrank to less than 50%, while on day 28, the hydrogel was completely degraded. This degradation rate of hydrogel was suitable for application in biomedical fields requiring long-term treatment, such as tissue repair. As shown in Figure 4c, at different time intervals, the tissue surrounding the gel was surgically separated and processed by H&E staining to examine the condition of tissue damage. Basically, no histological damage or immune response was found at any time point.

## 2.6. Safety Evaluation

The cytotoxicity of hydrogel mPEG<sub>45</sub>–PLAAla<sub>30</sub> in vitro was evaluated by methyl thiazolyl tetrazolium (MTT) assay and hemolysis test. In Figure 5a, L929 cells treated with the highest concentration of mPEG<sub>45</sub>–PLAAla<sub>30</sub> (100.0  $\mu\text{g mL}^{-1}$ ) for 24 h retained almost 100% viability, confirming its excellent biocompatibility. Moreover, the effect of different concentrations of gel on hemolysis was also investigated. In this part, hemolysis was measured spectroscopically according to previously reported methods [39]. As shown in Figure 5b, no hemolysis was detected in blood samples treated with all test concentrations of mPEG<sub>45</sub>–PLAAla<sub>30</sub>, which further confirmed the outstanding biocompatibility. Overall, the findings above revealed that the hydrogel showed little toxicity, both in vitro and in vivo. Therefore, it is a promising material for usage in clinical practice.



**Figure 5.** Biological compatibility of mPEG<sub>45</sub>–PLAAla<sub>30</sub>. (a) In vitro cytotoxicity of the mPEG<sub>45</sub>–PLAAla<sub>30</sub> toward L929 cells. Data were presented as mean  $\pm$  SD ( $n = 5$ ); (b) Hemolysis experiments of mPEG<sub>45</sub>–PLAAla<sub>30</sub>. Data are presented as mean  $\pm$  SD ( $n = 3$ ).

## 3. Materials and Methods

### 3.1. Materials

mPEG–OH, number-average molecular weight ( $M_n$ ) = 2000  $\text{g mol}^{-1}$ , was purchased from Sigma-Aldrich (St. Louis, MO, USA). The mPEG<sub>45</sub>–NH<sub>2</sub> was synthesized conforming to the previously reported protocol in our work [40]. THF and toluene were refluxed with sodium and distilled under nitrogen before usage. *N,N*-Dimethylformamide (DMF) was stored over calcium hydride (CaH<sub>2</sub>) and purified by vacuum distillation. All the other reagents and solvents were bought from Sinopharm Chemical Reagent Co. Ltd., Beijing, China, and used as obtained.

### 3.2. Phase Diagram

The sol–gel transition behavior of the copolymers in PBS (pH 7.4) was determined by inverting test method with a temperature increment of 2  $^{\circ}\text{C}$  per step. Samples with concentrations ranging from 3.0–7.0 wt. % were dissolved in PBS and stirred at 0  $^{\circ}\text{C}$  for 12 h. The copolymer solution (0.2 mL) was introduced into the test tube with an inner diameter of 10.0 mm. The sol–gel transition temperature was recorded if no flow was observed within 30 s after inverting the test tube. Each data point was the average of three measurements.

### 3.3. Dynamic Mechanical Analysis

Rheological experiments were performed on a US 302 Rheometer (Anton Paar, Graz, Austria). The copolymer solution was placed between parallel plates of 25.0 mm in diameter with a gap of 0.5 mm. To prevent the evaporation of water, the outer edge of the sandwiched sample was sealed by a thin layer of silicon oil. The data were collected under a controlled strain  $\gamma$  of 1% and a frequency of 1 rad s<sup>−1</sup>. The heating rate was 1 °C min<sup>−1</sup>.

### 3.4. In Vitro Gel Degradation

For this, 0.5 mL of mPEG<sub>45</sub>–PLA<sub>30</sub> hydrogel was incubated in PBS (5.0 wt. %) in vials (diameter = 16.0 mm) at 37 °C for 10 min. PBS solutions (pH 7.4) containing 0.2 mg mL<sup>−1</sup> elastase K or 0.2 mg mL<sup>−1</sup>  $\alpha$ -chymotrypsin were used as degradation media, and hydrogels incubated in PBS were only used as a control. Different solutions (2.0 mL) were added to the top of the gels at 37 °C and the entire medium was changed daily. The weight of the remaining gel was measured daily.

### 3.5. In Vivo Gel Degradation

SD rats (about 180.0 g, provided by Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were used for the detection of gel degradation in vivo. Rats were anesthetized by inhalation of ether before 0.5 mL of mPEG<sub>45</sub>–PLA<sub>30</sub> PBS solutions (5.0 wt. %) were injected into the dorsal subcutaneous area of the rats using a 21-gauge needle. The rats were sacrificed on day 7, day 14, day 21, and day 28, respectively, to monitor the degradation behavior of the gel. The experiments on animals were carried out according to the guide for the care and use of laboratory animals, provided by Jilin University, Changchun, China, and the procedure was approved by the local Animal Ethics Committee.

### 3.6. Cytotoxicity Measurement

The relative cytotoxicity was assessed by MTT viability assay against L929 mouse fibroblasts cells. L929 cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10.0% (*v/v*) FBS, penicillin (50.0 IU mL<sup>−1</sup>), and streptomycin (50.0 IU mL<sup>−1</sup>) at 37 °C in a 5.0% (*v/v*) carbon dioxide atmosphere. L929 cells with a density of 6000 cells per well were planted in 96-well plates in 180.0  $\mu$ L of DMEM. After incubation for 24 h, 20.0  $\mu$ L of copolymer solutions at different concentrations (31.3–1000.0  $\mu$ g mL<sup>−1</sup>) were added. L929 cells were incubated with copolymers for another 24 h before 20.0  $\mu$ L of PBS solution containing MTT (0.05 mg mL<sup>−1</sup>) was added and incubated for a further 4 h. Then, the media was replaced with 160.0  $\mu$ L of dimethyl sulfoxide (DMSO). The absorbance of the solution was measured on a Bio-Rad 680 microplate reader (Hercules, CA, USA) at 490 nm. Cell viability (%) was calculated according to the following Equation (1).

Measurements were done in five replicates.

$$\text{Cell viability (\%)} = A_{\text{sample}} / A_{\text{control}} \times 100, \quad (1)$$

where  $A_{\text{sample}}$  and  $A_{\text{control}}$  denote the absorbances of sample and control, respectively.

## 4. Conclusions

In this work, thermosensitive hydrogels mPEG<sub>45</sub>–PLA<sub>n</sub> with three different DP (14, 22, and 30) were synthesized by ROP of L-Ala NCA monomer initiated by mPEG<sub>45</sub>–NH<sub>2</sub>. The effect of different DP on the solution–gel transition was investigated. It is worth noting that mPEG<sub>45</sub>–PLA<sub>30</sub> had a lower sol–gel transition temperature than mPEG<sub>45</sub>–PLA<sub>22</sub>, attributed to the longer hydrophobic segment. In addition, mPEG–PLA<sub>n</sub> hydrogel had good stability and high mechanical strength after gelation. Moreover, mPEG–PLA<sub>n</sub> hydrogel did not cause any tissue damage, inflammatory reaction, or hemolysis reaction during degradation, confirming its good biocompatibility. In summary,

the mPEG–PLA hydrogel designed in our study had improved biocompatibility, appropriate degradation, and gelation ability. Therefore, the polypeptide hydrogels showed promise for application in tissue repair and regeneration, cell 3D culture, and treatment of cancer. Moreover, by loading functional drugs, the hydrogels can also be used for postoperative recovery and anti-infection. Based on the above findings, polypeptide thermosensitive hydrogels have broad prospects in the biomedical field.

**Supplementary Materials:** The following are available online.

**Author Contributions:** W.X. and W.W. conceived and designed the experiments; J.H. performed the experiments; J.H., X.Z. and X.F. analyzed the data; and Y.H. contributed reagents and materials. J.H. initiated and wrote this article; X.F. helped to write and corrected the manuscript; and Y.H. discussed and suggested ideas for improvement of this article.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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**Sample Availability:** Samples of the compounds are available from the authors.



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Review

# Smart and Functional Conducting Polymers: Application to Electrorheological Fluids

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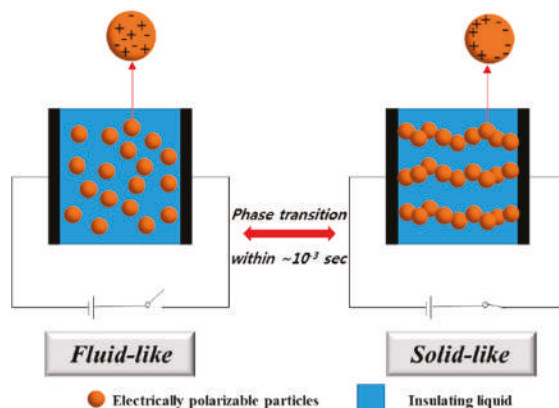
**Abstract:** Electro-responsive smart electrorheological (ER) fluids consist of electrically polarizing organic or inorganic particles and insulating oils in general. In this study, we focus on various conducting polymers of polyaniline and its derivatives and copolymers, along with polypyrrole and poly(ionic liquid), which are adopted as smart and functional materials in ER fluids. Their ER characteristics, including viscoelastic behaviors of shear stress, yield stress, and dynamic moduli, and dielectric properties are expounded and appraised using polarizability measurement, flow curve testing, inductance-capacitance-resistance meter testing, and several rheological equations of state. Furthermore, their potential industrial applications are also covered.

**Keywords:** conducting polymer; composite; electrorheological; smart fluid; viscoelastic

## 1. Introduction

Smart functional materials that can detect and identify external (or internal) stimuli, including electricity, light, heat, magnetic fields, stress, strain, and chemicals [1], have gained attention due to their various engineering applications, such as vibration controls [2], detection [3], electronics [4], and drug delivery [5]. Among these smart functional materials, electrorheological (ER) suspensions consisting of electro-responsive particles and an insulating fluid, such as silicone or mineral oil [6–8], play a crucial role. By engaging an electric field, the electro-responsive functional particles begin to polarize within milliseconds and the initially dispersed particles aggregate to build a chain form, following an external electrical field direction because of an induced dipolar attractive force. Thus, the microstructure of the ER suspension changes from a liquid-like to a solid-like form, as shown in Figure 1. Therefore, their physical and rheological behaviors, such as steady shear viscosity, shear stress, dynamic modulus, and yield stresses, and even stress relaxation changes according to the applied electric fields; however, these changes can be reversed by removing the field. Most ER fluids suit the Bingham fluid model, which illustrates that a fluid has its own yield stress, and the flow motion of the fluid impedes when an input external shear stress is lower than the yield stress [9]. ER fluids with this controllable and tunable phase change have a very good market prospect and wide applications, such as dampers [10], ER haptic devices [11], shock absorbers, microfluidics [12–14], clutches [15], ER finishing devices [16], and tactile displays [17].

ER fluids have been the subject of research and exploration since their discovery [18]. Furthermore, research to find fibrillation structures between two applied electrodes with an apparent increase of the viscosity of ER fluids have also been conducted. Klass and Martinek [19] introduced a dielectric principle, and a water-associated electrical double-layer model, to explain that the critical factor of the ER effect was the molecule in the wet-base ER fluids. The particles were polarized and distorted when the extra electric field was applied and then the water molecules made an attractive bridge between dispersed particles, thereby showing a higher surface tension.



**Figure 1.** Schematic diagram of electrorheological (ER) behavior with an external electrical field.

Furthermore, ER materials cover a wide range of organics and inorganics particles, including polymers and their organic or inorganic composites, with their particle size ranging from nanometers to micrometers with multiple shapes, such as spherical, fibrous, sheet, and rod-like [20,21].

In this study, we focus on anhydrous ER materials with various conducting polymers and their nanocomposites. Conducting polymers possess conjugated repeating units, which can be appraised by some important variables, such as ionization potential, electron affinity, band gap, and bandwidth [22–24]. By regulating these parameters the thermal, electrical, and optical properties of polymers can be easily enhanced [25,26]. In particular, the electrical conductivity and thermal or mechanical stability of nanocomposites, which have improved mechanical property, dispersion stability, or physical characteristics, and can be easily controlled by various fabrication techniques [27].

Sundry conducting polymers have been employed as ER materials, including polyaniline (PANI) [28,29], polypyrrole (PPy) [30], poly(*p*-phenylene) (PPP) [31–33], poly(3,4-ethylenedioxythiophene) (PEDOT) [34], and so on. This study focuses on the ER effect of conducting polymeric materials and their composites, including synthesis methods and typical characteristics, using various rheological measurement methods. Furthermore, according to the viscoelastic behaviors, such as flow curves, shear viscosity, yield stresses, and dynamic modulus, the ER effect of polymeric materials dispersion is compared under an external electrical field with various strengths.

## 2. Polymeric ER Materials

Oxides or non-oxide inorganic particles were introduced in ER fluids at the initial stages of study. However, the dispersion stability was poor because of the large density variation between the dispersed particles and the base oil. Therefore, organic particles gained attention, especially conducting polymers, which are provided with conjugated  $\pi$  bonds along with a high dielectric constant, thereby exhibiting excellent polarization capabilities under applied electric field strengths. Moreover, these types of polymers can be comprehensive as ER materials because they have highly polarizable functional groups on the molecular backbone.

### 2.1. PPy

Among the various conductive polymers, PPy is a research hotspot because of its high electrical conductivity, simple method of preparation [35], ease of surface modification [36], excellent environmental stability [37], and ion-exchange capacity [38]. In addition, it can be easily produced in a large quantity at a room temperature using various solvents, including water. PPy is an excellent conductive polymer; thus, it can be applied in ER fluids.

Kim and Song [33] dispersed PPy in mineral oil as an ER fluid and controlled the amount of oxidant and surfactant while synthesizing PPy particles to investigate the impacts on their ER effect. They observed that as the amount of oxidant and surfactant increases the yield stress increases at the initial stage but decreases after passing culmination because of the enhanced particle polarization. Using thermo-oxidative treatment in air, Xia et al. [39] tuned the electrical conductivity of the PPy nanofibers (shown in Figure 2b) to that of a nanofiber-based ER fluid. Its yield stress and shear modulus were stronger compared to those from a typical granular-shaped PPy based ER suspension, because the fiber-like particles can ameliorate the dispersion stability of the suspension. Many studies have been conducted on preparing conducting PPy-based composite materials to surmount the poor processability of PPy particles.

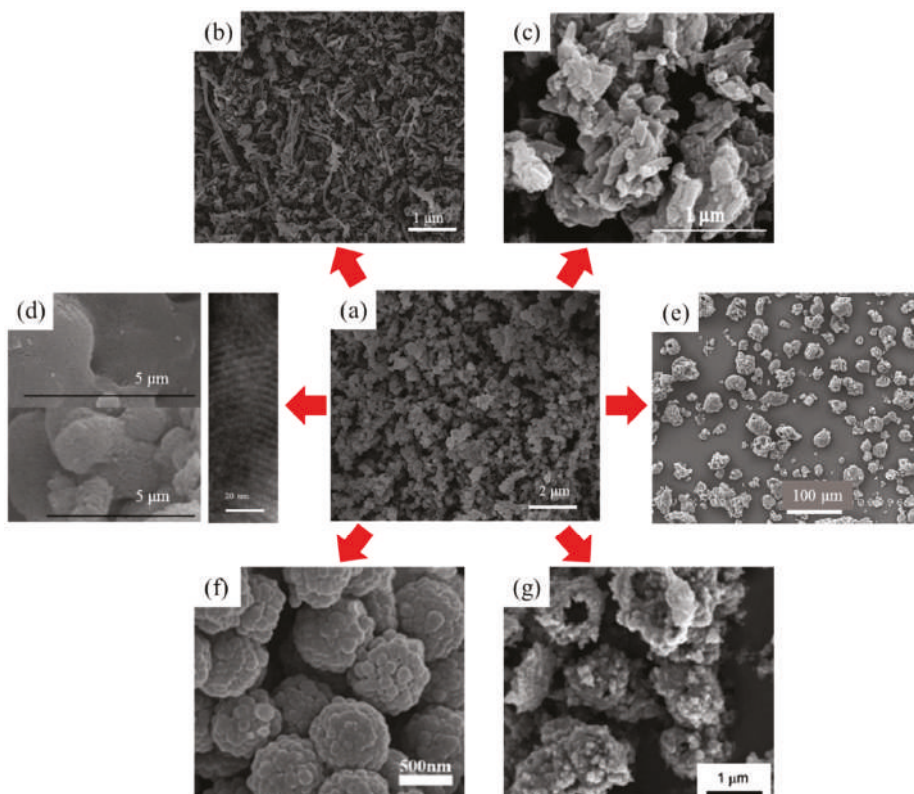
Moreover, while the morphology of the conducting polymers is well-known to be related to their synthetic conditions, such as temperature, pH, oxidant species, oxidant/monomer ratio, polymerization type, and so on, shape- or size-related morphology of the dispersed particles became an important parameter in the rheological output of ER fluids. Specifically, regarding the effect of a hierarchical form on ER properties, sea urchin-like conducting polymeric particles are known to exhibit both better sedimentation stability than those with a smooth surface and better rheological behaviors. They are expected to show larger drag forces and inter-particle frictions with their increased surface area compared to that of the smooth-surfaced particles. Therefore, an improved ER efficiency with larger yield stress can be expected in the same experimental conditions. Moreover, compared to the ER fluid of spherically shaped particles, the systems with fiber-like particles were also observed to exhibit better ER characteristics and enhanced suspension stability because the elongated shape possesses a bigger induced-dipole moment and greater inter-particle interaction.

Concurrently, clays are widely used to compose polymer composites not only because of their large specific surface area and good mechanical properties, but also because of their chemical, thermal, and dimensional stability and low cost [40]. Halloysite (HNT) is a type of clay mined from natural deposits and has a predominantly hollow tubular structure. HNT has been widely used in electronics, catalysis, separation, and functional materials because of its uniquely versatile surface features. Jang et al. [41] synthesized PPy/HNT nanocomposites (Figure 2c) and observed that adding HNT is an effective method to improve PPy conductivity and properties of PPy-based ER fluids. Furthermore, montmorillonite (MMT) clay is widely used in conducting polymer nanocomposites because their particle size is smaller than 10  $\mu\text{m}$  and they can be easily intercalated. Cations, such as  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , in its lamellar surface enable interactions with monomers ensuring reasonable conductivity of the associated electric signal. Following testing, Kim et al. [42] observed that PPy-dodecylbenzenesulfonic acid (DBSA)/clay nanocomposites have better insulation and thermal stability. PPy was also inserted into hexagonal channels of pore-expanded MCM-41 [43], whose insulating channel can improve the ER response of the PPy suspension (Figure 2d).

Not only clay, but also conventional polymers were introduced in conducting polymer/clay nanocomposites to overcome the limitation of poor processability, prepare conducting polymer-based composite materials, and improve the sedimentation of ER suspensions. Kim et al. [44] assembled PPy-polycaprolactone (PPy-PCL) composites (Figure 2e) to improve the mechanical and electrical properties of PPy, along with enhancing their ER response. Furthermore, Kim and Park [45] reported an ER response by coating PPy on polyethylene particles, thereby enhancing polarization by increasing the particle surface conductivity.

Moreover, compared to single-component counterparts, core-shell structured particles often exhibit better physical and chemical properties. A conducting shell can be polarized instantaneously; thus, its ER response can be improved. In addition, an insulating core or an insulating shell in the composite particles is designed to obtain a lower current density, by which the practicability and environmental protection of the materials can be improved [46,47]. Kim et al. [48] synthesized PPy-coated silica core-shell structured nanoparticles (Figure 2f), while Sedláčik et al. [49] synthesized titanium oxide ( $\text{TiO}_2$ )/PPy core-shell structured nanoparticles (Figure 2g) and analyzed the steady-state

and oscillatory flow behaviors. Both sets of results showed that the novel core-shell structure possessed predominant maneuverability and ER response.



**Figure 2.** SEM morphologies of polypyrrole (PPy) and its composites: (a) granular PPy and (b) PPy nanofibers, reprinted with permission from [39]. Copyright Elsevier, 2011; (c) PPy-HNT particles, reprinted with permission from [41]. Copyright Springer Nature, 2014; (d) PPy/MCM-41 particles, reprinted with permission from [43]. Copyright Elsevier, 2010; (e) PPy-polycaprolactone (PPy-PCL) composites, reprinted with permission from [44]. Copyright Springer Nature, 2007; and, core-shell structures of (f) PPy-silica, reprinted with permission from [48]. Copyright RSC Publishing, 2016 and (g) PPy-TiO<sub>2</sub>, reprinted with permission from [49]. Copyright Springer Nature, 2011.

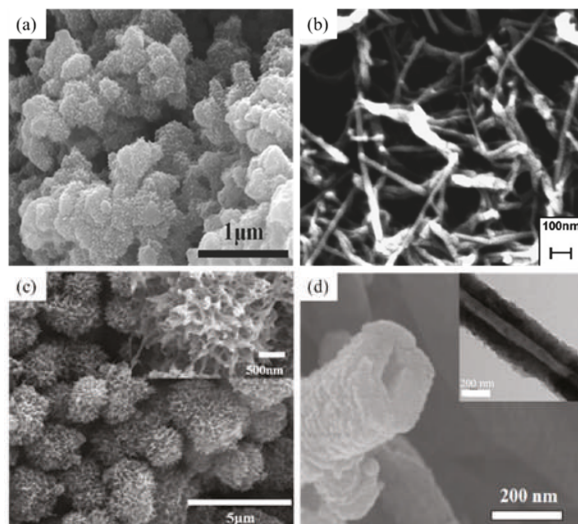
## 2.2. PANI

Among various whole conductive polymers, PANI is considered an essential candidate material for ER fluids because of its ease of synthesis, low price, chemical and thermal stabilities, and sensitive response to easily controllable electrical conductivity, which can be steered by a reversible doping/dedoping process [50]. Most morphologies of a synthesized PANI are irregular. However, using some methods, such as interfacial polymerization [51] and rapid mixing method [52], and using a surfactant or template [53] to direct PANI growth, we can obtain various shapes of fibers, tubes, spheres, and even urchin-like particles (shown in Figure 3).

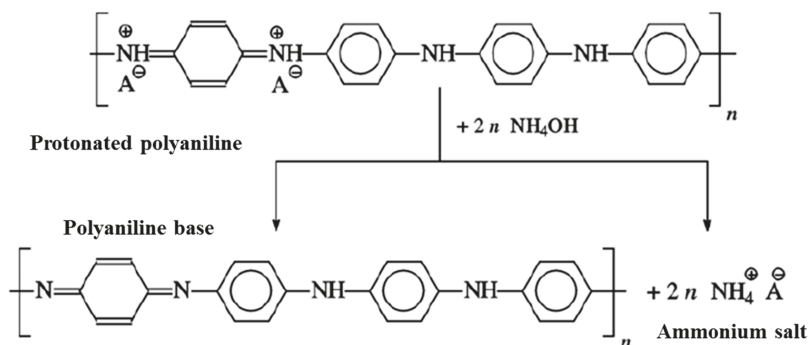
For ER fluid applications, the high conductivity of PANI is undesirable because of the high leakage currents passing through the suspension with the electric field [54]. Thus, the protonated PANI needs to be changed to a PANI base by treating it with an ammonium hydroxide solution. The electrical properties of the protonated PANI and its corresponding bases can be controlled at a molecular level



by partial protonation and acid selection of substitution of the PANI ring, or copolymerization of aniline with substituted aniline or other monomers, as shown in Scheme 1. In addition to the powder, PANI can be deposited on almost any object in the reaction mixture during aniline oxidation, or as a colloid when the reaction mixture contains a stabilizer.



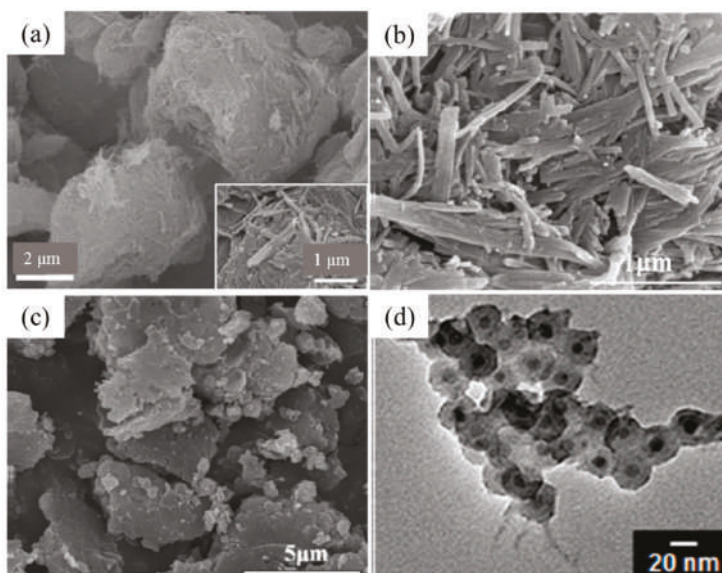
**Figure 3.** SEM images of synthesized polyaniline (PANI): (a) spheres, reprinted with permission from [55]; Copyright Spring Nature, 2013; (b) fibers, reprinted with permission from [52]. Copyright American Chemical Society, 2009; (c) urchin-like, reprinted with permission from [56]. Copyright John Wiley and Sons, 2009; and (d) nanotubes, reprinted with permission from [57]. Copyright American Chemical Society, 2006.



**Scheme 1.** Conductive protonated PANI is converted to a non-conducting PANI base by deprotonation with ammonium hydroxide.

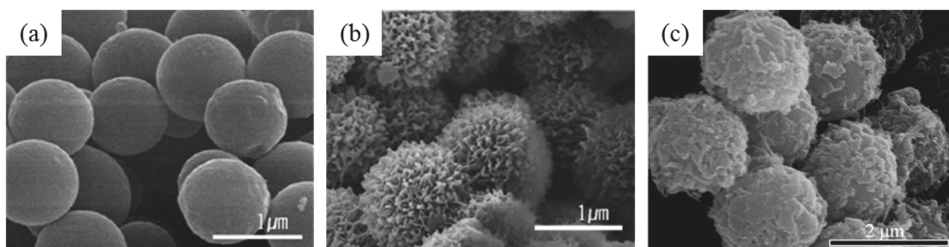
To improve the ER response of PANI particles and obtain an appropriate semiconducting regime for an ER application, multifarious inorganics, such as clays,  $\text{TiO}_2$ , silica, and  $\text{BaTiO}_3$ , and even magnetic materials, such as carbonyl iron or  $\text{Fe}_3\text{O}_4$  (shown in Figure 4) were introduced. Furthermore, some non-conducting core polymers form a core-shell structure with PANI as a shell (shown in Figure 5). For example, as one type of clay, attapulgite (ATP) or palygorskite, which is a kind of crystalline hydrated magnesium silicate with a fiber shape, has been introduced for PANI/clay composites. It not only possesses a large specific surface area but also cation exchange capacity and a reactive hydroxyl group on its surface [58]. Han et al. [59] synthesized PANI/ATP composite materials

and then estimated their ER responses, observing that they were a feasible and efficacious method for obtaining good mechanical strength, and easy processing of composite ER material. Furthermore, sepiolite (SPL), another species of natural silicate clay with a fibrous morphology that has a large surface area and porosity showing its mechanical reinforcing capability [60], has also been introduced. Owing to that, Jang and Choi [61] used SPL synthesizing with PANI to obtain a neoteric ER material. As a multi-functional physical cross-linker, laponite, a synthetic clay material, which was adopted to stabilize emulsions because of its uniform and small dimensions, can lead to non-covalent cross-linking on the clay/polymer interface [62]. Jun et al. [63] combined PANI and laponite confirming that this composite displayed a typical ER behavior. Noh et al. [64] reported neoteric  $\text{Fe}_3\text{O}_4/\text{SiO}_2/\text{PANI}$  nanoparticles, which can be applied in both ER and magnetorheological (MR) fluids. In addition, they observed that the ER and MR responses were significantly improved under both electric and magnetic fields.



**Figure 4.** SEM and TEM photos of: (a) PANI/ATP, reprinted with permission from [59]. Copyright Elsevier, 2017; (b) PANI/SPL, reprinted with permission from [61]. Copyright Elsevier, 2015; (c) PANI/laponite, reprinted with permission from [63]. Copyright Elsevier, 2015; and (d)  $\text{Fe}_3\text{O}_4/\text{SiO}_2/\text{PANI}$  nanoparticles, reprinted with permission from [64]. Copyright Royal Society of Chemistry, 2017.

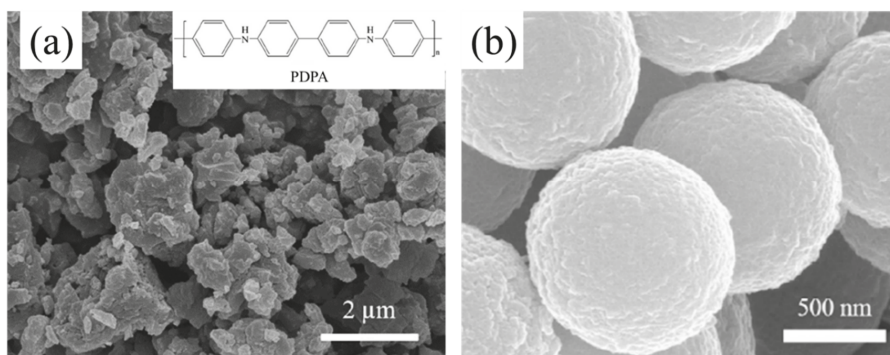
Moreover, PANI has also been applied comprehensively with various polymeric shells. It is not sufficiently sophisticated to form a PS/PANI nanosphere, due to  $\pi$ - $\pi$  stacking attractive interactions between an aniline monomer and sulfonating polystyrene (PS) using concentrated sulfuric acid. Kim et al. [65] compared two types of PS/PANI-based ER fluids whose PANI shell was either smooth or urchin-like. In Lee et al.'s work [66], the glycidyl methacrylate (GMA) was used to graft an aniline monomer with poly(methyl methacrylate) PMMA to form a PANI/PMMA core-shell structure. However, in Zhang et al.'s report [67], the core-shell PGMA/PANI composites were synthesized and applied in ER fluids.



**Figure 5.** SEM images of: (a) smooth PANI@PS and (b) urchin-like PANI@PS, reprinted with permission from [65]. Copyright RSC Publishing, 2015; and (c) PGMA/PANI composite, reprinted with permission from [67]. Copyright Elsevier, 2013.

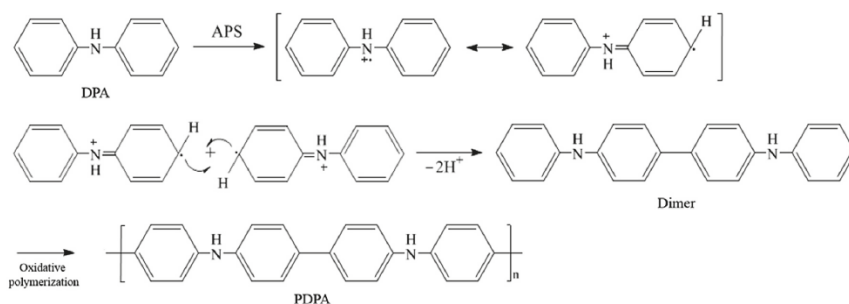
### 2.3. Other Conducting Polymers

In addition to either PPy or PANI, various other conducting polymers and their derivatives were applied in ER fluids. Although PANI has its own advantages in terms of controllable conductivity, environmental stability, and simple synthesis, it also exhibits limitations, such as poor solubility in various organic solvents. To overcome these defects, various derivatives were introduced [68]. Poly(diphenylamine) (PDPA) as a derivative of PANI, possesses not only a relatively low conductivity, which helps avoid cause short-circuiting and can be directly applied in an ER fluid, but also produces improved solubility in organic solvents with better thermal stability [69]. Kim et al. [70,71] at first synthesized normal PDPA nanoparticles for an ER fluid, and then further fabricated a novel core-shell-type PDPA/PS structure (shown in Figure 6), thereby observing that both these materials were suitable for ER fluids.



**Figure 6.** SEM images of (a) Poly(diphenylamine) (PDPA), reprinted with permission from [70]. Copyright Elsevier, 2017 and (b) PDPA/PS core/shell structure, reprinted with permission from [71]. Copyright Elsevier, 2017.

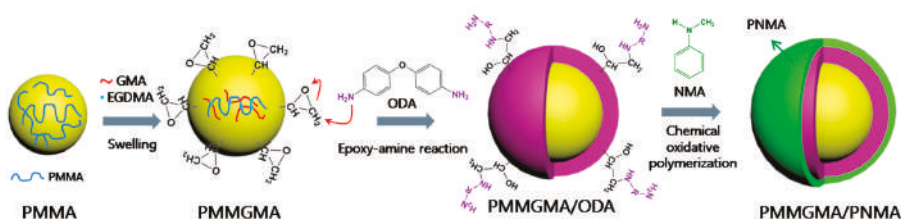
The chemical formula of a *p*-phenylenediamine (PDA) is similar that of an aniline. It inspires its ER application with its polymeric form [72] along with its versatile synthetic methods to prepare poly(*p*-phenylenediamine) (PPDA), whose schematic diagram is shown in Scheme 2) particles as compared to PANI. Plachy et al. [73] tried to modify PPDA by heat treatment to achieve appropriate conductivity; however, they observed that the ER efficiency decreased as the carbonization temperature increased. Cao et al. [74] fabricated poly(*p*-phenylenediamine)/graphene oxide (PPDA/GO) composite particles, and observed that both PPDA/GO particles and PPDA-based ER fluids exhibited significant ER characteristics.



**Scheme 2.** Schematic diagram of PDPA synthesis via an oxidative polymerization.

It can be also noted that by introducing proper substituents on polymeric backbones, functionalized PANI could be fabricated, providing extra tuning capability of their chemical and electrical characteristics [75,76].

Recently, poly(*N*-methylaniline), as a derivative of PANI, has piqued the interest of many researchers as, compared to PANI, it possesses much lower electrical conductivity. Thereby, it could be directly applied to the ER fluid without further treatment. Moon et al. [77] used a simple method to fabricate a core-shell typed poly(methyl methacrylate)/poly(*N*-methylaniline) (PMMA-PNMA). Figure 7 shows the synthetic fabrication of the PMMA-PNMA particles. Initially, the surface of the PMMA microspheres, which were fabricated through a dispersion polymerization process, was treated with glycidyl methacrylate, ethylene glycol dimethacrylate, and oxydianiline as swelling, crosslinking, and chemical-grafting agents, respectively. Further, the PNMA shell was coated by chemical oxidation polymerization. Their ER suspension based on the PMMA-PNMA particles demonstrated conventional ER behaviors in both simple shear flow and dynamic oscillation measurements for several different electric field strengths applied.

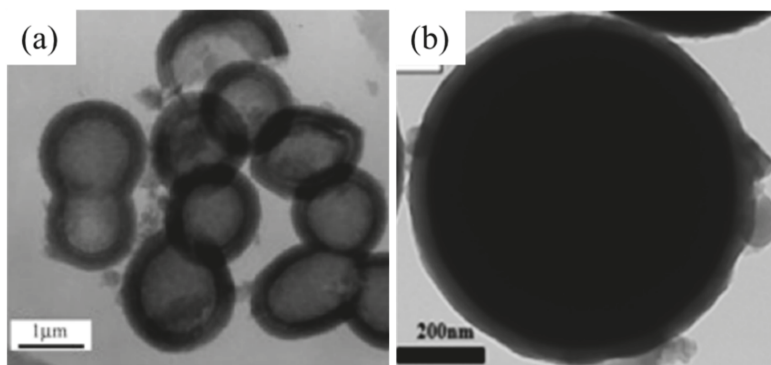


**Figure 7.** Schematic diagram of fabrication of poly(methyl methacrylate)/poly(*N*-methylaniline) (PMMA–PNMA) core-shell particles, reprinted with permission from [77]. Copyright Elsevier, 2015.

Polyindole (PIN) has attracted huge attention due to its good thermal stability and high redox activity [78]. Indole is composed of a benzene ring connected to the heterocyclic pyrrolic ring; thus, it was anticipated that it would show the properties of both PPy and PPP. Park et al. [79] synthesized emulsion-polymerized PIN nanoparticles and used these as an ER material. Sari et al. [80] synthesized PIN/polyethylene composites and observed that they were thermally more stable than PIN, and the ER suspensions was observed to be increasing with increases in the electric field.

Poly(*O*-anisidine) (POA), with a methoxy functional group ( $-\text{OCH}_3$ ) at an ortho position, lowers the mobility of electric charge carriers following the polymeric backbone, thereby lowering the electrical conductivity. Recently, POA was adopted as an ER material. Cheng et al. [81] used chemical oxidation polymerization to synthesize hollow POA microspheres with 1  $\mu\text{m}$  diameter in a sphere-like form. The hollow POA particles had low density, which is an advantage for a high-stability dispersed phase ER fluid. Furthermore, Lee et al. [82] fabricated a core-shell structure of POA-silica particles

using *N*-[(3-trimethoxysilyl)-propyl] aniline (PTMSPA) grafting agent and defined their ER properties (as shown in Figure 8).



**Figure 8.** TEM images of (a) hollow poly(*O*-anisidine) (POA), reprinted with permission from [81]. Copyright IOP Publishing, 2011 and (b) POA/silica core/shell structure, reprinted with permission from [82]. Copyright IOP Publishing, 2017.

Polyacene quinone radicals (PAQRs) are also a type of semi-conductive polymers, which possess the *p*-conjugated structure. They have been widely used and studied because of their better thermal stability, environmental stability, and the characteristic that their conductivity can be controlled by polymerization conditions (such as temperature, acenes, and anhydrides). Wang et al. [83] synthesized hollow PAQR sub-microspheres by a modified solvo-thermal method and then observed that their ER fluid exhibited conventional ER properties. Poly(naphthalene quinone) (PNQR) has a similar chemical *p*-conjugated structure as PAQR, and so also has an electrical conductivity and can be applied as an ER fluid material. Cho et al. [84] synthesized PNQR from the Friedel–Craft acylation and its flow behaviors when suspended in the silicone oil were analyzed using rheological equations of state from Bingham, De Kee-Turcotte, and Cho-Choi-Jhon (CCJ) equations.

Meanwhile poly(3,4-ethylenedioxythiophene)(PEDOT):poly-(styrenesulfonic acid) as a derivative of polythiophene, has attracted considerable attention because of its several advantages as a conducting polymer, such as processability in aqueous solutions, and better thermal and structural stability [85]. An et al. [86] fabricated core/shell typed polystyrene (PS)/PEDOT microspheres and reported their ER behaviors under an electrical field. On the other hand, Erol et al. [87] synthesized core/shell typed nano-rods with a titania core and conducting polymeric PEDOT shell. They were fabricated via covalent bonding to obtain a thin polymeric shell, making the interfacial interactions between the two components stronger. They found that the nanorod-shaped TiO<sub>2</sub>/PEDOT particles exhibited improved ER performance and storage moduli, as well as larger creep-recovery after stress loading, when compared to their particulate morphology.

Furthermore, to solve problems such as high conductivity and low thermal stability of PANI, aniline is often copolymerized with other conductive polymer monomers to form a more functional conductive polymer. Cho et al. [88] synthesized poly(aniline-*co*-pyrrole) (PAPP) particles (aniline:pyrrole = 1:1) via a chemical oxidation polymerization technique. It was proved that the copolymer has better thermal stability and more suitable conductivity than PANI as the dispersed phase in ER fluid. Pandey et al. [89] fabricated poly(aniline-*co*-*O*-anisidine) with a lower value of electrical conductivity by explaining the incorporated *O*-anisidine moieties in PANI backbones. Trlica et al. [90] also reported a poly(aniline-*co*-1,4-phenylenediamine) particles-based ER suspension with an applied external electric field, along with electrical characteristics of an ER suspension.



## 2.4. Composites

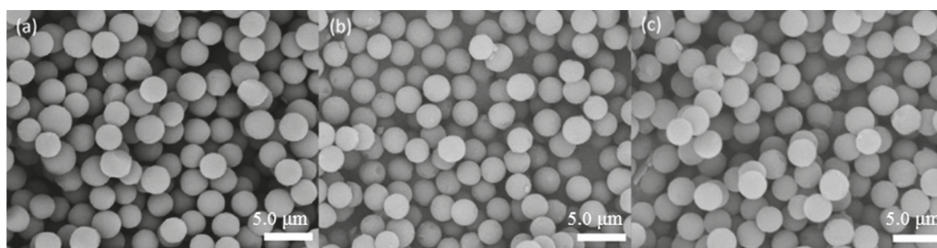
Various inorganic-conducting polymers have been introduced for their ER applications, and among these, recently graphene has attracted great interest in the fields of capacitors, electronic devices, sensors, and composites due to its unique structure and electrical characteristics, even in ER fluids [91]. Although graphene oxide (GO) which is the oxidized form of graphene has many defects during its preparation process, it contains functional components such as an hydroxyl group, epoxy group, and carboxyl group, making it more easily dispersed in water [92]. By simply compounding with other polymers, it can be modified to have multiple functions. Zhang et al. [93] synthesized PANI/GO composites by an in-situ oxidation polymerization method with a stable GO and reported its composite particle-based ER suspension showed a general ER property. On the other hand, Min et al. [94] fabricated core-shell typed PMMA/GO microspheres via a Pickering emulsion polymerization method with the GO as the solid surfactant, and studied their ER response. Cao et al. [74] synthesized poly(*p*-phenylenediamine)(PPDA)/GO composite particles via an in-situ oxidation polymerization technique of *p*-phenylenediamine in GO suspension, observing improved typical ER behavior when compared to PPDA particle-based ER fluid.

Furthermore, carbon nanotubes as another candidate for ER fluids have received a lot of consideration [95–97], although compared with single-walled carbon nanotubes (SWNT), multi-walled carbon nanotubes (MWNT) have been more widely used in the ER field due to their lower electrical conductivity. Jin et al. [98] produced MWNT-adsorbed PS and PMMA particles using different surfactants, such as anionic sodium dodecyl sulfate and sodium dodecylbenzene sulfonate, and cationic cetyl trimethylammonium bromide. Not only conductive carbon materials, but also many metal oxides, can be compounded with conductive polymers to form multifunctional materials showing remarkable ER effects. Fang et al. [99] synthesized magnetic nanoparticles ( $\text{Fe}_3\text{O}_4$ ) together with a conductive PPy using a simple fabrication method, and applied it to both ER and MR fluids.

## 2.5. Poly(Ionic Liquid)s

Poly(ionic liquid)s (PILs), initially evaluated as ion conductive polymers, refer to a new type of polymers in which ionic liquids (ILs) are linked through polymeric backbones to form macromolecular structures. They have attracted huge scientific interest not only because they have the suitable electrical conductivity and thermal stability from the ILs, but also because, like polymers, they have the advantages of good solid morphology, simple processing and good mechanical properties [100,101]. To activate the ER effect, classical polyelectrolytes need to adsorb a tiny amount of water to boost the local ion movement which will cause chemical corrosion, dielectric breakdown, and limited operation at higher temperatures. Nevertheless, because of fluorine counter-ions including tetrafluoroborate ( $\text{BF}_4^-$ ), hexafluorophosphate ( $\text{PF}_6^-$ ), and fluorinated imide ( $(\text{CnF}_{2n+1}\text{SO}_2)_2\text{N}^-$ ), PILs are generally organophilic and insoluble in water. Using a microwave-assisted dispersion polymerization technique, Dong et al. [102] synthesized mono-dispersed PIL particles possessing a styrenic backbone and different sizes of cationic/anionic parts (shown in Figure 9) and reported their enhanced anhydrous ER characteristics. Then they further synthesized several mono-dispersed poly[*p*-vinylbenzyl trimethylammonium] PIL microspheres possessing a similar diameter but different counter-anions. This showed that PILs with a smaller volume or counter-ion size exhibited higher ER behavior compared to PILs possessing organic counter-ions or inorganic counter-ions alone [103].



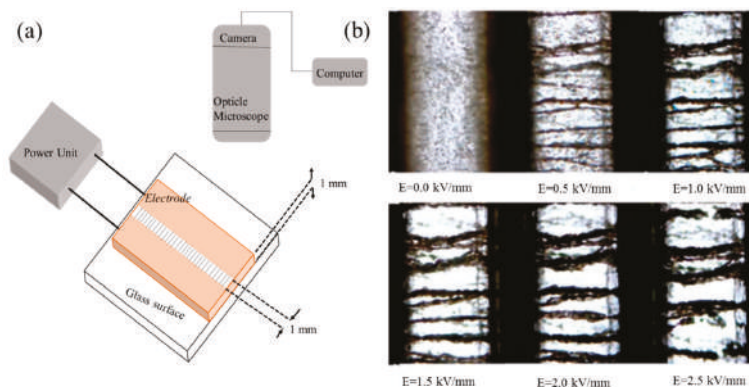


**Figure 9.** SEM photos of poly(ionic liquid)s (PIL) particles: (a) poly((*p*-vinylbenzyl trimethylammonium) (VTMA)<sup>+</sup>CF<sub>3</sub>SO<sub>3</sub>); (b) poly(VBTMA<sup>+</sup> (CF<sub>3</sub>SO<sub>2</sub>)<sub>2</sub>N<sup>−</sup>); and (c) poly((*p*-vinylbenzyltriethylammonium bis(trifluoromethane sulfonimide) (VBTEA)<sup>+</sup> (CF<sub>3</sub>SO<sub>2</sub>)<sub>2</sub>N<sup>−</sup>), reprinted with permission from [102]. Copyright Elsevier, 2016.

### 3. ER Characteristics

#### 3.1. Direct Observation

The electrical stimuli response characteristics of ER fluids can be directly detected by an optical microscope (OM) with an external electric field [84]. The polarization measuring device is presented in Figure 10a. The ER suspension was placed in the center of a transparent substrate connected to a DC high voltage power supply, and micro-structural changes were captured by the OM. Sever and Unal [104] prepared an ER fluid by dispersing nanocube-TiO<sub>2</sub>/poly(3-octylthiophene) in silicone oil and observing its polarizability and potential ER performance with this OM device technique. The particles were suspended randomly similar to a liquid state when  $E = 0$  kV/mm, while they were electrically polarized and migrated to organize a columnar structure parallel to a field direction when  $E \neq 0$  kV/mm (Figure 10b). The formation of this chain like-columnar structure caused ER fluid to exhibit a solid-like behavior, which was attributed to the polarization of the particles [8]. Furthermore, this columnar structure was found to be stronger and thicker, in accordance with the increment in applied  $E$  range from 0.5–2.5 kV/mm and could be maintained as long as an external electrical field applied, indicating that the increased  $E$  caused a tighter columnar structure. These results are consistent with OM studies carried out for various ER fluid materials [105–107].



**Figure 10.** (a) Schematic illustration of polarizability measuring device; (b) optical microscope (OM) images showing the polarization of nanocube-TiO<sub>2</sub>/poly(3-octylthiophene)-based ER fluid, reprinted with permission from [104]. Copyright John Wiley and Sons, 2016.

### 3.2. Flow Curve Behaviors

Stable steady shear flow curves in ER fluids can be established via a typical rotation rheometer test, like the controlled shear rate (CSR) method, which provides a basis for observing the yield stress of ER suspensions and electrical stimulus response behavior [21,108]. The flow curve of ER fluid produced from this CSR method exhibits shear stress ( $\tau$ ) and shear viscosity ( $\eta$ ) as a function of shear rate ( $\dot{\gamma}$ ).

In general, two types of interactions occur between dispersed ER particles in a steady shear flow; the electrostatic interaction caused by an external electrical field and a hydrodynamic interaction induced by a steady shearing flow [109]. The shear flow field results in shear deformation of the chain structures formed under an electric field; however, the broken structure tends to rebuild the chain simultaneously under an applied external electrical field. These phenomena of destruction and reorganization depend on the applied shear stresses and the electric field strengths. At a low shear rate regime, the electro-static attractive interaction becomes prominent, allowing the chain structure to reduce the effect from shear deformation [110]. As a shear rate increases, the breaking rate exceeds the recombination rate, so that the chain structure will eventually be destroyed and will exhibit a liquid-like behavior. However, a stronger electric field can result in a stronger electrostatic interaction which is sufficient to resist shear deformation, allowing the solid-like behavior of the ER fluid to re-emerge.

Based on the electrical stimuli-response behaviors of ER fluids in a shear flow field, many models have been introduced to qualitatively explain the flow curve. The Bingham model (Equation (1)), the simplest equation with two parameters, has been extensively employed as a viscoelastic equation to fit steady shear behaviors exhibited in ER fluids along with various suspensions and MR fluids [111]:

$$\begin{aligned} \tau &= \tau_y + \eta \dot{\gamma} & \tau &\geq \tau_y \\ \dot{\gamma} &= 0 & \tau &< \tau_y \end{aligned} \quad (1)$$

In Equation (1),  $\dot{\gamma}$  is a shear rate;  $\eta$  is a shear viscosity;  $\tau$  and  $\tau_y$  are shear and yield stresses, respectively. Piao et al. [112] explained shear stress behaviors of PS/PANI-based ER suspension using this Bingham fluid model, in which the flow curve, as shown in Figure 11a, was fitted by this model quite well. Its shear stress was observed to increase linearly with increased shear rate when  $E = 0$  kV/mm, which is similar to that of Newtonian fluids. Under an applied electrical field, the ER suspension demonstrated increments in shear stresses and exhibited a plateau behavior because of the formation of the columnar structure.

However, in the case of Figure 11b [59], shear stresses of the PANI@ATP-based ER suspension presented a weak concave shape in a low shear rate area, passed through a minimum value, and once again increased. This behavior could be explained by the fact that the reconstituted structure is not as complete as the structure formed before the steady shearing flow applied. Thus, they adopted the Cho-Choi-Jhon (CCJ) equation [84], which is an empirical formula with six parameters to express the ER characteristics in a whole shear rate range. This CCJ equation is given as follows:

$$\tau = \frac{\tau_y}{1 + (t_1 \dot{\gamma})^\alpha} + \eta_\infty \left( 1 + \frac{1}{(t_2 \dot{\gamma})^\beta} \right) \dot{\gamma} \quad (2)$$

The first term corresponds to the decrease in the shear stress at the low shear rate area, and the next term depicts a shear-thinning property at the high shear rate area. The  $t_1$  and  $t_2$  parameters are time constant, and  $\eta_\infty$  is a shear viscosity at the infinite shear rate. The exponent  $\alpha$  represents the shear stress decrease, and  $b$  is located between 0 and 1. This model was observed to be a good indication of the overall behavior of various ER fluids [61,87,113], including the reduced shear stress phenomenon.

In addition, the De Kee-Turcotte equation (Equation (3)) and Herschel-Bulkley equation (Equation (4)), as a modified version of the Bingham equation, have also been applied for various ER suspensions [114,115]:

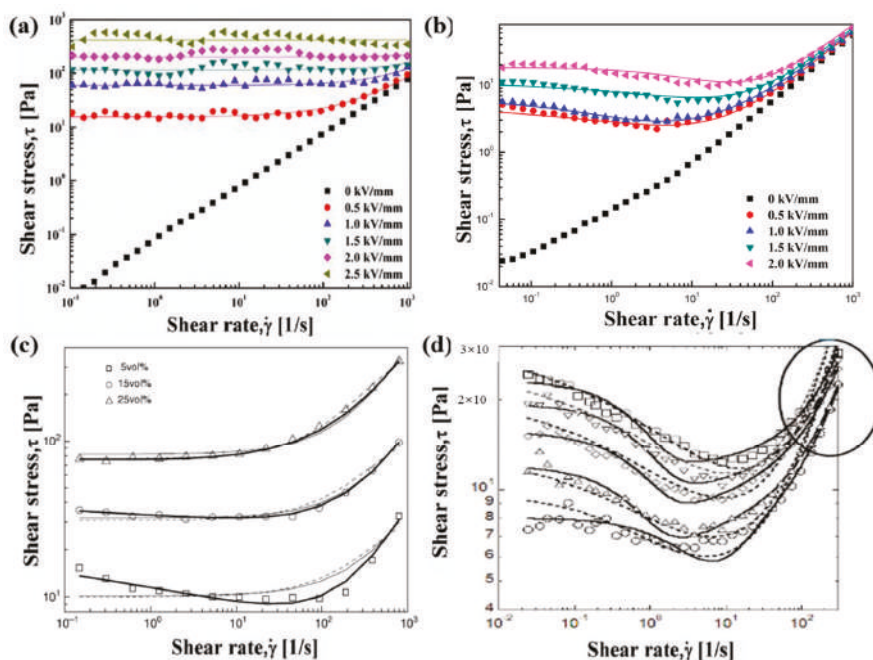
$$\tau = \tau_0 + \eta_1 \dot{\gamma} e^{-t_1 \dot{\gamma}} \quad (3)$$

$$\tau = \tau_0 + m \dot{\gamma}^n \quad (4)$$

where  $t_1$  is a time constant,  $m$  is a consistency index, and  $n$  is a flow index. Cho et al. [84] constructed the flow curve of a poly(naphthalene quinone)-based ER suspension for different particle concentration and electric field strengths, and interpreted its steady-state flow properties using three different equations from the Bingham, De Kee-Turcotte, and CCJ models. As presented in Figure 11c, the CCJ equation fitted the decrement of shear stress better for a shear rate range from 0.01–100 s<sup>−1</sup>; however, significant deviation was found at a low shear rate region compared to other two equations, especially for a lower volume fraction of poly(naphthalene quinone)-based ER fluid.

Based on the above models, Seo and colleagues [116] proposed a new rheological model (Equation (5)) that can depict the flow behavior of the ER suspensions over the full range of shear rates with four parameters.

$$\tau = \tau_{sy} \left( 1 - \frac{(1 - \exp(-a\dot{\gamma}))}{(1 + (a\dot{\gamma})^\alpha)} \right) + \eta \dot{\gamma} \quad (5)$$



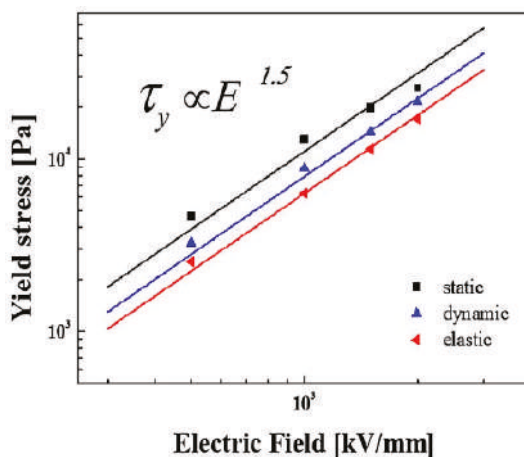
**Figure 11.** Shear stress vs. shear rate for: (a) PS/PANI-based ER suspension (solid lines for Bingham equation), reprinted with permission from [112]. Copyright Elsevier, 2017; (b) PANI@ATP-based ER suspension (solid lines for CCJ equation), reprinted with permission from [59]. Copyright Elsevier, 2017; (c) poly(naphthalene quinone)-based ER fluid (dashed lines for Bingham equation, solid lines for De Kee-Turcotte equation, and bold solid lines for CCJ equation), reprinted with permission from [84]. Copyright American Chemical Society, 2012; and (d) PMMA/PANI-based ER fluid (dotted and solid lines for CCJ equation and four-parameter equation), reprinted with permission from [117]. Copyright Elsevier, 2005.

This four-parameter model was introduced to predict static yield stress, while the CCJ and Bingham models were based on dynamic yield stress. In Equation (5),  $a$  is a time constant,  $\tau_{sy}$  represents a static yield stress, and  $\alpha$  is a power-law index relating to the shear thinning. In Figure 11d, Seo and Seo [117] analyzed the flow curves of PMMA/PANI-based ER suspension using the CCJ equation and their proposed equation. At a low shear rate regime, the shear stresses predicted by the new model converged to yield stress, while the prediction of the CCJ model continued to increase as the shear rate decreased. Moreover, at the high shear rate area, the prediction of the CCJ equation deviated from the experimental data, while the new equation prediction was considered to match the experimental data better.

### 3.3. Yield Stress

Yield stress, as a key parameter of rheological properties in suspensions, can provide information about the performance of the ER fluids depending on electrical field strength, volume fraction, and dielectric properties of the particles. Many test methodologies have been applied to study yield stress characteristics, corresponding to different yield stress values including dynamic, elastic, and static yield stresses [118].

As shown in Figure 12, Lim et al. [119] studied dynamic, static, and elastic yield stresses vs. electrical field strength for a PANI-nanotube-based ER suspension, estimated from controlled shear rate, controlled shear stress, and dynamic oscillatory frequency tests, respectively. The results showed that the static yield stress had the highest value because the static state that included more aggregate without shear rate control, as compared to the ER fluid under the dynamic condition. The elastic yield stress is obtained from the maximum shear stress required to achieve full recovery after the shear stress is removed, which is the lowest. Nonetheless, these three types of yield stress as a function of electric field strength presented equivalent behaviors.

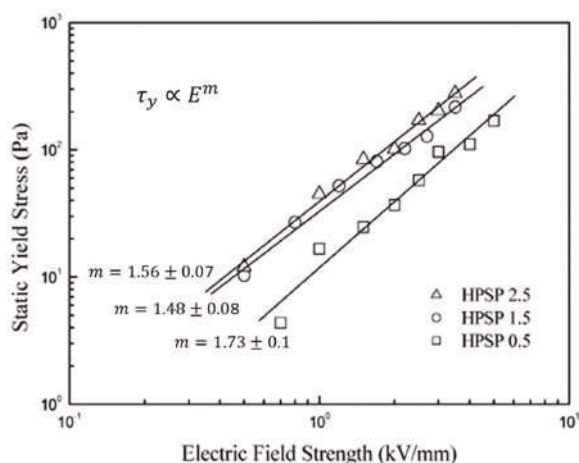


**Figure 12.** Static, dynamic, and elastic yield stresses as a function of electrical field strength for PANI-nanotube-based ER suspension, reprinted with permission from [119]. Copyright Elsevier, 2017.

As mentioned above, the electric field strength dependent yield stress has been the focus of many studies on ER characteristics. Klingenberg et al. [6] introduced an electrostatic polarization model and calculated the functional relation between yield stress and electrical field strength as follows:

$$\tau_0 = 18\varphi\epsilon_0\epsilon_c\beta^2E_0^2f_m \left[ 1 - \frac{(\pi/6)^{1/2}}{(L/a) \tan \theta_m \varnothing^{1/2}} \right] \quad (6)$$

where  $\varphi$  represents a volume fraction and  $L$  represents the electrode separation.  $f_m$  and  $\theta_m$  are the maximum dimensionless restoring force and angle, respectively. This expression means that the dynamic yield stress is essentially proportional to the squared electrical field strength. However, although some experiments confirmed this result [120,121], the effects of the particular material, concentration, shape, and electric field strength will cause the index to deviate from 2.0 [122,123]. As shown in Figure 13, Sung et al. [124] observed that the yield stress of a highly substituted potato starch phosphate (HPSP)-based ER fluid was not proportional to  $E^2$ , which deviated from the polarization model because of the non-spherical shape of HPSP.



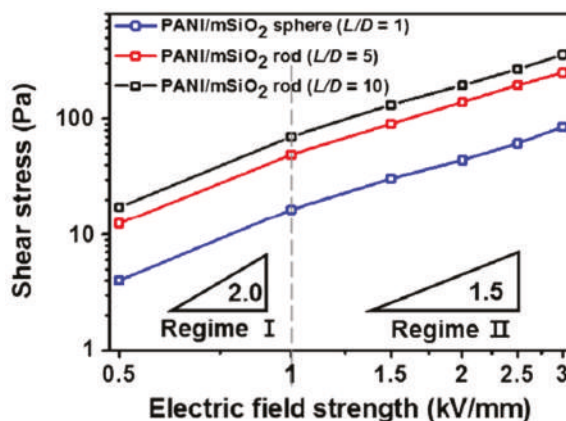
**Figure 13.** Yield stress vs. electrical field strength for highly substituted potato starch phosphate (HPSP)-based ER suspension with different molar ratios, reprinted with permission from [124]. Copyright American Chemical Society, 2005.

Concurrently for conducting suspensions under a high electric field strength, the ER characteristic would be affected by a conductivity mismatch and an interaction between dispersed particles and dispersing oil, rather than by their dielectric constant mismatch. Davis et al. [125] investigated the effect of field-dependent fluid conductivity and proposed that the yield stress appeared to approach  $E^{3/2}$  under high electric field strength (consistent with Figure 12) [126].

Moreover, as shown in Figure 14, the yield stresses of PANI/mSiO<sub>2</sub>-based ER suspensions can be separated into two different areas in the entire electric field strength ranges [127]. Choi et al. [128] proposed a critical electrical field strength ( $E_c$ ) and developed the following simplified equation to represent a hybrid yield stress for a wide electrical field strength area:

$$\tau_y = \alpha E^2 \left( \frac{\tanh \sqrt{E/E_c}}{\sqrt{E/E_c}} \right) \quad (7)$$

$E_c$  could be obtained from an intersection between two power-law slopes for a low and a high electrical field area. The parameter  $\alpha$  is dependent on a particle volume concentration and a dielectric constant of the ER suspension. Equation (7) contains two limiting behaviors of yield stress:  $E^2$  for  $E \ll E_c$  and  $E^{1.5}$  for  $E \gg E_c$ . As presented in Figure 14, the ER characteristics of PANI/mSiO<sub>2</sub> corresponded to the non-linear conductivity model where the indices are 2 below  $E_c$  and 1.5 above  $E_c$ . Many other studies have proved the rationality of this equation [129,130].

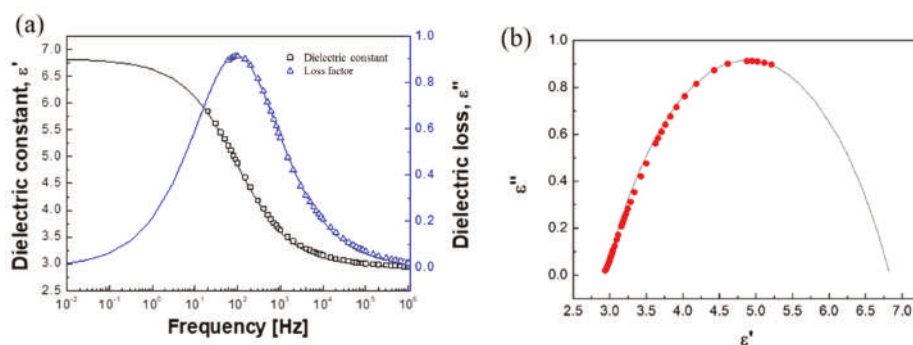


**Figure 14.** Yield stress of PANI/mSiO<sub>2</sub>-based ER suspension as a function of electrical field strength, reprinted with permission from [127]. Copyright Elsevier, 2016.

#### 4. Dielectric Analysis

A high dielectric constant shows a useful effect on the ER response because of the more effectively achievable polarizability. In general, the dielectric property is measured by an inductance capacitance resistance (LCR) tester. Figure 15 represents both the dielectric spectrum and the Cole–Cole curve of POA-silica composites [82]. The permittivity is frequency-dependent and can be expressed in its complex form involving permittivity ( $\epsilon'$ ) and the loss factor ( $\epsilon''$ ):  $\epsilon^* = \epsilon' + i\epsilon''$ . The polarizability is described as the permittivity difference of  $\Delta\epsilon = \epsilon_0 - \epsilon_\infty$  and relaxation time ( $\lambda = \frac{1}{2\pi f_{max}}$ ), which are obtained by fitting the dielectric spectra with the Cole–Cole formula.

$$\epsilon^* = \epsilon' + i\epsilon'' = \epsilon_\infty + \frac{\Delta\epsilon}{(1 + i\omega\lambda)^{1-\alpha}} \quad (8)$$



**Figure 15.** (a) Dielectric spectrum and (b) Cole–Cole plot of silica/POA based ER suspension, reprinted with permission from [82]. Copyright IOP Publishing, 2017.

In Equation (8),  $\omega$  is a frequency and  $(1 - \alpha)$  expresses the relaxation time distribution for a whole frequency area. It has been observed that a large ER efficiency is related to a large  $\Delta\epsilon$  and a dielectric loss peak is placed between  $10^2$  and  $10^5$  Hz. When  $\alpha$  is zero, Equation (8) is reduced to the Debye's single relaxation time model. In Dong's report [103], Equation (8) was used to compare different sizes of poly[*p*-vinylbenzyl trimethylammonium] based PILs possessing a similar diameter but different counter-anions. The results are shown in Table 1, with the counteranions of TfO,  $\Delta\epsilon$  having the largest value. Furthermore, in the subsequent ER response experiment, the PILs with TfO also exhibited the



best ER effect, thereby showing that PILs with a smaller volume or size of counter-ions demonstrated a higher ER response.

**Table 1.** Dielectric properties of PIL suspensions [103].

Sample	$\epsilon_0$	$\Delta\epsilon$	$\lambda$
P[VBtMA][PF <sub>6</sub> ]	4.70	1.70	0.002
P[VBtMA][BF <sub>4</sub> ]	5.01	1.88	0.0061
P[VBtMA][TFSI]	5.14	1.96	0.0009
P[VBtMA][TfO]	5.11	2.01	0.00049

## 5. Engineering Applications

With yield stress generated under external electrical field strengths applied, ER fluids have the advantages of reversible continuous control and fast response and so have been widely adopted in vibration control, tactile display, sensor, and other applications for different modes. In a flow mode, two electrodes are set, and the vibrational control is realized by tuning the fluid motion between the two electrodes. In a shear mode, one electrode rotates freely relative to the other, thereby controlling the vibration by tuning the shear force. In a squeeze mode, the electrode gap becomes larger and the ER suspension is extruded by the normal force.

Further detailed applications using ER fluids include the torsional vibration clutch, brake, damper, optical material polishing, tactile devices, and so on. The controllable ER characteristics can be used as power transfer agents with functions similar to friction in conventional clutches; thus, much research has been devoted to the application of this special object in automotive clutches. Madeja et al. [131] investigated the practical ranges of a hydrodynamic clutch by applying an electric field. To control braking force distribution, Choi et al. [132] introduced a vehicle ER valve-based anti-block brake system (ABS). Until now, ER fluids have relatively low yield stress under 8 kPa; thus, they are not used as hydraulic actuators. However, with the development of giant ER fluids [133], because the yield stress of the ER actuator can reach 130 kPa, it is expected to produce ER actuator.

An ER fluid can be precisely controlled by tuning the electrical field intensity; thus, it can also be used in polishing the tactile devices of optical materials. Kuriyagawa et al. [16] used an ER solution for the first time in the fabrication of optical lenses. Compared with MR polishing, ER polishing has the advantages of a uniform electric field and simple electrode structure. Su et al. [134] used an ER fluid to fabricate wheeled integrated electrodes for micro-optical components.

Moreover, Taylor et al. [135] developed a force display device using a 1-degree of freedom (1-DOF) ER brake. Recently, Han et al. [136] reported a 4-DOF haptic device using ER fluids for a minimally invasive surgery.

## 6. Conclusions

In this study, we discussed various modified and unmodified conductive polymers, their composites with special structural properties (fiber, core-shell spherical or non-spherical particles, and so on), and their ER responses. Considerable progress has been made in the introduction of new and synthetic methods, and in their corresponding ER characteristics. ER materials with ideal chemical and physical characteristics can be prepared by interfacial oxidation, micro-emulsion, Pickering emulsion, polymer grafting, the co-precipitation hydrothermal method, the sol-gel method, and other methods. Among these, the study of PANI and PPY is the most extensive and comprehensive, not only in terms of the morphology and properties of the polymers themselves, but also in terms of the results of their combination with other organic or inorganic compounds. In recent years, many new conductive polymers, such as PDA, PDPA, PIN, and POA, and their composites with different conductivity properties, have been widely used in ER fluids.

The power law between yield stresses and electric field strengths of various conducting polymer-based ER fluids, with its exponent between 1.5 and 2.0, is explained based on both a polarization

model with an exponent of 2.0 and a conduction model with an exponent of 1.5. Nonetheless, to cover various conducting polymer-based ER suspensions in which the exponents do not follow one of the two mechanisms mentioned above, a universal yield stress equation was designed to properly correlate their dependence. In addition, both the CCJ and four-parameter models exhibited better fitting performance for the shear stress curve of the ER suspensions under applied electrical fields in the range of shear rate as compared with that of the traditional Bingham fluid model. Finally, recent applications of ER fluids, such as in damper systems, control systems, haptic devices, polishing systems, and so on, were summarized for potential commercialization of various engineering devices.

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Review

# A Perspective on Reversibility in Controlled Polymerization Systems: Recent Progress and New Opportunities

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**Abstract:** The field of controlled polymerization is growing and evolving at unprecedented rates, facilitating polymer scientists to engineer the structure and property of polymer materials for a variety of applications. However, the lack of degradability, particularly in vinyl polymers, is a general concern not only for environmental sustainability, but also for biomedical applications. In recent years, there has been a significant effort to develop reversible polymerization approaches in those well-established controlled polymerization systems. Reversible polymerization typically involves two steps, including (i) forward polymerization, which converts small monomers into macromolecule; and (ii) depolymerization, which is capable of regenerating original monomers. Furthermore, recycled monomers can be repolymerized into new polymers. In this perspective, we highlight recent developments of reversible polymerization in those controlled polymerization systems and offer insight into the promise and utility of reversible polymerization systems. More importantly, the current challenges and future directions to solve those problems are discussed. We hope this perspective can serve as an “initiator” to promote continuing innovations in this fairly new area.

**Keywords:** controlled polymerization; reversible polymerization; sustainable polymers

## 1. Introduction

The fundamental concept of reversibility has been widely utilized to drive the development of new polymeric materials, which can display distinct but reversible change in properties upon receiving a stimulus [1,2]. In light of this, a library of reversible materials based on polymers have recently been achieved, including self-healing materials bearing reversible-covalent linkages [3–8], recyclable materials such as vitrimers [9–11], polymer networks enabling reversible sol-gel transitions [12–14], architecture-transformable polymers [15], and covalent or metal organic frameworks harnessing reversible bonds [16–21]. Despite the tremendous success in the aforementioned polymer systems, little attention has been paid to achieving reversible polymerizations. Compared with self-immolative polymers, which can only undergo one-way depolymerization, a reversible polymerization typically features reversible transformations between polymers and original monomers [22]. Indeed, step-growth polymerizations relying on reversible-covalent chemistry, particularly Diels–Alder chemistry, have provided an approach to reversible polymers that favors forward polymerization at room temperature and tends to depolymerize at high temperatures (i.e., 120 °C) [23]. However, those polymers prepared by step-growth polymerizations typically have very broad molecular weight

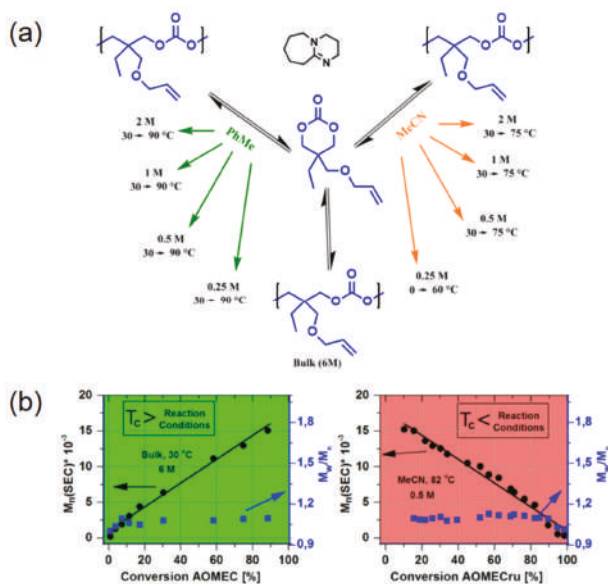
distributions and small molecular weights, limiting their potentials in certain applications that require precise polymer chain length, complicated architectures, and high molecular weights [24–29].

In the last two decades, the rapid advent of controlled and living polymerizations has offered polymer scientists a powerful synthetic toolbox for accessing polymers with predetermined molecular weights, well-defined architectures, and narrow distributions in molecular weights [30–35]. In addition, the excellent tolerance of functional groups in controlled polymerization systems has enabled us to achieve advanced polymer materials with desired functions and properties [36–41]. In general, controlled polymerization techniques can be categorized into three common classes. The first one involves ring-opening polymerization (ROP) of cyclized monomers (e.g., caprolactones, lactides, and *N*-carboxyanhydrides) in the presence of a nucleophilic initiator and a catalyst (metal or organic) [42–47]. The second category highlights the well-developed applications of controlled anionic/cationic polymerizations, which have led to industrial production of thermoplastic elastomers [30]. The last class focuses on reversible-deactivation radical polymerization (RDRP) methodologies, which are capable of polymerizing vinyl monomers, such as acrylates, methacrylates, and styrene in a controlled manner [48–51]. To date, three mainstream RDRP techniques, including atom-transfer radical polymerization (ATRP) [52,53], reversible addition–fragmentation transfer (RAFT) polymerization [54–57], and nitroxide-mediated polymerization have been developed [58]. Among them, ATRP and RAFT are receiving the most attention, due to their unique advantages, such as mild polymerization conditions, broad monomer scope, and ease of end-group functionalization [59]. These controlled polymerization systems are enjoying tremendous success in producing polymers that accommodate both industrial use (supported by ROP and anionic/cationic polymerizations) and academic research. However, the studies related to depolymerization are scarce, because depolymerization was typically considered as a side reaction that would lessen the performance—e.g., the mechanical properties of polymer materials [60]. While this is true in the pursuit of maximizing lifetime or long-term stability of polymer materials, serious pollution problems have arisen from the lack of degradability in commercial polymer plastics under common conditions [61]. Sustainable polymers, such as degradable polyesters deriving from biomass resources, represent a promising platform for environmental remedy. However, those polymers currently suffer from high manufacturing cost and low mechanical properties compared to vinyl polymer-based materials from petroleum resources. Moreover, the degradation of these sustainable polyesters is typically one-way, resulting in non-polymerizable fragments that are not useful for the regeneration of new materials. To fully achieve sustainability in polymer materials, recent attentions have been shifted to the development of new methods for depolymerizing polymers back into original monomers under accessible conditions [60,62–71]. These regenerated monomers can be recycled and further repolymerized to obtain new polymer materials. From this perspective, we aim to first critically assess the state-of-the-art toolbox for achieving reversible polymerization in controlled polymerization systems (Scheme 1). Recent examples on reversible polymerizations will be classified according to their controlled polymerization mechanisms, i.e., ROP and RDRP (Scheme 1 and Table 1). In addition, we believe it is important to assess the current challenges of reversible polymerizations that can trigger polymer chemists to solve this issue. Finally, potential applications deriving from this fairly new concept will be predicted and discussed.



one-pot reaction, involving the oligomerization of trimethylolpropane allyl ether, diethyl carbonate, and NaH, followed by an in-situ anionic depolymerization. It was observed that the depolymerization can occur beyond the ceiling temperature of the polyester at a certain polymer/monomer concentration, suggesting the reversible nature of the polymerization of AOMECE. However, there was still room to improve, since the oligomers were synthesized simply by condensation reaction rather than ROP, and the degree of polymerization was only from 1 to 7.

Inspired by this pioneering work, Albertsson's team continued the study on AOMECE and first demonstrated the reversible ROP of this six-membered cyclic carbonate monomer in 2016 [64]. It was concluded that the equilibrium between controlled ROP of AOMECE and controlled ring-closing depolymerization (RCDP) of poly(AOMECE) were dictated by various reaction parameters, such as monomer concentration, reaction temperature, and even solvents. In their approach, AOMECE was polymerized by ROP in the presence of an organocatalyst—that is, 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) either in bulk or in different solvents (Figure 1a). Various temperatures were used to evaluate the equilibrium conversion for calculating the ceiling temperature at certain polymerization conditions. According to the thermodynamic principle, ROP of AOMECE was favored at a temperature lower than the ceiling temperature. As the reaction temperature was higher than the ceiling temperature, RCDP of poly(AOMECE) dominated. It is worth noting that the molecular weights increased/decreased linearly as a function of monomer conversion in the cases of both ROP and RCDP (Figure 1b). Moreover, the molecular weight distribution of all the evolving polymers remained as low as 1.1, which further verified the exceptional control over the course of both polymerization and depolymerization.

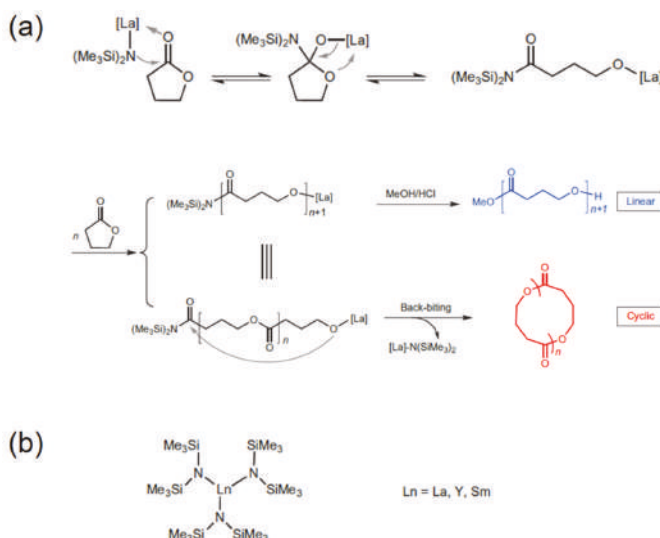


**Figure 1.** Reversible polymerization in ring-opening polymerization systems. (a) Reversible ROP of 2-allyloxymethyl-2-ethyltrimethylene carbonate (AOMECE) at various reaction conditions; (b) Correlations of molecular weights, polydispersity index, and monomer conversions under two different polymerization conditions. Reproduced with permission from [64].

As one of the best suitable biomass-derived compounds to replace petroleum-derived chemicals,  $\gamma$ -butyrolactone ( $\gamma$ -BL) and its polymer P $\gamma$ BL have great potential as sustainable materials [68,70]. However,  $\gamma$ -BL has been commonly considered to be “non-polymerizable” due to its low strain energy, which has thereby rendered the ROP of  $\gamma$ -BL extremely challenging. Starting from 2016, Chen et al. has conducted extensive research on ROP of  $\gamma$ -BL, and have now developed several

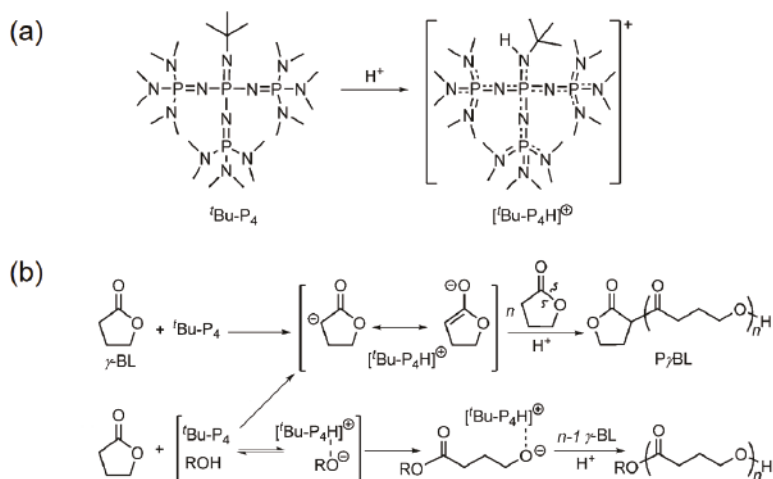


exciting synthetic methodologies to achieving P $\gamma$ BL by ROP. In agreement with Albertsson's viewpoint on the thermodynamic basis, Chen anticipated that reaction conditions should be modulated to achieve successful ROP, including a much lower reaction temperature than the ceiling temperature, a high initial monomer concentration, and most importantly, a robust catalyst. In their first work associated with  $\gamma$ -BL, the ROP of  $\gamma$ -BL was carried out at  $-40$  °C in the presence of a lanthanide (Ln)-based coordination polymerization catalyst [68]. The polymerization was capable of affording P $\gamma$ BL with  $M_n$  as high as  $30,000 \text{ g mol}^{-1}$  and up to 90% monomer conversion. In addition, the polymer topology (e.g., linear or cyclic) can also be controlled by varying the initiator structure and the feeding ratio of raw materials (Figure 2). From the sustainable perspective, a quantitative depolymerization of P $\gamma$ BL was realized by heating the purified polymers for 1 h at higher temperatures (i.e., 220 or 300 °C) than the ceiling temperature. Interestingly, the P $\gamma$ BL that was dissolved in appropriate solvents depolymerized much more rapidly in the presence of an organocatalyst (e.g., 1,5,7-triazabicyclo[4.4.0]dec-5-ene) or metal catalyst (e.g.,  $\text{La}[\text{N}(\text{SiMe}_3)_2]_3$ ), even at room temperature.



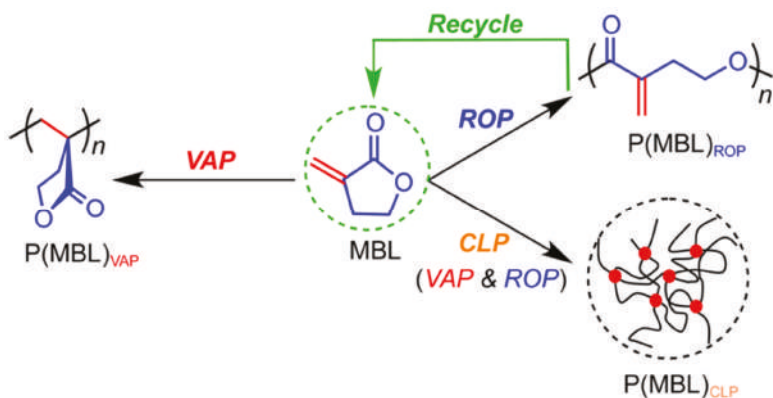
**Figure 2.** Reversible polymerization in ring-opening polymerization systems. (a) Proposed mechanism for lanthanide-catalyzed ROP of  $\gamma$ -BL in the preparation of both linear and cyclic P $\gamma$ BL; (b) Chemical structure of lanthanide catalyst. Reproduced with permission from [68].

In parallel, Chen and coworkers also discovered a metal-free ROP strategy to produce P $\gamma$ BL, using a super-basic organocatalyst with abbreviation tert-Bu-P $_4$  (Figure 3a) [71]. It should be noted that the catalyst itself was able to initiate the ROP of  $\gamma$ -BL at  $-40$  °C, by abstracting protons from  $\gamma$ -BL to form highly reactive enolate species (Figure 3b). However, the monomer conversion was limited up to 30.4%, due to the possible interference of  $[\text{tert-Bu-P}_4\text{H}]^+$  and an anionic dimer. Furthermore, the ROP performance was greatly enhanced when tert-Bu-P $_4$  was added, along with a suitable alcohol serving as the initiator (e.g., BnOH). With the help of the alcoholic initiator, the monomer conversion reached as high as ca. 90%, and the corresponding P $\gamma$ BL possessed a molecular weight of  $26,700 \text{ g mol}^{-1}$ . Notably, the P $\gamma$ BL prepared by this organocatalyzed ROP was completely recyclable, and can depolymerize back to  $\gamma$ -BL upon heating at 260 °C for 1 h.



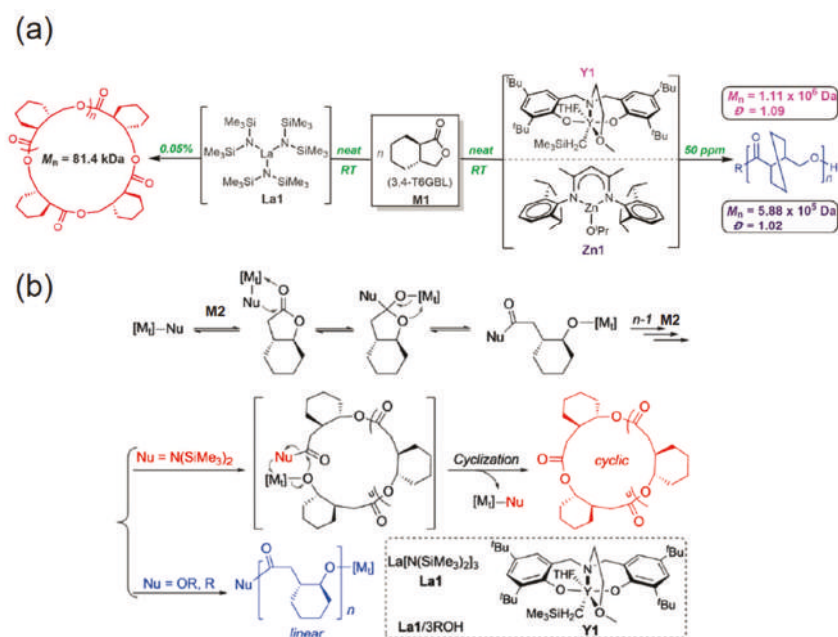
**Figure 3.** Reversible polymerization in ring-opening polymerization systems. (a) Structure and basicity of  $t\text{-Bu-P}_4$ ; (b) proposed mechanism for the  $t\text{-Bu-P}_4$ -catalyzed ROP of  $\gamma\text{-BL}$  (i) with and (ii) without alcohol as an initiator. Reproduced with permission from [71].

Encouraged by the success of preparing fully-recyclable  $\text{PyBL}$  via ROP with either metal (La, Y) or organocatalysts, Chen's group proceeded to apply this unique polymerization process to an enriched variety of monomers. ROP of  $\alpha$ -Methylene- $\gamma$ -butyrolactone (MBL), a small molecule derived from biomass and regarded as a potential alternative to the petroleum-based MMA, was subsequently investigated (Figure 4) [69]. Since the monomer comprises a non-strained five-membered lactone and a highly reactive exocyclic  $\text{C}=\text{C}$  double bond, many knee-jerk studies were exclusively focused on traditional vinyl addition polymerization (VAP) [74,75]. In Chen's work, the lanthanide (Ln)-based coordination polymerization catalyst was utilized, leading to an unsaturated polyester  $\text{P}(\text{MBL})$  with  $M_n$  up to  $21,000 \text{ g mol}^{-1}$  through the ROP process. Remarkably, by adjusting the reaction conditions, such as the catalyst (La)/initiator (ROH) ratio and temperature, three pathways of the MBL polymerization can be realized independently, including conventional VAP, ROP, and crosslinking polymerization. As was foreseeable, only the polymers resulted from the ROP pathway were fully recyclable.



**Figure 4.** Reversible polymerization in ring-opening polymerization systems. Polymerization of  $\alpha$ -Methylene- $\gamma$ -butyrolactone (MBL) following three distinct mechanisms. Reproduced with permission from [69].

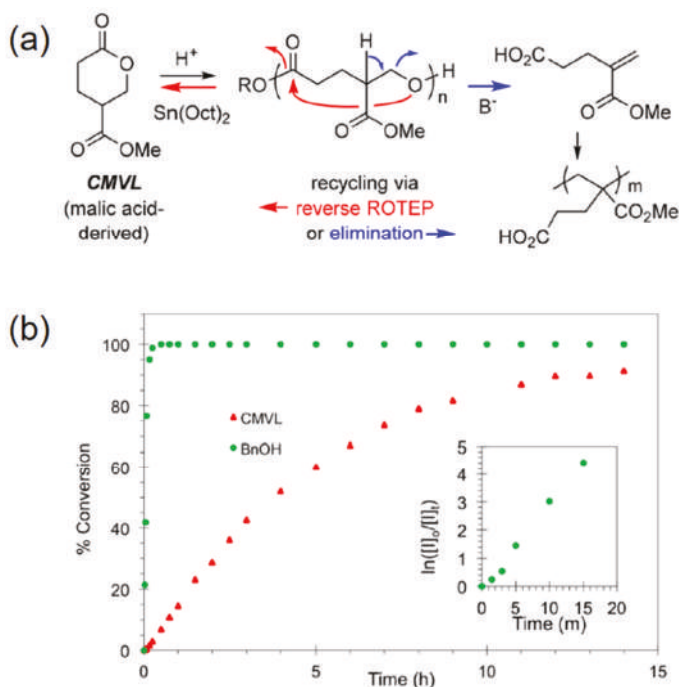
Despite the notable achievement in lanthanide (La, Y) or superbase (tert-Bu-P<sub>4</sub>) catalyzed ROP of  $\gamma$ -BL and its derivatives, the undesirable low temperature (i.e.,  $-40\text{ }^{\circ}\text{C}$ ) to implement the process still remained as one of biggest hurdles for industry use. Moreover, the as-synthesized polymers suffered from limited thermostability and crystallinity. Hence, the exploration of new materials with both superior properties and energy economy are still in demand. Recently, Chen's group proposed a  $\gamma$ -BL derivative (abbreviated as 3,4-T6GBL), with a cyclohexyl ring transfused to the five-membered lactone at the  $\alpha$  and  $\beta$  positions [70]. This monomer can be polymerized by using the coordinative insertion ROP catalysts, producing linear or cyclic polymers with high molecular weights at room temperature (Figure 5a). Following this finding, Chen et al. extended the scope of ROP of another  $\gamma$ -BL derivative (4,5-T6GBL), where the cyclohexyl ring was fused at the  $\beta$  and  $\gamma$  (or 4,5) positions of the BL ring, giving rise to linear/cyclic polymers at room temperature (Figure 5b) [67]. A controlled polymerization behavior was observed as the molecular weights of evolving polymers, which increased linearly with the monomer conversions. As expected, in both studies the resulting polymers possess enhanced thermostability and could be quantitatively recycled back to their original building monomers by either thermolysis or chemolysis.



**Figure 5.** Reversible polymerization in ring-opening polymerization systems. (a) Metal catalyzed ROP of 3,4-T6GBL (M1) to linear and cyclic polymers; (b) Proposed pathways for metal catalyzed ROP of 4,5-T6GBL (M2) to linear and cyclic polymers. Reproduced with permission from [67,70].

Very recently, Hoyer et al. described the synthesis of a novel substituted polyvalerolactone from a malic acid derived monomer, 4-carbomethoxyvalerolactone (CMVL) [66]. In their work, this six-membered ring monomer was blended with a diol (1,4-benzenedimethanol) and an organic acid (diphenyl phosphate) at an ambient temperature, finally forming a semicrystalline material with a molar mass up to  $71,000 \text{ g mol}^{-1}$ . Notably, the resulting polymer can be either depolymerized back into its original precursor monomer or degraded into acrylate-type analogues (Figure 6a). The former process was catalyzed by tin octanoate ( $\text{Sn}(\text{Oct})_2$ ), providing 87% monomer recovery; while the latter was promoted by DBU with a comparable yield. In particular, a substantial kinetics study showed that CMVL was polymerized smoothly and reached ca. 90% conversion after 15 h (Figure 6b). It also

revealed that nearly all of the initiator was consumed within 20 min. This “fast initiation” can be regarded as one of the characteristic behaviors of controlled polymerization systems.



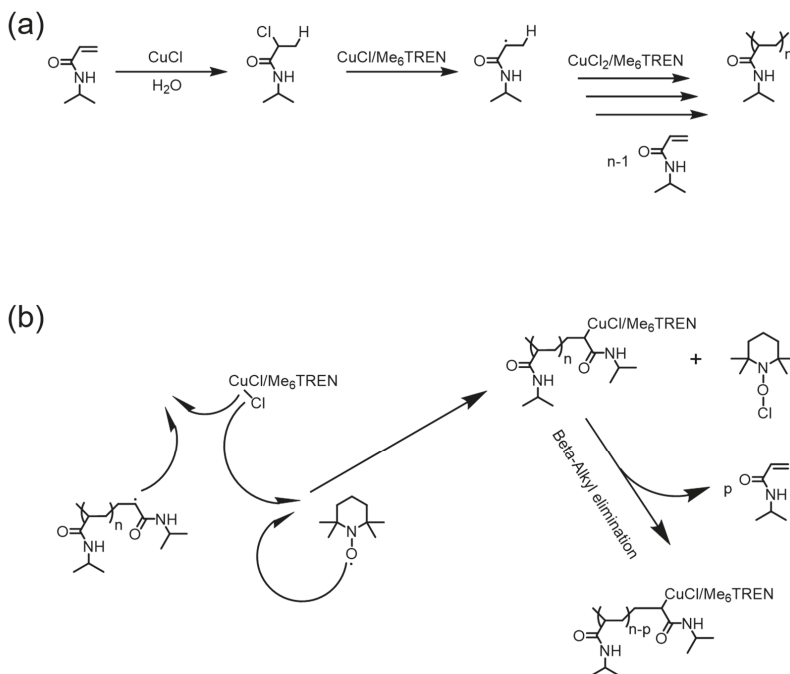
**Figure 6.** Reversible polymerization in ring-opening polymerization systems. (a) Acid-catalyzed ROP of 4-carbomethoxyvalerolactone (CMVL) and the divergent chemical recycling; (b) kinetics of the ROP of CMVL in the presence of BnOH as an initiator. Reproduced with permission from [66].

### 3. Reversible Polymerization in Reversible-Deactivation Radical Polymerization Systems

Although reversible polymerizations in ROP systems have shown great promise in next-generation sustainable polymer materials, the major market for commodity polymers is still occupied by vinyl polymers, due to their low cost in manufacturing. Vinyl polymers are derived from petroleum, a non-renewable resource. Therefore, the recycling of used vinyl polymer materials such as plastics has immense merits, not only in global waste reduction, but also for petroleum sustainability [76]. In this section, recent examples in depolymerization of vinyl polymers derived from RDRP systems will be discussed. We hope those timely developments will prompt more innovative thinking with regard to plastic recycling via a reversible polymerization approach.

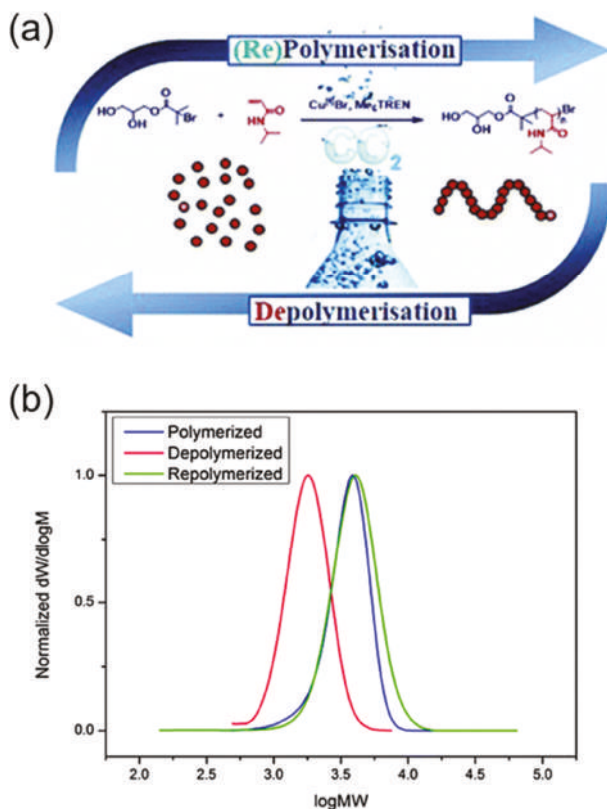
In 2012, Zhu et al. reported the first example of reversible polymerizations in an RDRP system [62]. In their pioneering work, vinyl polymerizations of several acrylamide monomers, including *N*-isopropylacrylamide (NIPAM) and *N,N*-dimethylacrylamide (DMA), were successfully achieved in the presence of CuCl and tris(2-dimethylaminoethyl)amine (Me6TREN) (Figure 7a). Very intriguingly, they unexpectedly observed a phenomenon of depolymerization when radical inhibitors like 2,2,6,6-tetramethylpiperidinoxy (TEMPO) or 1,4-benzoquinone (BQ) were added to the ongoing polymerization system, with the initial purpose of terminating radical polymerizations. To further elucidate the role of the copper catalyst in the depolymerization process, a control experiment with regard to a conventional radical polymerization, using 2,2'-azobisisobutyronitrile (AIBN) as radical initiator, was carried out in the absence of a copper catalyst and ligands. TEMPO was added during the conventional radical polymerization, resulting in only the termination of polymerization,

without any noticeable depolymerization. Those results unequivocally verified that a copper catalyst is essential in depolymerization. Therefore, a depolymerization mechanism based on  $\beta$ -alkyl elimination from the copper (II) coordination center was proposed (Figure 7b).



**Figure 7.** Reversible polymerization in copper-mediated polymerization systems. (a) Proposed mechanism of CuCl/ tris(2-dimethylaminoethyl)amine (Me<sub>6</sub>TREN)-based radical polymerization of *N*-isopropylacrylamide (NIPAM); (b) 2,2,6,6-tetramethylpiperidinoxy (TEMPO)-induced depolymerization mechanism via  $\beta$ -alkyl elimination. Reproduced with permission from [67].

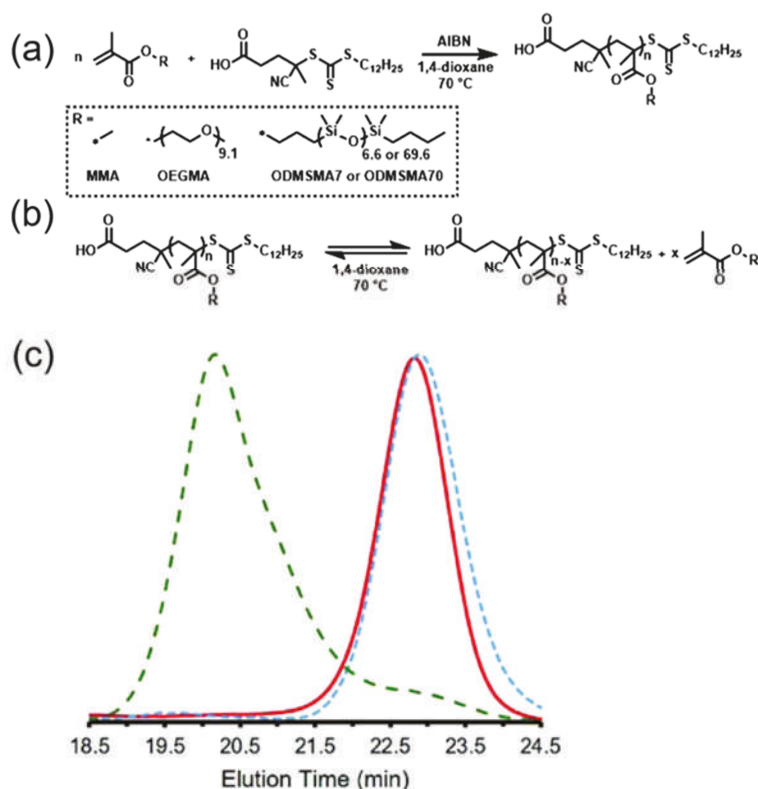
In a similar demonstration of reversible polymerization mediated by a copper catalyst, Haddleton and coworkers were able to prepare well-defined polyacrylamides and polyacrylates through aqueous copper-mediated radical polymerization in the presence of dissolved CO<sub>2</sub> (Figure 8a) [65]. In the case of a NIPAM monomer, the forward polymerization adopted rapid reaction kinetics, achieving full monomer conversion within 10 min. Thereafter, a significant in-situ depolymerization occurred to an extent of 52%, and thereby led to the regeneration of the NIPAM monomer, which was systemically confirmed by proton nuclear magnetic resonance (NMR), gel permeation chromatography (GPC), and electron ionization-mass spectroscopy. Importantly, this recycled NIPAM can be repolymerized upon deoxygenation of the resulting solutions, illustrating the reversibility of the polymerization (Figure 8b). Furthermore, the scope of reversible copper-mediated polymerization was extended to *N*-hydroxyethyl acrylamide (HEAm) and 2-hydroxyethyl acrylate (HEA), demonstrating the versatility of this system. However, it should be noted that the mechanism of depolymerization, as well as the role of CO<sub>2</sub> in the depolymerization process, was not identified in their study.



**Figure 8.** Reversible polymerization in copper-mediated controlled polymerization system. (a) A schematic illustration of aqueous reversible polymerizations mediated by a copper catalyst and  $\text{CO}_2$ ; (b) Gel permeation chromatography GPC traces of original poly (*N*-isopropylacrylamide) (PNIPAM) (blue), depolymerized PNIPAM (red), and repolymerized PNIPAM (green). Reproduced with permission from [65].

In the aforementioned RDRP systems (Vide supra), the depolymerization phenomenon was only observed during the course of the polymerization. However, it is arguably more interesting from the materials point of view if one can depolymerize a polymer post-synthesis or after the manufacturing process. In very recent work described by Gramlich, a set of brush polymers consisting of oligo-ethylene glycol or oligo-dimethylsiloxane side chains were prepared by traditional RAFT polymerization in the presence of AIBN at 70 °C (Figure 9a) [60]. After polymerization, those polymers were thoroughly purified by repeated precipitations, to ensure the removal of residual monomers and initiators. Upon purification, thermally-induced depolymerization of the as-synthesized polymer was conducted in dilute dioxane solutions, leading to the regeneration of vinyl monomers until reaching the monomer's inherent equilibrium monomer concentration. Importantly, the residual polymers exhibited high chain-end fidelity by retaining the trithiocarbonate moiety after depolymerization, allowing for further reinitiation and repolymerization via a RAFT mechanism (Figure 9b,c).





**Figure 9.** Reversible polymerization in a RAFT system. (a) Preparation of brush-type polymers via RAFT polymerizations of methacrylate monomers; (b) depolymerization and repolymerization of RAFT polymers; (c) GPC trace of original RAFT polymer (red solid line), depolymerized polymer (blue dotted line), and repolymerized polymer (green dotted line). Reproduced with permission from [60].

#### 4. Closing Remarks

The current success in reversible polymerizations has enabled us to think about many new possibilities in future polymer science. However, many challenges still remained to be addressed, hampering the further translation of this new concept into real-world applications. One apparent hurdle is how to achieve good control over the depolymerization process. While all the examples covered in this perspective are related to controlled polymerizations, allowing for a predictable degree of forward polymerizations, little information was provided to reveal the kinetics of the depolymerization process, especially those involved in controlled radical polymerization. Indeed, previous literature has placed much focus on the start and endpoint of depolymerization (in other word, the highest degree of depolymerization). Notwithstanding, kinetic study will shed more light on the fundamental mechanisms of reversible polymerizations, and if one can predetermine and control the degree of depolymerization by changing several reaction parameters, such as time, temperature, catalyst/initiator loading, polymer/monomer concentrations, among others. Moreover, the ability to tune the depolymerization rate under normal conditions is expected to open the door to many interesting applications (in addition to sustainable materials)—for example, self-healable materials, and sustained release systems, which require slow and controllable depolymerization. It is worth noting that the concurrent depolymerization approaches are typically associated with

harsh conditions (e.g., high temperatures, metal catalysts), significantly impeding the translation of this concept into biomedical uses. In light of this, the continuing pursuit of new depolymerization methodologies that can be implemented under mild and physiological conditions will be important towards bio-related applications.

Another challenge stems from the relatively low efficiency in depolymerizations, particularly those in RDRP systems. In comparison with ROP-based depolymerization systems, which mainly rely on breaking weak polyester backbones, the energy input necessary for reversing vinyl polymer backbones (i.e., carbon–carbon single bonds) back to vinyl monomers is considerably higher. To our best knowledge, the highest reported depolymerization conversion in RDRP systems was only 71% when N-hydroxyethyl acrylamide was involved in copper(0)-mediated reversible polymerizations [65]. In the RAFT mediated depolymerization approach, only 30% of monomers can be regenerated after heating the diluted RAFT polymer solutions at 70 °C under a vacuum for several days [60]. From the viewpoint of potential industrial applications, insufficient depolymerization could dramatically increase the cost deriving from separating regenerated monomers from residual polymers. Therefore, we envision that more attention will be paid to detailed mechanism study and the rational design of depolymerization systems, with the goal of achieving high depolymerization efficiency (such as the effort for lowering equilibrium monomer concentration). Moreover, we believe that mathematical tools, such as modeling and simulations of reversible polymerizations, should play a key role in prediction of the dynamics, final products, and optimal conditions in reversible polymerizations [77]. While the concept of reversible polymerizations is still in its infant stage, it is anticipated that the future development in this area will not only deepen our understanding of fundamental depolymerization mechanisms, but also promote many new opportunities and applications in polymer science and engineering.

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Review

# Polymeric Co-Delivery Systems in Cancer Treatment: An Overview on Component Drugs' Dosage Ratio Effect

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**Abstract:** Multiple factors are involved in the development of cancers and their effects on survival rate. Many are related to chemo-resistance of tumor cells. Thus, treatment with a single therapeutic agent is often inadequate for successful cancer therapy. Ideally, combination therapy inhibits tumor growth through multiple pathways by enhancing the performance of each individual therapy, often resulting in a synergistic effect. Polymeric nanoparticles prepared from block co-polymers have been a popular platform for co-delivery of combinations of drugs associated with the multiple functional compartments within such nanoparticles. Various polymeric nanoparticles have been applied to achieve enhanced therapeutic efficacy in cancer therapy. However, reported drug ratios used in such systems often vary widely. Thus, the same combination of drugs may result in very different therapeutic outcomes. In this review, we investigated polymeric co-delivery systems used in cancer treatment and the drug combinations used in these systems for synergistic anti-cancer effect. Development of polymeric co-delivery systems for a maximized therapeutic effect requires a deeper understanding of the optimal ratio among therapeutic agents and the natural heterogeneity of tumors.

**Keywords:** polymeric nanoparticles; stimuli-sensitive polymers; co-delivery systems; synergistic effect; nucleic acid delivery; chemotherapy

## 1. Introduction

Cancer, next to heart disease, ranks as the second leading illness-related cause of death worldwide with a growing incidence and mortality. It is one of the most challenging-to-treat diseases due mainly to inefficient pharmacologically active agents and the complexity of cancer. To date, chemotherapy has been widely used and has been the most efficient and successful treatment method in clinical practice. However, there are three major issues limiting the therapeutic efficacy of chemotherapy. First, most of chemotherapeutic agents have poor solubility that leads to deficiencies like low bioavailability, rapid blood/renal clearance, and nonspecific targeting, with significant undesirable side effects on healthy tissues. Second, non-uniform biodistribution limits the localization of drugs at the tumor site and leads to consequent demands for higher doses that have unacceptable toxicity. Above all, genetic variations that control survival and apoptotic pathways are involved in the development of cancers. Targeting an individual pathway with conventional chemotherapy is often unsuccessful in eradicating all cancer cells and results in multidrug resistance (MDR) over the course of treatment.

Several alternative approaches to overcome these problems associated with traditional chemotherapy have been established. Much attention has been focused directly on drug combination approaches with the aims of more effective treatment and decreased side effects [1–3]. In general, combination chemotherapy involves the simultaneous administration of two or more drugs with non-overlapping toxicities and dissimilar mechanisms of action so as to inhibit multidrug resistance. Combination therapy can overcome the toxicity of single drug therapy by targeting various signaling pathways. Lately, combination therapy regimens have been intensively studied, and the results of clinical practice have demonstrated synergistic effects that are greater than the sum of the individual drug effects and less systemic toxicity associated with the delivery of lower drug doses.

However, combination regimens are still limited by a low rate of successful outcomes and significant side effects due to low bioavailability of drugs and their nonuniform biodistribution. To take advantage of possible synergy between drugs, they must attain effective molar ratios at the tumor sites that are often hard to reach by conventional administration methods due to differences in injection schedules, pharmacokinetic properties, metabolism, and non-uniform biodistribution.

One strategy for delivery of drugs to the tumor site at the desired molar ratio involves the merging of nanotechnology with pharmacology and thereby take advantage of the nanoscale structures that carry multiple drugs, allow tuning of drug release, and modify biodistribution and pharmacokinetic characteristics of chemotherapeutic agents [4,5]. Such co-delivery systems may be used to not only regulate the dosages and the ratio of chemotherapeutic agents at the tumor site. They may also improve the efficacy of anticancer drugs through enhanced water solubility of hydrophobic molecules, lower toxicity, and higher stability, which prolongs blood circulation time to enhance accumulation in tumor tissues. Further enhancement of therapeutic efficacy can be achieved by taking the advantage of stimuli-responsive drug delivery systems equipped with target-activated moieties [6,7].

Co-delivery systems ideally possess the potential for encapsulation of both hydrophobic and hydrophilic drugs. Platforms for co-delivery systems should be designed to carry both traditional chemotherapeutics and cell regulatory molecules, such as nucleic acids [6]. Although much progress has been made with nanotechnology-based co-delivery systems, there are several problems to be considered in formulating an ideal drug delivery system including those associated with encapsulating drugs with a variety of solubilities and physicochemical properties, elevating drug concentrations within tumor tissues and regulating their sequential drug release.

To date, considerable efforts have been made to develop nano-particulate co-delivery systems for combination chemotherapy [6,8]. Various nanocarriers have been investigated, including lipid nanoparticles [9], liposomes [10–12], dendrimers [13] and polymeric nanoparticles [14]. More attention has been paid to polymeric nanoparticles mainly because of their potential to carry both hydrophobic and hydrophilic drugs, favorable controlled drug release characteristics, low toxicity, high stability and a prolonged circulation time which ultimately enhances accumulation in tumor targets.

Many research and review papers involving co-delivery of therapeutic agents by polymeric nanoparticles in cancer therapy have been published [15–17]. However, therapeutic agents are often administered separately instead of simultaneously using a polymeric nanoparticle capable of delivering both agents [18]. Additionally, to the best of our knowledge, there is no comprehensive review addressing the effects of the drug ratio in co-delivery systems, a likely significant parameter promoting a synergistic therapeutic effect. The scope of this review is distinct from generalizations of about polymeric co-delivery systems for chemotherapy. Our aim is to address the dosage, cell type, mechanism, and their efficacy relationships that need to be considered in designing co-delivery systems.

## 2. Polymer Types Used in Preparing Co-Delivery Systems

### 2.1. Block Co-Polymer Conjugates

Amphiphilic polymers self-assemble into nanoparticles that are ideal co-delivery systems for both hydrophilic and hydrophobic drugs (Table 1). These polymers are usually obtained by conjugating together polymers with diverse properties. The block co-polymers formed inherit the properties of each block, thus integrating advantages of various blocks into a single system. Depending on the chemical composition, block co-polymers are prepared by conjugating hydrophobic and hydrophilic polymers together through physical/chemical interactions or by modifying hydrophilic polymers with hydrophobic lipid moieties. These synthetic block co-polymers self-assemble into polymeric nanoparticles or micellar nanoparticles for co-delivery purposes. In order to minimize toxicity and side effects, the major materials used are biodegradable polymers: chitosan [19], poly(lactic acid) [20], gelatin [21], poly[N-(2-hydroxypropyl) methacrylamide] (HPMA) [22] and their copolymers, poly(lactide-co-glycolide-co-caprolactone) [23] and poly(lactide-co-glycolic acid) [24]. Their total degradation can occur in the body and can reach the kidney threshold for excretion [25].

Six classic methods for obtaining polymeric nanoparticles have been described: nanoprecipitation [26], emulsion-diffusion [27], emulsification-coacervation [28], double emulsification [29], surface polymerization [30], and layer-by-layer methods [31]. In addition, multifunctional polymeric structures with precisely defined morphology can be obtained by a controlled atom transfer radical polymerization (ATRP) method [32].

Polymeric systems are grouped below by sensitivity to changes in temperature, pH, light, redox potential, and other special factors in their environment. Stimuli-sensitive polymers have become one of the most prominent solutions for anti-cancer therapy. The unique properties of polymers allow them to change their accumulation and drug release profile depending on the surrounding conditions. They are used to target drugs, bioactive substances and genes. These systems selectively deliver therapeutic agents to target tissues, cells and cell compartments to release their cargos [33–35]. By doing so, the pharmacological properties, release profile and therapeutic outcomes are improved compared to delivery as free drugs.

### 2.2. Thermo-Sensitive Polymers

Polymeric nanoparticles formulated with thermo-sensitive polymers has been applied to activate and control the release of active ingredients after reaching the target site. For example, overheating a cancer by activating magnetic cargo-loaded polymeric nanoparticles with a local magnetic field [36]. Thermo-sensitivity has been one of the most commonly used stimulating features for biomedical applications [37]. There are two types of polymers distinguished by their phase distribution. The first type, called UCST (upper temperature of the critical solution), passes between phases during cooling. In the second type LCST (lower critical temperature of the solution), this transition occurs with increasing temperature. Systems with UCST are more prevalent for polymers soluble in an organic solvent, while systems with LCST exist mainly for polymers dissolved in aqueous solvents. The solubility of polymers in organic solvents is due to short-range van der Waals interaction. The solubility of polymers in water is related to hydrophobic and hydrophilic interactions and the formation of hydrogen bonds. For polymeric drug delivery systems, mainly aqueous solvents are used [38–40].

**Table 1.** Types of stimuli-sensitive polymers commonly used as co-delivery systems.

Types	Polymers	Dosage Form *	Drug 1	Drug 2	Cell Line	Ref.
pH-sensitive	PDEA-PDMA-PEG	M	siBcl-2	DOX	Hep G2	[41]
	PEG-PLL-PAsp	NP	siBcl-2	DOX	Hep G2	[42]
	Trimethyl Chitosan	NP	IL-2	DOX	SMMC7721	[43]
	PEI-PLA/PEG-PAsp	NP	siSurvivin	PCT	A549	[44]
	PEO-b-PCL	M	siMDR-1	DOX	MDA-MB-435	[45]
Redox-sensitive	PDP-PDHA	NP	shSurvivin	DOX	MCF7 /ADR	[46]
	PEI-CD	NP	TRAIL pDNA	DOX	SKOV-3	[47]
	PEG2k-CLV-Dox / miRNA-34a-S-S-PE	MM	miRNA-34a	DOX	MCF7, HT1080	[48]
	miPEG-PCL-SS-Dox / miPEG-PCL-SS-DTX	MM	DOX	DTX	MCF7	[49]
	Poly(acrylic acid)	H	DOX	CDDP	MCF7 /ADR	[50]
Thermo-sensitive	Gambogic acid-poly(amido amine)s	M	DTX	MMiP9shRNA	MCF7	[51]
	PEG-PLG-PDMA/PMA	NP	siMDR-1	DOX	MCF7	[52]
	DH <sub>700</sub> -MF-13.5/MDocLF	HMM	DOX	DTX	CT-26	[53]
	DHmPEG-b-PELG	H	IL15	CDDP	B16F0-RFP	[54]
	PLGA-PEG-PLGA	H	DOX, MTX	CDDP	Saos-2, MG-63	[55]
MMP-sensitive	PLGA-DOX/PEO-PPO-PEO	NP	DOX	IFN $\gamma$	B16F10	[56]
	PECT	HM	DOX	131I	Hep G2	[57]
	PEG2k-CLV-Dox / miRNA-34a-S-S-PE	MM	miRNA-34a	DOX	MCF7, HT1080	[48]
	PEG-pp-PEI-DOPE	M	siSurvivin	PCT	A549 T24	[58]
	PEG-PLA, G0-C14	NP	VEGF siRNA	PCT	HT-1080, A375, PC-3	[59]
Magnetic-responsive	PLGA /TPGS/OA	NP	TPGS	DOX	MCF7, MCF7 /ADR	[60]
	ASA-MNP $\beta$ s-CDDP / mPEG-PLL-FA	NP	CDDP	TPP2 DNA	HNE-1, NP69	[61]
	PCL /P(NIPAAm-co-HEMA-co-MAA-co-TMSPMA)	NP	DOX	MTX	MCF7	[62]

\* MM—mixed micelles; M—micelles; NP—nanoparticles; H—hydrogels; HM—micelles entrapped in hydrogel, HMM—mixed micelles entrapped in hydrogel.  
 PECT—poly( $\epsilon$ -caprolactone-co-1,4,8-trioxal[4,6]spiro-9-undecanone)-poly(ethylene glycol)-poly( $\epsilon$ -caprolactone-co-1,4,8-trioxal[4,6]spiro-9-undecanone).

### 2.3. pH-Sensitive Polymers

The sensitivity of polymers to pH has been utilized in polymeric nanoparticles designed for drug administration via the gastrointestinal tract (pH ranges from 2–4 in the stomach to about 6.8 in the gut), in solid tumors where the interstitial space is more acidic due to hypoxia, with a pH of about 6.5 compared to a blood plasma pH of about 7.4. These pH-sensitive polymers can be weak bases (more polar in an acid environment in a protonated form) or weak acids (more polar in a basic environment in deprotonated form). Polymers abundant with primary amines are sensitive to low pH (pH 5.0) and can facilitate the endosomal escape of drugs. Additionally, pH differences between normal tissue and tumor tissue can create conditions for use of pH-sensitive polymeric drug delivery systems with enhanced targeting and reduced side effects [63,64].

### 2.4. Redox-Sensitive Polymers

The redox potential differences in the tumor microenvironment inspired the idea of building a redox reaction-sensitive polymeric system for cancer treatment. Redox sensitivity is usually used in cases where changes of redox potential occur in inflamed tissues compared to healthy tissues. Changes in redox potential in cancer tissues are due to the production of reactive oxygen species by activated macrophages. Oxidatively degradable polymers, such as arylborone based on acid esters (which after oxidation become phenols and boric acid), or dialkyl sulphide-based polymers (which after oxidation become more hydrophilic), have been used as delivery systems for drugs to inflamed tissues [65]. The disadvantage of these polymers is that the level of reactive oxygen species is often not enough to fully oxidize the polymer so that the drug/gene cannot be released [66,67]. Drug delivery systems also use polymers that react to light exposure and the presence of certain ions or organic molecules including sodium alginate [68]. These types of polymer are applied mainly for diagnostic purposes [69].

It can be said that designing a polymeric drug system with micro-environmentally sensitive polymers is a “smart” strategy. Combining multiple therapeutic agents that inhibit tumor growth through different pathways into one system is also a “smart” strategy. Many polymeric systems have shown promising effects in cancer therapy based on these two ideas.

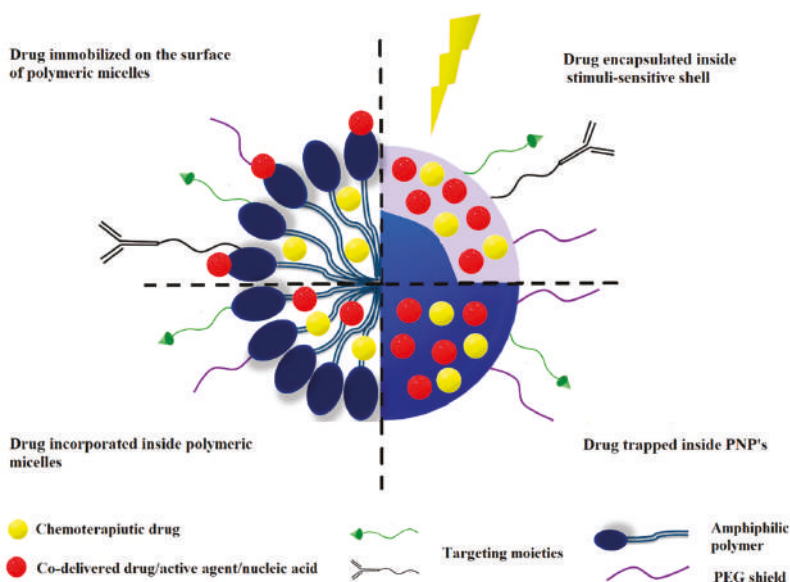
## 3. Application of Polymeric Nanoparticles as Co-Delivery Systems

### 3.1. Polymeric Nanoparticles for Co-Delivery of Chemotherapeutics

The aim of combining chemotherapeutics is to achieve an additive or even a synergistic effect. By targeting different pathways, combination therapy delivered by polymeric nanoparticles makes cancer cells more susceptible to the delivered therapeutic agents. Four commonly used approaches for co-delivery of therapeutic agents using polymeric nanoparticles are shown in Figure 1. Most drugs are passively loaded in polymeric nanoparticles according to their hydrophilicity. Hydrophobic molecules can be loaded in the hydrophobic moieties of micelles or polymersomes, and hydrophilic molecules are trapped in the hydrophilic compartments. Some drugs, such as nucleic acids, are co-loaded on the surface of the polymeric nanoparticles by electrostatic forces or chemical conjugations. Another approach is to directly conjugate drugs with the polymer through ester, amide or disulfide bonds [70]. Different types of polymeric nanoparticles can be further modified with targeting moieties.

Doxorubicin (DOX), a chemotherapeutic anthracycline, has been used clinically for treatment of several hematologic malignancies and solid tumors including breast cancer [71]. However, using DOX alone usually causes serious side effects in normal tissues, especially cardiotoxicity [72]. The molecular mechanism of DOX-induced cardiotoxicity is still unclear [73,74]. However, it has been postulated that it is caused by conversion of quinone into free radicals of half quinone, which in turn initiates cascading reactions leading to production of reactive oxygen and nitrogen in the body [75,76]. To increase the antitumor effect of DOX by overcoming cells resistance and, at the same time toning down the

cardiotoxicity, DOX is often used in combination with other chemotherapeutics, nucleic acids and antibodies in cancer therapy [77,78].



**Figure 1.** Drug loading in polymeric nanoparticles used as co-delivery systems in cancer treatment.

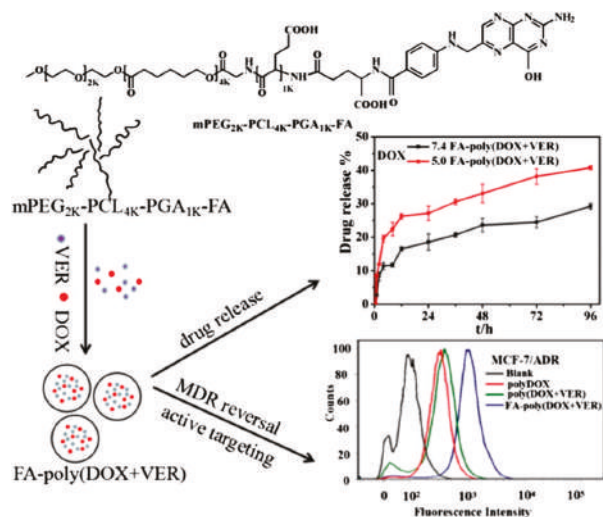
The combination of DOX with other anticancer drugs aims to achieve a synergistic effect of the combined drugs or improve their biodistribution. Doxorubicin is often combined with Paclitaxel (PCT). The hydrophobic delivery systems containing PCT are based on the entrapment of the drug in their hydrophobic core. The combination of DOX and PCT in mPEG-PLGA polymeric nanoparticles made by a dual emulsion method has allowed a better anti-cancer effect *in vivo* and a much faster release of the drug from the carrier in an acidic environment [79]. A similar formulation was also used to destroy tumor stem cells [80]. The double-reacting nanoparticles built of four polymers approved by the Food and Drug Administration (FDA) for medical use were composed of poly (D-, L-lactide-co-glycolide) (PLGA), Pluronic F127 (PF127), chitosan, and hyaluronic acid (HA). Combining PLGA and PF127 forms a more stable and homogeneous nanoparticles than with PLGA or PF127 alone. HA was used as a targeting moiety towards cancer stem cells to reduce drug resistance associated with the dormant metabolic state. As a result of the combination of both drugs, the anti-cancer effect was amplified ~500 times compared with a simple mix of the two drugs [80]. Similar polymeric co-delivery systems were also used that enclosed the hydrophobic irinotecan (CPT) and hydrophilic DOX HCl, which inhibited the activity of topoisomerase I and II and exhibited an enhanced therapeutic effect on breast and brain tumors [81].

Another strategy, used to deliver camptothecin (CPT) and doxorubicin, was to form a graft copolymer with side drug segments that form nanostructures using a protein folding pathway [82]. The graft copolymer was constructed by direct polymerization of g-camptothecin-glutamate N-carboxyhydride (Glu (CPT)-NCA) at multiple sites on the PEG-based main chain via open ring polymerization (ORP). Only the conjugated CPT is hydrophobic and served as the main driving force during the assembly process. When exposed to water, the copolymer, together with DOX, curls to form monodisperse nanolayers for the delivery of the two drugs. PEG coated nanocarriers exhibit good stability and are internalized by various cancer cell lines through the lipid raft and clathrin-mediated endocytosis pathways without premature leakage. These nanolayers generated high synergistic activity of the CPT and DOX in various tumor cell lines. The *in vivo* study confirmed that the nanolayers can



show strong accumulation at tumor sites and result in significant anti-tumor activity in a lung cancer xenograft model compared to free drugs [82].

To overcome the drug resistance of tumors, redox-sensitive polymeric nanogels (<100 nm) based on poly(acrylic acid) were designed. Doxorubicin and cisplatin were enclosed in the nanogels by chelation, electrostatic interaction and  $\pi$ - $\pi$  stacking interaction. Compare to free drugs, nanogels delivered more drugs to MCF-7/ADR cells. Considerable accumulation in cancerous tissues was observed in biodistribution experiments. In vitro anticancer studies showed better cell killing activity of the nano drug delivery system. All the data indicated that the combination therapy was more effective with reduced side effects [50]. Combinational delivery of DOX and verapamil in pH-sensitive polymeric nanoparticles based on co-polymer methoxy-poly(ethylene glycol)2k-poly( $\epsilon$ -caprolactone)4k-poly(glutamic acid)1k (mPEG2k-PCL4k-PGA1k-FA) demonstrated a high drug release efficiency in tumor environment. The system was reported to overcome the multidrug resistance and improve the anti-cancer effect in MCF7/ADR cells (Figure 2) [83]. Other strategies to overcome the drug resistance of a tumor include regulation of the level of multidrug resistant protein [84]. Combining chemotherapeutics with gene therapy in polymeric nanoparticles for cancer treatment has received extensive attention [85,86].



**Figure 2.** Folate-modified pH-sensitive co-delivery system of FA-poly(DOX+VER) polymer assembly exhibits obvious pH-sensitivity, high active targeting ability, strong multidrug resistance reversal and the enhanced therapeutic effect. Reproduced with permission from Li et al., Journal of Colloid and Interface; Elsevier, 2016 [83].

### 3.2. Polymeric Nanoparticles for Co-Delivery of Nucleic Acid Therapeutics and Chemotherapeutics

Multiple genetic targets have been established for cancer treatment over the past several decades. Based on the genetic links associated with tumor progression and development, nucleic acid therapeutics, such as siRNA, plasmid DNA (pDNA), miRNA, and antisense oligonucleotides, have provided highly attractive approaches to downregulate tumor-associated proteins or to recover the function of tumor-suppressing pathways. However, because of their high molecular weight, large amounts of negative charge, and enzyme-induced degradation, nucleic acids are very unstable in the systemic circulation and can barely penetrate the cellular membrane. Thus, intensive efforts have been made to develop delivery systems for nucleic acid therapy [87]. Here, combinations of chemotherapeutics together with siRNA, pDNA, and miRNA delivered by polymeric nanoparticles for cancer treatment are discussed.

### 3.2.1. Co-Delivery of siRNA and Chemotherapeutics with Polymeric Nanoparticles

One of the most widely used nucleic acid molecules used in preclinical and clinical studies has been small interfering RNA (siRNA). Cationic charged polymers, including chitosan [88,89], poly(ethylenimine) [90], poly(amidoamine) (PAMAM) dendrimer [13,91], and poly [2-(*N,N*-dimethyl aminoethyl) methacrylate] (PDMAEMA) [92,93], are capable of complexing with siRNA through electrostatic interaction, preventing degradation of siRNA and enhancing delivery of siRNA across the cell membrane. These polymers can be further modified by conjugating with other polymeric compartments to form an amphiphilic polymer with the ability to deliver siRNA and chemotherapeutics simultaneously.

Chitosan is a non-toxic and efficient vector for siRNA delivery. Surface modification with cationic chitosan by either absorption or covalent binding is a good strategy to enable the traditional drug delivery system to deliver siRNA as well [94]. Wang et al. coated chitosan on PLGA (50:50) nanoparticles loaded with DOX and grafted with a co-delivery system for both DOX and siRNA for an epidermal growth factor receptor [95]. Targeted with angiopoep-2, the co-delivery system induced a 13% higher cell apoptosis rate than a PLGA nanoparticle loaded with DOX alone.

Cao et al. conjugated low molecular weight PEI with poly( $\epsilon$ -caprolactone) (PCL) through disulfide or ester covalent linkages [96]. This amphiphilic PEI-PCL self-assembled into a micellar structure. Doxorubicin was loaded into the PEI-PCL micelles using a chloroform/water mixture with sonication. Bcl-2 siRNA was complexed with PEI-PCL micelles followed by further modification with PEG chains to decrease the cytotoxicity of the nanoparticles. This DOX and siBcl-2 co-delivery system induced a 60% decrease in cell viability 96 h after treatment in a Bel7402 cell line. However, the cell viability decreased by only 40% when siScramble was used instead of siBcl-2, indicating a synergistic effect with the co-delivery of DOX and siBcl-2. With further modification of the DOX plus siBcl-2 loaded nanocarrier using folic acid, the cell viability decreased to 5%.

Another co-delivery system for siRNA and chemotherapy based on PEI was reported by Navarro et al. [97]. To prepare the amphiphilic molecule, PEI was conjugated with a dioleoylphosphatidylethanolamine (DOPE) moiety. Micellar nanoparticles formed by PEI-DOPE reversed the multidrug resistance in MCF7/ADR cells with delivery of P-glycoprotein siRNA (siP-gp) with DOX. Similarly, stearic acid was attached to PEI (1.8 kDa and 10 kDa) as a hydrophobic compartment by Huang et al. [98]. The combination of DOX and VEGF siRNA (siVEGF) co-delivered by PEI-SA micelles produced a promising *in vivo* Huh-7 tumor growth inhibition effect over 30 days. Tang et al. constructed an amphiphilic polymer, polyethyleneimine-block-poly((1,4-butanediol)-diacrylate-*b*-5-hydroxyamylamine) (PEI-PDHA), based on PEI 1.8 kDa [46]. Together with PEG-PDHA, this polymer self-assembled into nanoparticles co-loaded with siSnail, siTwist, and PCT. Significantly, an enhanced cytotoxic effect was observed at a PCT concentration of 50  $\mu\text{g/mL}$ . The IC<sub>50</sub> of the nanoparticles loaded with the three agents was 54.7-fold lower than that of free PCT.

PAMAM dendrimer is another candidate for siRNA delivery. The siRNA molecules are complexed via the primary amines on the surface of PAMAM dendrimers. Hydrophobic compartments used to load hydrophobic drugs can also be attached on the molecules through chemical conjugation. Biswas et al. prepared a tri-block co-polymer PAMAM-PEG2k-DOPE by conjugation between G4 PAMAM and DOPE-modified PEG [99]. PAMAM-PEG2k-DOPE self-assembled into micellar nanoparticles that complexed siRNA on the surface via the PAMAM moiety and encapsulated DOX base into the hydrophobic core. This co-delivery system efficiently delivered siRNA and DOX into cells and downregulated green fluorescent proteins (GFP) used to monitor the transfection efficacy in C166-GFP cells.

Another promising polymer for siRNA and chemotherapeutics co-delivery is PDMAEMA. Modification in the backbone of PDMAEMA not only decreased the cytotoxicity of the polymer, but also gave it the ability to load both siRNA and hydrophobic drugs [93,100]. Zhu et al. synthesized the block co-polymer PDMAEMA-PCL-PDMAEMA by free radical reversible addition-fragmentation

chain transfer polymerization and assembled it into cationic micelles. PCT and siBcl-2 were delivered simultaneously using this system to PC3 cells, resulting in a 20% increased cytotoxicity than with free PCT after 24 h' treatment [100]. Wang et al. also delivered siBcl-2 together with DOX using a PEG-PDEA-PDMA-DDAT triblock co-polymer. Nanoparticles loaded with both DOX and siBcl-2 increased the cytotoxicity by 27.5% and 19.8% compared to nanoparticles loaded only with Dox or only siBcl-2, respectively [41].

### 3.2.2. Co-Delivery of pDNA and Chemotherapeutics with Polymeric Nanoparticles

Plasmid DNA (pDNA) was also delivered into cells after forming complexes with cationic polymers such as PEI, chitosan and PDMAEMA [101]. After modifying polymers with hydrophobic moieties, these polymers were widely applied for co-delivery of pDNA and hydrophobic chemotherapeutic agents. In 2006, Wang et al. developed PCT and interleukin-12 encoding pDNA co-delivery systems based on p(MDS-co-CES), which is poly{[(N-methyl-dietheneamine sebacate)-co-[(cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium bromide] sebacate}. PCT was loaded inside the hydrophobic core during self-assembly, and pDNA was complexed with the cationic moieties. P(MDS-co-CES) micelles co-loaded with PCT and pDNA resulted in a greater tumor reduction than treatment with the gene or drug alone in a 4T1 mouse breast cancer model [102].

An abundance of primary amines makes branched PEI an ideal candidate for interaction with the large number of phosphate groups found on pDNA. Similar to the PEI-PCL used for co-delivery of siRNA and DOX, PEI1.8k-PCL was also reported as a co-delivery system for pDNA and hydrophobic drug by Qiu et al. [103]. After loading with both DOX and pDNA encoding luciferase reporter gene, PEI-PCL self-assembled into nanoparticles. Higher gene transfection efficacy than PEI25k and enhanced cytotoxicity compared to DOX alone were achieved in HepG2 [103]. Based on this structure, Shi et al. crafted a triblock co-polymer which consisted of mPEG5k-PCL2k-PEI2k for the delivery of DOX and Msurvivin T34A encoding pDNA [104]. By reducing the proliferation of tumor cells through Msurvivin T34A-induced caspase-mediated apoptosis, the author showed a higher anti-tumor effect compared to micelles loaded with DOX or pDNA alone. The author also suggested that treatment with dual-loaded micelles may allow lower doses of chemotherapeutics while maintaining a similar therapeutic outcome and help mitigate the toxic effect associated with high dose chemotherapeutics [104].

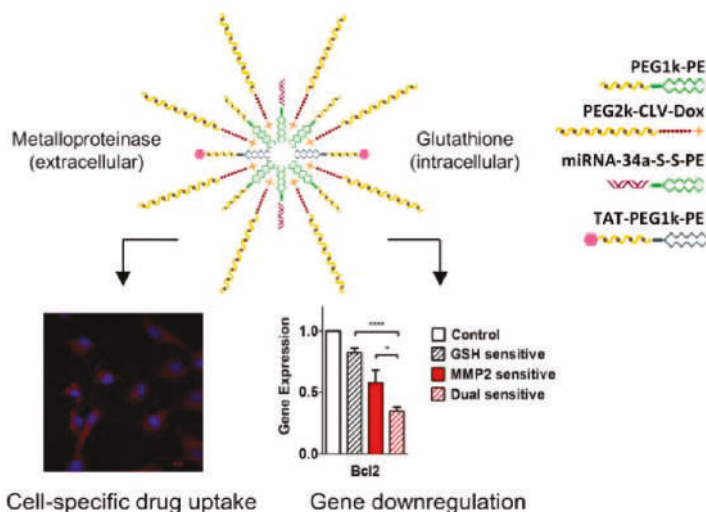
### 3.2.3. Co-Delivery of miRNA and Chemotherapeutics with Polymeric Nanoparticles

MiRNAs regulate the multiple molecular pathways associated with cancer development with a high degree of biochemical specificity and potency [85]. Combination of miRNA with small molecule therapeutics has provided an unprecedented opportunity to improve therapeutic efficacy in a broad range of human cancers [105–108]. Using vectors identical for siRNA delivery, modified cationic polymers have been widely applied for co-delivery of miRNA and small molecule drugs. Mitall et al. conjugated gemcitabine (GEM) and complexed miR-205 mimics on a mPEG-b-PCC-g-GEM-g-DC-g-CAT co-polymer, which self-assembled into polymeric micelles. GEM and miR-205 mimics were co-delivered at concentrations of 500 nM and 100 nM in vitro. Co-delivery of both drugs in polymeric micelles reduced tumor cell migration and restored chemosensitivity to gemcitabine in resistant MIA PaCa-2R pancreatic cancer cells. Intratumor injection of miR-205 (1 mg/kg)/gemcitabine (40 mg/kg) micelles to mice bearing MIA PaCa-2R xenografts potentially arrested tumor growth, whereas free gemcitabine or gemcitabine micelles had only a modest effect [109].

Hyaluronic acid-chitosan nanoparticles used to co-encapsulate DOX and miR-34a were reported by Deng et al. for treatment of triple negative breast cancer. Combinations of 100 nM of miR-34a and 0.1/0.5/2.5  $\mu$ M of DOX were investigated for their cytotoxic effect on MDA-MD-231 cells. Nanoparticles loaded with both drugs significantly increased cytotoxicity at all three DOX concentrations. A superior in vivo efficacy of the combination therapy with 5 mg/kg of DOX and

2 mg/kg of miR-34a was further demonstrated in mice bearing MDA-MB-231 subcutaneous xenografts following intravenous administration of the co-delivery nanoparticles every two days [110]. Similarly, Wang et al. prepared hyaluronic acid coated PEI-PLGA nanoparticles as a polymeric co-delivery system for miR-542-3p and DOX. A range of DOX concentrations from 0.25 µg/mL to 2 µg/mL was investigated for their therapeutic efficacy with 100 nM of miR-542-3p. The highest cytotoxicity increase, compared to nanoparticles loaded with DOX only, was at a combination of 2 µg/mL of DOX and 100 nM of miR-542-3p in triple negative breast cancer cell lines [111].

In addition to nanoparticles composed of cationic polymers, neutral nanoparticles have also been used to co-encapsulate miRNA and chemotherapeutics. As reported by Salzano et al., miRNA-34a was conjugated with phospholipids through a disulfide bond, and DOX was conjugated with PEG through a metalloproteinase 2 (MMP-2)-sensitive peptide (Figure 3). Conjugates were formulated into dual-stimuli sensitive micellar nanoparticles that can simultaneously deliver DOX and miRNA-34a. The combination of both drugs reduced HT1080 cell viability to 40% and 50% in monolayer and 3D spheroids tumor models, respectively [48]. Liu et al. incorporated miR-200c and docetaxel (DTX) into the PEG-gelatinase cleavable peptide-poly(ε-caprolactone) (PEG-pep-PCL) nanoparticles. The concurrent delivery of 10 mg/kg of DTX and 10 mg/kg of miR-200c markedly potentiated the anti-tumor efficacy of DTX in vivo [112].



**Figure 3.** MMP-2 and glutathione sensitive polymeric nanoparticles used for co-delivery of DOX and miRNA-34a. Reproduced with permission from Salzano et al., Small; John Wiley and sons, 2016 [48].

\*  $p \leq 0.05$ , \*\*\*\*  $p \leq 0.0001$ ,  $n = 3$ , error bars represent mean  $\pm$  SD.

#### 4. Dose/Efficacy Relationship within Co-Delivery Systems

Different combination delivery systems of anti-cancer drugs within a single polymeric vesicle have been discussed in previous sections. The aim of these polymeric system is to leverage the different mechanisms of the individual therapeutic agent for additive or synergistic therapeutic effects. However, eventual success is determined by several other important factors that deserve consideration in designing polymeric co-delivery systems. First, binding of pDNA onto polymeric nanoparticles affects the DOX loading and vice versa. Xu et al. reported that co-encapsulation of p53 pDNA and DOX within PLA coated PLGA microsphere resulted in significantly reduced encapsulation efficiency of DOX [113]. However, similar hurdles were less obvious in carriers loaded with RNA and DOX. Second, another factor that influences the therapeutic outcome can be ascribed to the drug release profiles of different payloads. Depending on the interaction between drugs and polymers, drug delivered by

hydrophobic interaction may be released much faster than those conjugated with polymers under physiological conditions [114]. Thus, the drug release profile of polymeric nanoparticles is one of the most crucial factors to consider when designing polymeric co-delivery systems [79,115]. Third, due to different mechanisms of therapeutic agents, the therapeutic efficacy of one drug may disguise the effect of others. Zhang et al. screened the IC<sub>50</sub> of DOX and curcumin (CUR) at different ratios using SMMC 7721 cells after 48 h treatments. The IC<sub>50</sub> value for DOX and CUR in cells treated with a combination of DOX/CUR at ratio of 1:10 decreased to 0.46  $\mu$ M and 4.65  $\mu$ M, respectively, compared to IC<sub>50</sub>'s of free DOX at 1.30  $\mu$ M and free CUR at 25.7  $\mu$ M [115]. Much research requires a focus on optimizing the drug ratio for the optimal synergistic effect prior to co-loading multiple drugs into nanoparticles.

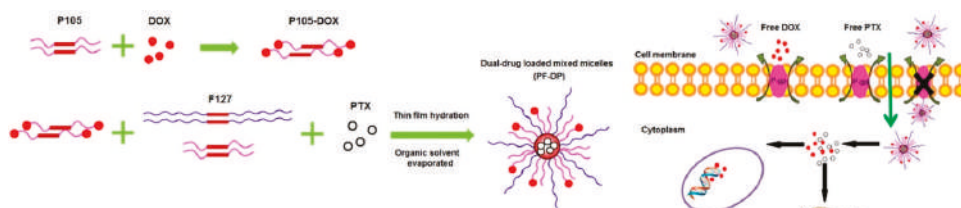
We would like to further elaborate on how drug combinations co-delivered are related to their therapeutic outcomes by concentrating on the dosing ratios between different therapeutic agents loaded in polymeric nanoparticles. Among the research published within the past decade, certain drug combinations have been widely used as models for developing co-delivery systems, including DOX, PCT, CUR, siBcl-2, siMDR-1. Although identical in drug combination, the therapeutic outcomes of polymeric co-delivery systems vary between cells and types of polymers. Here, we reviewed the factors that influence the therapeutic outcomes, with the aim of providing guidance in the design of polymeric co-delivery systems.

#### 4.1. Dose Combinations of Chemotherapeutics in Polymeric Co-Delivery Systems

##### 4.1.1. Doxorubicin and Paclitaxel Combinations

Combining two chemotherapeutics in a single carrier has been a popular approach in designing polymeric co-delivery systems. To validate the efficacy of these systems, a lot of work has to be done to characterize the relationship between the therapeutic outcome with two agents and the ratio of the two agents. In Table 2, drug combinations, at which synergistic anti-cancer effect was achieved, were listed. DOX and PCT are two classic chemotherapeutic agents that have been applied extensively in various polymeric systems. Combination delivery of both DOX and PCT are attractive for their achievement of a higher therapeutic outcome. Wang et al. loaded hydrophilic DOX and hydrophobic PCT in methoxyl PEG-PLGA co-polymer nanoparticles. The mPEG-PLGA co-loaded with both DOX and PCT produced greater tumor growth inhibition *in vitro* than mPEG-PLGA loaded with either DOX or PCT at the same concentrations. Moreover, the highest anti-tumor efficacy was achieved when DOX and PCT were loaded at a concentration ratio of 2:1 using three different types of tumor cell line [79].

Xu et al. prepared an amphiphilic poly(ethylene glycol)-poly(L-lactic acid) (PEG-PLA) diblock co-polymer and incorporated DOX and PCT into the ultrafine PEG-PLA fibers by an “emulsion-electronspinning” method [116]. The authors showed a lower cell viability and higher percentage of cell cycle arrest when DOX and PCT were delivered at a concentration ratio of 1:1 to rat glioma C6 cells 72 h after treatment. Chen et al. conjugated Pluronic P105 with DOX and encapsulated PCT into the hydrophobic core formed by P105-DOX and Pluronic F127 as a co-delivery system for hydrophobic DOX and PCT (Figure 4). The ratio between DOX and PCT was 2:3, when a higher *in vitro* cytotoxicity was observed in both MCF7/ADR and KBv cell lines. An increased apoptotic event, S phase cell cycle arrest and enhanced spheroid growth inhibition were observed in MCF7/ADR cells. The *in vivo* study also effected an efficient tumor growth inhibition over 14 days at this drug ratio [117]. Similarly, Ma et al. investigated the performance of pH-sensitive Pluronic F127-grafted chitosan for delivery of DOX together with PCT *in vivo*. In their study, 25 mg/kg of DOX and 20 mg/kg of PCT were used. All these studies used a concentration ratio between DOX and PCT of about 1:1 [118].



**Figure 4.** Co-delivery of DOX and PCT in polymeric nanoparticles consist of P105 and Pluronic F127 into MCF7/ADR cells. Reproduced with permission from Chen et al., International Journal of Pharmaceutics; Elsevier, 2015 [117].

However, other groups have held a different opinion on the optimal drug ratio between DOX and PCT loaded in polymeric vehicles. Duong et al. also prepared a PEG-PLGA based co-polymer system for delivery of hydrophobic DOX base and PCT. In addition, folic acid and TAT peptide were included to enhance the cellular interaction between PEG-PLGA micelles and a human oral cavity carcinoma KB cell line. After screening several different combinations of DOX and PCT and calculated their effectiveness using Calcsyn software, they demonstrated a better synergistic effect of DOX and PCT at a concentration ratio of 1:0.2 than of a concentration ratio of 1:1 [119]. This ratio is supported by Lv et al.'s study. In their case, an amphiphilic deoxycholate decorated methoxy poly(ethylene glycol)-b-poly(l-glutamic acid)-b-poly(l-lysine) triblock co-polymer (mPEG-b-PLG-b-PLL) was synthesized and developed as a nano-vehicle for co-delivery of DOX and PCT. The DOX and PCT co-loaded nanoparticles at a concentration ratio of 4:1 showed an obvious synergistic effect. The  $CI_{50}$  value was approximately 0.57, indicating co-delivery of DOX and PCT had evident superiority in tumor growth inhibition as compared with free drug combinations [120]. In an animal study, 4 mg/kg of DOX and 1 mg/kg of PCT loaded in nanoparticles were given to animals every four days. An efficient tumor growth inhibition was observed over 18 days in a A549 xenograft tumor model. The tumor volume of the co-loaded nanoparticles-treated group was only 9.0% of the control group at the 18th day, which was 3.2-fold, 6.3-fold, and 2.4-fold smaller than when treated with free DOX, free PCT and free DOX + PCT, respectively [120].

Although various drug combinations were used among these different cases to achieve a synergistic effect, one conclusion shared by these results is that control of the amount of DOX used is a prominent factor. DOX has a faster release rate than PCT, and the release of DOX facilitates the release of PCT. No significant synergistic effect was observed when PCT was used in excess [119]. Additionally, PCT inhibited the tumor growth by stabilizing the microtubule during cell mitosis. Cells were arrested rather than entering an apoptotic pathway when treated with DOX. Thus, synergistic effects can hardly be observed 24–48 h after treatment because the effect of DOX overrode the effect of PCT [79].



Table 2. Combinations of chemotherapeutics delivered in polymeric co-delivery systems.

Polymers	Drug 1	Concentration 1	Drug 2	Concentration 2	Cell line	Ref.
HA	CPT	0.05–5.0 µM	DOX	0.22–0.5 µM	BT-474,	[81]
HA	CPT	0.04–0.45 µM	DOX	0.02–0.4 µM	bEnd.3	[81]
PLL-PTX <sup>1</sup> , HA30k-GEM <sup>2</sup>	GEM	1 × 10 <sup>−4</sup> –1.0 mM	PCT	1 × 10 <sup>−4</sup> –1.0 mM	SCK	[121]
PEG-soyPC-PLGA)	DOX	1–50.0 ng/mL	triptolide (TPL)	0.05–1 folds over DOX	HuCC11	[122]
PEG-PLGA	CUR	5–15 µM	Chrysin	15–45 µM	KB	[123]
DOX-PEG-GEM	DOX	0.001–100 µM	GEM	0.001–100 µM	Caco-2	[124]
TPGS-PAE <sup>3</sup>	DOX	0.031–1.0 µM	CUR	0.312–1.0 µM	SKOV-3, MCF-7, MDA-MB-231	[115]
PEG-P(Glu)-P(Phe) <sup>4</sup>	PCT	0.0041–3.0 µg/mL	CDDP	0.94–60 µg/mL	SMMC7721	[125]
PSn(P2VP-b-(PAA-g-PNIPAM)) <sup>5</sup>	PCT	1–15 µg/mL	camptothecin	1–15 µg/mL	HeLa, A549	[126]
PLGA	PCT	10 µM	tetrandrine	10 µM	A549	[127]
Polyphosphazene	DOX	12.5 µg/mL	CQ	1:1 and 2:1 over DOX	A2780	[128]
mPEG-PLGA	DOX	Various ratios	PCT	Various ratios	MCF7/ADR and HL60/ADR	[79]
P(MeOx-b-BuOx-b-MeOx) <sup>6</sup>	PCT	0–1 µg/mL	alkylated CDDP	0–2.5 µg/mL	A549, HepG2, B16	[129]
PEG-P(Glu)-P(Phe)	PCT	CDDP/PCT = 10:1	CDDP	0–10 µg/mL	LCC-6-MDR, A2780 A2780/CisR	[130]
PHBV-PLGA	Ox	5 mg/kg	In vivo			
HA	CPT	2 mg/kg	5-FU	25 mg/kg	CT26	[131]
PLL-PTX, HA30k-GEM	GEM	108.8 µg/animal	DOX	1.05 mg/kg	4T1	[81]
Pluronic F127-chitosan	DOX	25 mg/kg	PCT	54 µg/animal	HuCC11	[121]
TPGS-PAE	DOX	1 mg/kg	CUR	20 mg/kg	Healthy rat	[118]
PEG-P(Glu)-P(Phe)	PCT	3 mg/kg	CDDP	10 mg/kg	SMMC 7721	[115]
Polyphosphazene	DOX	15 ng/animal	CQ	15 ng/animal	A549	[125]
PPBV <sup>7</sup>	PCT	4 mg/kg	CUR	10 mg/kg	MCF7/ADR	[128]
P(MeOx-b-BuOx-b-MeOx)	PCT	20 mg/kg	alkylated CDDP	20 mg/kg	MCF7	[132]
PEG-P(Glu)-P(Phe)	PCT	4 mg/kg	CDDP	4 mg/kg	A2780/CisR	[129]
					A2780/Luc	[130]

<sup>1</sup> Poly (l-lysine)-carboxylate pacitaxel. <sup>2</sup> Hyaluronic acid-gemcitabine. <sup>3</sup> D-α-tocopheryl poly(ethylene glycol) 1000-block-poly (β-amino ester). <sup>4</sup> Poly(ethylene glycol)-b-poly (l-glutamic acid)-b-poly(l-phenylalanine). <sup>5</sup> Star-graft quarterpolymers, composed of hydrophobic polystyrene and pH-sensitive poly(2-vinylpyridine)-b-poly(acrylic acid). <sup>6</sup> Poly(2-methyl-2-oxazoline-block-2-butyl-2-oxazoline-block-2-methyl-2-oxazoline). <sup>7</sup> Poly(ethylene glycol)-benzoic imine-poly(γ-benzyl-L-aspartate)-b-poly(1-vinylimidazole) block copolymer.

#### 4.1.2. Doxorubicin and Curcumin Combinations

Another classis combination is DOX and CUR. CUR was believed to inhibit the tumor growth by causing cell cycle arrest [133,134], inducing an apoptotic signal [135,136], reversing multidrug resistance [137] and inhibiting the activation of NF- $\kappa$ B [138,139]. The exact molecular mechanisms of curcumin-induced apoptosis in cancer cells varied and depended on the cell type and dose used [140]. Application of CUR as an adjuvant in co-delivery systems has aroused great interest [141].

Zhang et al. combined pro-apoptotic, anti-angiogenic activities in pH-sensitive nanoparticles prepared with D- $\alpha$ -tocopherol poly(ethylene glycol)1k-block-poly ( $\beta$ -amino ester) (TPGS-PAE) polymers. The authors optimized the concentration of DOX and CUR at a 1:10 ratio. When exposed to 0.25  $\mu$ M of DOX together with 2.5  $\mu$ M of CUR, a 45% increased cytotoxicity occurred over that treated with DOX alone after 48 h in human SMMS 7721 liver cancer cells. The same ratio was also used in an in vivo study, where 1 mg/kg of DOX and 10 mg/kg of CUR were delivered within TPGS-PAE nanoparticles given intravenously once every other day. The CUR + DOX loaded nanoparticles induced a tumor weight suppression of 73.4%, compared to the 32.6% in a free CUR + DOX group [115].

Yan et al. also investigated the performance of a DOX CUR co-delivery system in a human liver cancer cell line, Hep G2. They came to a contradictory conclusion from Zhang's. regarding the drug ratio between DOX and CUR needed to reach a synergistic effect [142]. They prepared a redox-responsive micelle composed of a glycyrrhetic acid-modified chitosan-cystamine-poly( $\epsilon$ -caprolactone) co-polymer (PCL-SS-CTS-GA). DOX and CUR were loaded in the PCL-SS-CTS-GA nanoparticles at mole ratios of DOX:CUR = 1:1, 2:1 and 3:1. A synergistic effect was observed only at ratio of 2:1 and 3:1 in a Hep G2 cell line [142]. This result was similar to that reported by Zhang et al., who conjugated DOX with methoxy-poly(ethylene glycol)-aldehyde (mPEG-CHO) and encapsulated CUR into the hydrophobic core formed within the mPEG-DOX micelles. They used a 2.5-fold higher concentration of DOX compared to CUR corresponding to a mole ratio of DOX:CUR = 1.6:1. In both Hep G2 and HeLa cell lines, an enhanced cytotoxic effect was observed in the mPEG-DOX-CUR nanoparticle-treated group compared to cells treated with DOX or CUR alone [143].

In other studies where CUR was utilized in an attempt to overcome multidrug resistance, Wang et al. studied the effect of co-delivery of DOX and CUR in mice bearing MCF7/ADR or 4T1 tumors. The polymeric micelles for DOX and CUR co-delivery were based on two diblock co-polymers, D- $\alpha$ -tocopherol polyethylene glycol succinate (TPGS) and PEG2k-DSPE. Cells were incubated with polymeric micelles containing both drugs at a 1:1 ratio (mole ratio: DOX:CUR = 1:1.6) for 48 h. Over a concentration range of DOX of 0.5  $\mu$ g/mL to 40  $\mu$ g/mL, the maximum cytotoxicity increase was observed at 10  $\mu$ g/mL. An 18.3-fold increase of apoptotic events was also observed at 10  $\mu$ g/mL of DOX in the group treated with polymeric micelles loaded with DOX and CUR compared with the one treated with DOX only. A formulation co-loaded with 5 mg/kg of DOX and 5 mg/kg of CUR injected intravenously every two days resulted in a significantly lower tumor volume after a 10-day treatment than those treated with either DOX or CUR alone [144]. Duan et al. co-encapsulated 0.12  $\mu$ g/mL of DOX and 0.2  $\mu$ g/mL of CUR (mole ratio: DOX: CUR = 1:2.6) in chitosan/poly(butyl cyanoacrylate) nanoparticles to reverse the multidrug resistance in MCF7 ADR cell lines. They reported that polymeric nanoparticles loaded with both drugs induced a higher cell growth inhibition and a significantly greater downregulation of MDR-1 protein at 48 h after treatment [145].

Overall, the mole ratio of CUR and DOX reported for these polymeric delivery systems had a broad range, suggesting that in the presence of multiple tumor inhibiting pathways, CUR acts differently depending on the cell line. Thus, it's hard to predict an effective universal ratio for combination treatment with CUR plus DOX. Additionally, the release profile of CUR differs markedly between polymeric delivery systems [146,147]. In some cases, DOX is directly conjugated with the polymer, while in other cases, DOX and CUR are both passively encapsulated. The release profile and types of tumor are key points to be taken into consideration during the design of polymeric co-delivery systems using CUR.

#### 4.1.3. Paclitaxel and Cisplatin Combination

The use of a combination of PCT and cisplatin (CDDP) is another classic and popular co-delivery strategy. Cisplatin, a member of the platinum-containing anticancer drugs, has been used intensively for the treatment of various solid tumors, particularly in advanced stages. There are different mechanisms that account for the effect of CDDP. It is well-known as a non-specific DNA-modifying agent that induces cell apoptosis by interaction with nuclear DNA to inhibit the transcription and replication of DNA. The clinical application of CDDP is limited because of its high dose-dependent toxicity, drug resistance, and low bioavailability. PCT, on the other hand, a number of the taxane family, works as a microtubule-stabilizing chemotherapeutic agent. It is known that PCT can also inhibit platinum–DNA adduct repair and enhance apoptosis of cisplatin-resistant tumor cells [148]. However, it enhances the nephrotoxicity of CDDP. Nowadays, the combination therapy including CDDP and PCT is still a popular clinical regimen used as a the first-line chemotherapeutic agent for advanced cancer treatment.

To date, few works have dwelt on the study of co-delivery of the CDDP/PCT, due mainly to the hydrophilic nature of CDDP and hydrophobicity of PCT, which has made the loading step very challenging [149,150]. One strategy to overcome this problem is to use different cisplatin prodrugs to facilitate drug loading [151,152]. Polymeric nanoparticles, particularly micelles with the ability to carry both hydrophilic and hydrophobic drugs represent a promising candidate for the co-delivery of CDDP and PCT. Mi et al. used a D- $\alpha$ -tocopherol-co-poly(ethylene glycol)1k succinate (TPGS)-cisplatin prodrug (TPGS-CDDP) along with docetaxel to improve CDDP loading into Herceptin-decorated PLA-TPGS nanoparticles. The highest drug loadings achievable for CDDP, PCT, and Herceptin using this approach were  $3.5 \pm 0.1$ ,  $9.0 \pm 0.5$ , and  $73.1 \pm 5.8$   $\mu\text{g}/\text{mg}$ , respectively [151]. The presence of polymers with the ability to form a chelate with CDDP, such as PEG or poly-glutamic acid in the copolymer structure, can also enhance drug loading capacity [125,129,130]. In He et al.'s work, the drug loading capacity of PCT and CDDP attainable with FA-PLGA-PEG micelles has been reported to be  $5.83 \pm 0.04\%$ ,  $4.68 \pm 0.07\%$ , respectively, at a polymer/drug ratio of 40:2:4 (polymer:CDDP:PCT) [153].

It is important to note that, the ratio of drug loading in the carrier plays a determinant role in achieving the highest therapeutic efficacy for combination therapy. According to Wan et al., the actual drug ratios in the tumor did not differ significantly from the drug ratios in the initial co-loaded drug formulations [129]. Thus, assuring the drugs loading for polymeric nanoparticles in an appropriate ratio would be of great importance in formulating a treatment. He et al. showed that in A549 and M109 cells, the effectiveness of co-delivery of NPs, with a CDDP/PCT concentration ratio of 1:2, was approximately twice that of CDDP [153]. Moreover, co-delivery of NPs, with a CDDP/PCT concentration ratio of 1:2, exhibited the greatest anti-tumor activity among the two varieties of lung cancer cell. Although in general, dual drug-loaded nanoparticles with a higher ratio of PCT exhibited the greatest response, there are some reports indicating the best response corresponds to an equal drug ratio [152].

Time-dependency is another factor affecting the synergism of CDDP/PCT co-delivery systems [125]. This phenomenon was related to the release behavior of CDDP and PCT from the carrier. In single drug-loaded nanoparticles, drug is quickly released, while in a dual drug-loaded carrier system, chelation of PCT with CDDP prevented an initial burst release and resulted in a lower growth inhibition effect. However, over time, more PCT was released, and the combination effect became manifest. The in vivo studies on co-delivery systems for CDDP and PCT also clearly revealed improved pharmacokinetics and biodistribution in the blood and tumor of either one or both drugs compared to single drug micelles [129].

#### 4.2. Dose Combinations of Nucleic Acid Therapeutics and Chemotherapeutics in Polymeric Co-Delivery Systems

The rationale for combination of nucleic acid therapy with chemotherapeutics in a single platform to ensure that cells will be simultaneously exposed to two types of damage has been discussed

previously. The dose combination between nucleic acids and chemotherapeutics delivered has large effects on the synergistic anti-cancer efficacy of such co-delivery systems (Table 3). Nucleic acid therapy induces tumor inhibitory effects via different molecular pathways than traditional chemotherapeutics. Plasmid DNA requires delivery into the nucleus followed by translation into therapeutic proteins and the oligonucleotide requires interference with the mRNA or proteins in various pathways to generate their effect. Thus, nucleic acid therapy takes a longer time than chemotherapeutics before the tumor growth inhibition effect can be observed. The effect of chemotherapeutic agents can also disguise the one from nucleic acid therapy. Therefore, controlling the ratio between nucleic acid molecules and chemotherapeutics involving different mechanisms and types of pathways in polymeric systems is critical for an ideal synergistic effect.

#### 4.2.1. Inducing Apoptosis through Delivery of TRAIL

Interfering with the apoptotic pathway by either enhancing the pro-apoptotic effect or inhibiting the anti-apoptotic effect with nucleic acid therapies is a promising cancer treatment. One of the most attractive pro-apoptotic pathways for the treatment of cancer is through the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). TRAIL is a potent stimulator of apoptosis, and tumor cells are significantly more sensitive to TRAIL-induced apoptosis than normal cells [154]. However, numerous cancer cells exhibit a certain amount of resistance to TRAIL-induced apoptosis. Thus, combining TRAIL protein with traditional chemotherapeutics could increase the therapeutic efficacy in TRAIL-resistant cancer cells [155,156]. Lee et al. co-loaded both TRAIL protein and DOX in polymeric nanoparticles self-assembled from a biodegradable cationic co-polymer P(MDS-co-CES) to achieve a synergistic cytotoxic effect in cancer cells. A synergistic effect of a 40% enhanced cytotoxicity was observed with nanoparticles loaded with 10 nM of TRAIL and 0.8  $\mu$ M of DOX compared to nanoparticles loaded with each drug alone in TRAIL-resistant SW480 colorectal carcinoma cells [157]. The same group also used P(MDS-co-CES) for co-delivery of TRAIL and PCT. In the study, various human breast cancer cell lines were exposed to 10 nM of TRAIL and 1.67  $\mu$ M of PCT co-loaded in P(MDS-co-CES) polymeric nanoparticles. A 25% enhanced cytotoxic effect was achieved compared to single drug-loaded treatments [158].

In addition to delivering TRAIL in polymeric nanoparticles, Fan et al. delivered plasmid DNA encoded for TRAIL together with DOX in  $\beta$ -cyclodextrin-polyethyleneimine (PEI-CD) supramolecular nanoparticles (SNP). The PEI-CD SNP loaded with 0.5  $\mu$ g/mL of DOX and 2.5  $\mu$ g/mL of TRAIL pDNA (pTRAIL) induced many more apoptotic events than individual drug treatment in SKOV-3 cells after 48 h treatment. At the same drug ratio, SKOV-3 ovarian tumor-bearing mice received a combination treatment of 6  $\mu$ g of DOX and 30  $\mu$ g of pTRAIL twice per week. After 18 days, a significantly higher tumor growth inhibition was observed in those mice [47]. In another study, DOX was complexed with pORF-hTRAIL, which was then complexed with polyamidoamine-PEG-T7 (PAMAM-PEG-T7) through electrostatic interaction. A significant synergistic effect occurred both *in vitro* and *in vivo* when 12.5-fold more DNA than DOX by weight was loaded into this platform. Each Bel-7402 tumor-bearing mouse was treated with 50  $\mu$ g of DNA in combination with 4  $\mu$ g of DOX [159].

**Table 3.** Combinations of nucleic acids therapeutics and chemotherapeutics delivered in polymeric co-delivery systems.

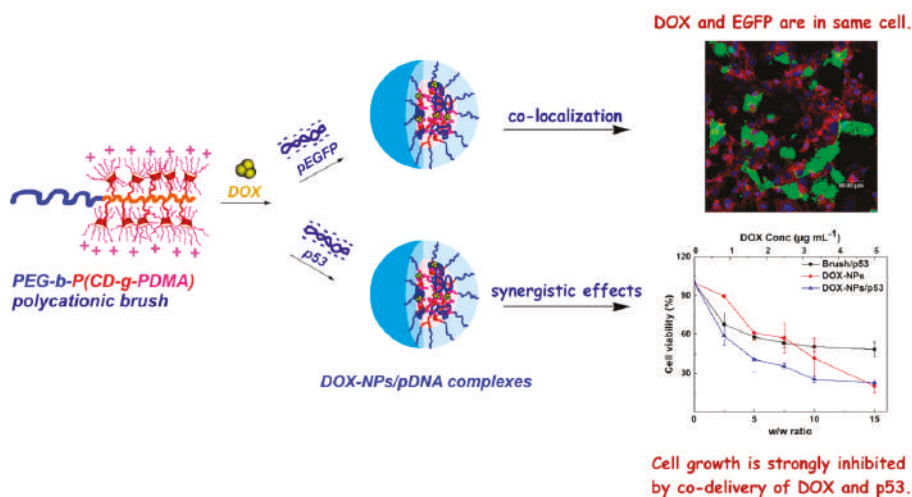
Polymers	Drug 1	Concentration 1	Drug 2	Concentration 2	Cell Line	Ref.
F(MDS)-co-CES F(MDS)-co-CES PEL-CD PLGA-PLA PDMAEMA-PMPD PEI-PCL/FA-PEG-PCA PEI-PCL/FA-PEG-PCA PDEA-PDMA-PEG PEG-PLL-PASP F(MDS)-co-CES PDMAEMA-PCL-PDMAEMA PEI-CyD PEG-pp-PEI-DOPE PEI-PLA/PEG-PASP PEO-b-PCL PEI-DOPE PAMAM-PEG-DOPE PAMAM-PEG-DOPE NSC-PLL-PA PEG-PLG-PDMAEMA DMAE-PLGA HA/PEI-PLGA PEG-PLGA-PLL PEG-PCC-GEA-DC-CAT PCL-PEG-PHIS	TRAIL	10 nM	DOX	0.8 µM	SW480-TR	[157]
	TRAIL	10 nM	PCT	1.67 µM	MCF7, T47D, MDA-MB-231	[158]
	TRAIL pDNA	2.5 µg/mL	DOX	0.5 µg/mL	SKOV-3	[47]
	p53 gene	4 µg/mL	DOX	0.9 µg/mL	Hep G2	[160]
	siBcl-2	20 nM	DOX	3 µg/mL	MCF7	[161]
	siBcl-2	25 nM	DOX	50 nM	Bel-7402	[96]
	siBcl-2	20 nM	DOX	0.5 µg/mL	C6	[162]
	siBcl-2	20 nM	DOX	1.69 µM	Hep G2	[41]
	siBcl-2	20 nM	DOX	0.6 µg/mL	Hep G2	[42]
	siBcl-2	20 nM	PCT	100/400 nM	MDA-MB-231	[102]
	siBcl-2	188 nM	PCT	0.58 µM	PC3	[100]
	shSurvivin	2 µg/mL	PCT	0.6 µg/mL	SKOV-3	[163]
	siSurvivin	150 nM	PCT	12–24 nM	A549 T24	[58]
	siSurvivin	20 nM	PCT	0.096 µg/mL	A549	[44]
In vitro	siMDR-1	100 nM	DOX	5 µg/mL	MDA-MB-435	[45]
	siMDR-1	100 nM	DOX	1 µg/mL	MCF7/ADR	[97]
	siMDR-1	125 nM	DOX	1.7 µg/mL	A2780/ADR	[91]
	siMDR-1	125 nM	DOX	0.43 µg/mL	MCF7/ADR	[91]
	siMDR-1	100 nM	DOX	5 µg/mL	Hep G2/ADM	[164]
	siMDR-1	100 nM	DOX	3 µg/mL	MCF7	[52]
	siMDR-1	100 nM	DOX	11.6 µg/mL	MCF7/ADR	[165]
	miR-542-3p	100 nM	DOX	2 µg/mL	MDA-MB-231	[111]
	miR-7	100 nM	PCT	0.01 µg/mL	HO8910pm	[166]
	miR-205	100 nM	GEM	500 nM	MIA PaCa-2R, CAPAN-1R	[109]
	siVEGF	100 nM	PCT	2 µg/mL	MCF7	[167]
In vivo	TRAIL pDNA	30 µg/animal	DOX	6 µg/animal	SKOV-3	[47]
	pORF-hTRAIL	50 µg/animal	DOX	4 µg/animal	Bel-7402	[159]
	p53	1.5 mg/kg	DOX	2 mg/kg	MCF7	[161]
	siBcl-2	1.6 µg/kg	DOX	22.5 µg/kg	C6	[162]
	siBcl-2	200 µg/kg	DOX	1 mg/kg	Hep G2	[42]
	Msurvivin	5 mg/kg	DOX	4 mg/kg	B16-F10	[104]
	shSurvivin	6 µg/animal	PCT	20 µg/animal	SKOV-3	[163]
	shSurvivin	2 mg/kg	PCT	10 mg/kg	A549	[168]
	shSurvivin	2 mg/kg	DOX	6 mg/kg	MCF7/ADR	[169]
	siSurvivin	20 nM	PCT	0.096 µg/mL	A549	[44]
	siMDR-1	0.2 mg/kg	DOX	0.5 mg/kg	Hep G2/ADM	[164]
	siMDR-1	1.2 mg/kg	DOX	2 mg/kg	MCF7/ADR	[170]
	IL-36γ	50 µg/animal	DOX	5 mg/kg	4T1.2	[171]
	IL-2	1.2 µg/animal	DOX	2 mg/kg	SMMC7721	[171]
F(MDS)-co-CES Trimethyl Chitosan PLGA/Pluronic F127 PEG-PLGA-PLL PEG-PCC-GEA-DC-CAT PCL-PEG-PHIS PEI-SA	IL-2	5 µg/animal	PCT	10 µg/animal	4T1	[102]
	IL-2	2.5 µg/kg	PCT	10 mg/kg	B16-F10	[172]
	miR-7	2 mg/kg	PCT	6 mg/kg	HO8910pm	[166]
	miR-205	1 mg/kg	GEM	40 mg/kg	MIA PaCa-2R	[109]
	siVEGF	5 mg/kg	PCT	1.2 mg/kg	MCF7	[167]
	siVEGF	0.3 mg/kg	DOX	0.45 mg/kg	Huh7	[98]

#### 4.2.2. Increasing Apoptosis by Restoring p53

A similar combination was also used to restore the function of the tumor suppressor, p53. Xu et al. treated Hep G2 cells with a combination of 2  $\mu\text{g/mL}$  of p53 gene and 0.9  $\mu\text{g/mL}$  of DOX. Chitosan-p53 nanoparticles and DOX molecules were co-loaded in double-walled microspheres that consisted of a PLGA core surrounded by a poly(L-lactic acid) (PLA) shell. Overall, the combined DOX and p53 treatment enhanced cytotoxicity and increased activation of caspase-3 compared to either DOX or p53 treatment alone [160].

Li et al. used a combination of DOX and p53 plasmid at 3  $\mu\text{g/mL}$  and 4  $\mu\text{g/mL}$ , respectively, to induce a synergistic anti-cancer effect in breast cancer. Both drugs were loaded in micelles formed from a star-shaped polymer consisting of a cationic poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA) shell and a zwitterionic poly[N-[3-(methacryloylamino) propyl]-N,N-dimethyl-N-(3-sulfopropyl) ammonium hydroxide] (PMPD) corona that was grafted from a polyhedral oligomeric silsesquioxane (POSS)-based initiator. A high tumor cell apoptosis in MCF7 cells occurred in vitro and extensive tumor growth inhibition was observed over 28 days with 1.5 mg/kg of DOX and 2 mg/kg of p53 plasmid administrated every five days [161].

Usually, 2–4  $\mu\text{g/mL}$  of plasmid DNA was used in co-delivery with chemotherapeutics to achieve a synergistic effect. But the ratio between chemotherapeutics and pDNA ranged from 1–10. Chen et al. investigated the response of cell viability in MCF7 cells to DOX-loaded polymeric nanoparticles complexed with different ratios of p53 pDNA (Figure 5) [173]. An enhanced cell growth inhibition was observed at weight ratios between DOX-NP and p53 pDNA from 5–10. This enhanced effect was not observed at weight ratios higher than 10 or lower than 5. These results suggested that the ratio between chemotherapy and pDNA for a synergistic effect could be affected by a diversity of cell lines and types of cationic polymers. Especially because that, as previously discussed, the complexation of pDNA with polymeric nanoparticles could affect the loading of chemotherapeutics [113]. Investigating the pDNA complexation effect on drug loading, drug release profile is essential in evaluating the anti-cancer performance of pDNA and chemotherapeutics-loaded polymeric co-delivery systems.



**Figure 5.** Co-delivery of DOX and pDNA in cationic polymeric nanoparticles with co-localization of cargos and enhanced tumor cell growth inhibition. Reproduced with permission from Chen et al., Polymers; MDPI, 2019 under the license CC BY 4.0 [173].



#### 4.2.3. Decreasing Anti-Apoptotic Effect through Downregulation of Bcl-2

Another popular candidate used within co-delivery systems with pro-apoptotic effects has been siBcl-2. Overexpression of Bcl-2 family proteins suppresses the cell death induced by chemotherapeutics [174]. Thus, it's particularly interesting to pursue possible synergistic effects derived from the downregulation of Bcl-2 and administration of chemotherapeutics. Cao et al. proposed loading DOX and siBcl-2 into PEI-PCL micelles using a chloroform/water mixture under sonication. The micelles were then coated with folic acid-conjugated poly(ethylene glycol)-block-poly(glutamic acid) (FA-PEG-PGA) after complexation of siBcl-2. Together with 20 nM of siBcl-2, 0.05  $\mu$ M of DOX was delivered to Bel-7402 liver cancer cells. The PEI-PCL/FA-PEG-PGA micelles co-loaded with DOX and siBcl-2 induced a 60% increased cytotoxicity 96 h after treatment [96]. From the same group, Cheng et al. investigated the therapeutic efficacy of PEI-PCL/FA-PEG-PGA micelles loaded with siBcl-2 and DOX using C6 glioma cells. They demonstrated that 25 nM of siBcl-2 achieved a higher knockdown effect than that at 12.5 nM. Most importantly, the knockdown effect of Bcl-2 became saturated at concentrations higher than 25 nM. The combined therapeutic outcome of DOX (22.5  $\mu$ g/kg) and siBcl-2 (1.6  $\mu$ g/kg) treatment in vivo highlighted the importance of combined therapy of DOX and siRNA for tumor growth inhibition [162].

Wang et al. also investigated the combination of DOX and siBcl-2 in another human liver cancer cell line, Hep G2, using a PDEA-PDMA-PEG co-polymer. They also used 20 nM of siBcl-2 but increased the DOX concentration to 1.69  $\mu$ M. Under this circumstance, co-delivery of both drugs increased the cytotoxicity by 27.5% and 19.8% compared to nanoparticles loaded with DOX and siBcl-2, respectively [41]. Instead of DOX, PCT has also been delivered together with siBcl-2. Wang et al. studied the synergistic effect between siBcl-2 and PCT in triple negative breast adenocarcinoma MDA-MB-231 cells. In the presence of 20 nM siBcl-2, cell viability decreased from 78% to 59% and from 58% to 39% at PCT concentrations of 100 nM and 400 nM, respectively [102].

However, other groups have used a higher concentration of siBcl-2. For example, Zhu et al. treated PC3 human prostate cancer cells with a combination of 188 nM of siBcl-2 and 0.58  $\mu$ M of PCT. The drugs were delivered using micellar nanoparticles composed of PDMAEMA-PCL-PDMAEMA triblock co-polymer. They reported a synergistic effect of about 20% increased cytotoxicity compared to free PCT at 24 h after treatment [100]. Such differences in the concentration of siBcl-2 used may have resulted from the responses to siBcl-2 seen in different cell lines. Instead of screening for the response of cells to siBcl-2, most groups have used a standard siRNA concentration of 100 nM in their studies [42,175].

#### 4.2.4. Decreasing the Anti-Apoptotic Effect through Downregulation of Survivin

Survivin is one of the most frequently occurring antiapoptotic proteins seen in cancerous tissues (i.e., breast, colon, pancreas, and lung). Its main mechanism of action depends on inhibition of caspase activation [176]. Through its action, survivin leads to increased proliferation of tumor cells [177]. Wang et al. developed a DOX, PCT, and survivin co-delivery system using a nano-emulsion composed of a methoxy-poly (ethylene glycol) block copolymer (mPEG-PLGA) and e-polylysine (EPL). The core of the nano-emulsion was DOX, and the PCT was enclosed in the hydrophobic layer. EPL on the surface of the nano-emulsion complexed siRNA by electrostatic interaction. Experiments in mice bearing a B16-F10 melanoma tumor showed a synergistic tumor growth inhibition effect from DOX (8.6 mmol/kg), PCT (17.2 mmol/kg), and survivin-siRNA (1.5 mg/kg) [178].

In Shi et al.'s report where the block copolymer mPEG-PCL-g-PEI was used for co-delivery of doxorubicin and Msurvivin T34A plasmid, a synergistic effect of DOX (4 mg/kg DOX) and Msurvivin T34A plasmid (5 mg/kg) was demonstrated in mice bearing a B16-F10 melanoma, both in subcutaneous and lung metastases models. Although they obtained only a slightly higher antitumor activity when compared to free DOX, they effectively reduced systemic toxicity of the treatment [104]. Survivin shRNA-encoding plasmid was also delivered to SKOV-3 cells by self-assembled supramolecular micelles composed of  $\beta$ -cyclodextrin-polyethylenimine (PEI600-CyD)

and 2-amineadamantine-conjugated PCT (Ada-PCT) by Hu et al. They proved that simultaneously administrated PCT and shRNA at concentrations of 0.6  $\mu\text{g/mL}$ , 2  $\mu\text{g/mL}$  in vitro and 6  $\mu\text{g/animal}$ , 20  $\mu\text{g/animal}$  in vivo, respectively, induces significantly higher cell apoptosis and inhibits tumor growth [163].

Another co-delivery pluronic system P85-PEI/TPGS/PCT/shSur containing survivin hairpin RNA was developed to treat A549 human lung cancer. The purpose of this study was to overcome paclitaxel resistance. Simultaneously administrated PCT (10 mg/kg) and shSur (2 mg/kg) showed enhanced efficacy of anticancer activity including higher PTX-induced apoptosis and cells arrested in G2/M phase [168]. A combination of DOX and survivin shRNA was also investigated by Tang's group for its effect in overcoming multidrug resistance. In their work, a pH-sensitive polymer based on poly(b-amino ester), poly[(1,4-butanediol)-diacrylate-b-5-polyethylenimine]-block-poly[(1,4-butanediol)-diacrylate-b-5-hydroxy amylamine] (PDP-PDHA) was synthesized. Nanoparticles containing 6 mg/kg DOX and 2 mg/kg shRNA were administered to MCF7/ADR tumor-bearing mice. The authors successfully raised the accumulation of DOX and shSur in the tumor tissue, resulting in a tumor growth inhibition of 95.9% after 21 days [169].

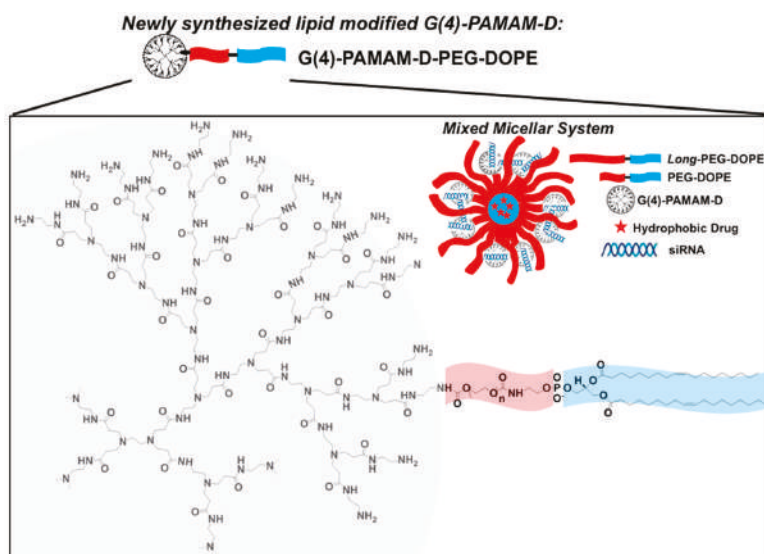
#### 4.2.5. Increasing Intracellular Drug Accumulation by Inhibiting Drug Efflux

P-glycoprotein (P-gp), encoded by the MDR-1 gene, overexpressed in many types of human cancers, contributes to the multidrug resistant effect. Downregulation of P-gp has been associated particularly with enhancing the therapeutic outcome of chemotherapeutics. Thus, it is a popular inclusion in co-delivery systems for its synergistic effect. Xiong et al. reported the co-delivery of siMDR-1 and DOX using polymeric micelles formed by poly(ethylene oxide)-block-poly( $\epsilon$ -caprolactone) (PEO-b-PCL) amphiphilic block co-polymers to improve the anticancer effect in the multidrug drug resistant human breast cancer cell line (MDA-MB-435/LCC6MDR1). Micelles containing 5  $\mu\text{g/mL}$  of DOX and 100 nM of siMDR-1 led to a maximum of ~70% cell growth inhibition at 72 h after treatment [45]. Navarro et al. also demonstrated that the downregulation of P-gp led to the inhibition of DOX efflux activity resulting in an enhanced cytotoxicity of DOX in the MCF7/ADR cell line. The combination used in their study was 1  $\mu\text{g/mL}$  of DOX and 100 nM of siMDR-1. Drugs were delivered in polymeric nanoparticles consisting of PEI modified DOPE [97].

Zhang et al. prepared polymeric micelles based on N-succinyl chitosan-poly-L-lysine-palmitic acid (NSC-PLL-PA) for co-delivery of DOX and siRNA targeting P-gp. The study revealed that the therapeutic efficacy was close to the maximum when the siRNA concentration reached about 100 nM. This finding indicated that 100 nM was sufficient to downregulate P-gp expression, increase intracellular DOX concentration, and maximize the therapeutic effects. The cytotoxicity results at 48 h after treatment also indicated that a synergistic effect was achieved at 5  $\mu\text{g/mL}$  of DOX and 100 nM of siMDR-1. Increasing the concentration of DOX disguised the effect of siMDR-1, leading to a more than 80% cytotoxic effect among all groups. Additionally, micelles loaded with 0.5 mg/kg of DOX and 0.2 mg/kg of siMDR-1 given to tumor-bearing mice every three days showed a significant tumor growth inhibition over 24 days [164]. Other groups also reported synergistic effects derived from co-delivery of siMDR-1 with DOX using polymeric nanoparticles at similar concentration combinations. For example, Xu et al. co-delivered 3  $\mu\text{g/mL}$  of DOX and 100 nM of siP-gp in polymeric vehicles prepared from triblock copolymers, folate/methoxy-poly(ethylene glycol)-block-poly(L-glutamate-hydrazide)-block-poly(N,N-dimethylaminopropyl methacrylamide) (FA/m-PEG-b-P(LG-Hyd)-b-PDAPMA) to MCF7 breast cancer cells [52]. And Misra et al. overcame multidrug resistance in cells by co-delivering 11.6  $\mu\text{g/mL}$  of DOX and 100 nM of siMDR-1 using dimethyldidodecylammonium bromide (DMAB)-coated PLGA nanoparticles in the MCF7 ADR cell line [165]. All these studies demonstrated the enhanced therapeutic efficacy against cancer using polymeric co-delivery systems.

In our previous work, micellar nanoparticles consisting of PAMAM-PEG2k-DOPE and PEG5k-DOPE were investigated as a co-delivery system for both hydrophobic drugs and siRNA

(Figure 6). Combinations of DOX and siMDR-1 at different concentrations were applied to multidrug resistant cell lines A2780/ADR and MCF7/ADR [91,99]. The synergistic effect of the co-delivery system was observed in MCF7/ADR and A2780/ADR when treated with 125 nM of siMDR-1, 0.43  $\mu$ M of DOX, or 125 nM of siMDR-1, 1.7  $\mu$ M of DOX, respectively. The cytotoxicity results also suggested that co-delivery of siMDR-1 and DOX achieved an increased anti-cancer effect when delivery of siMDR-1 was followed by DOX treatment separately. Since downregulation of P-gp alone does not cause significant tumor growth inhibition, delivery of siMDR-1 together with intracellular delivery of chemotherapeutics is required for an ideal therapeutic effect. However, inadequate downregulation amount of P-gp or excess of chemotherapeutics could impair the performance of co-delivery systems targeting inhibition of drug-efflux. An optimal concentration ratio should be established for desired therapeutic outcomes.



**Figure 6.** Schematic structure of mixed dendrimer micelles composed of PAMAM-PEG2k-DOPE and PEG5k-DOPE in co-delivery of DOX and siMDR-1. Reproduced with permission from Pan et al., European Journal of Pharmaceutics and Biopharmaceutics; Elsevier, 2019 [91].

#### 4.2.6. Inhibiting Tumor Growth by Altering Immune Responses

Among many strategies for co-delivery systems, being able to modulate the natural immune response against cancer cells is one of the most promising challenges. Chen et al. designed a co-delivery system of DOX + IL-36 $\gamma$ /POEG-st-Pmor with an improved anti-metastatic effect in a mouse breast cancer lung metastasis model with 4T1.2 cells. DOX 5 mg/kg and IL-36 $\gamma$  plasmid 50  $\mu$ g per animal synergistically enhanced the immune response (type I) by increasing the IFN- $\gamma$  positive CD4+ and CD8+ T cells [171]. Wu et al. also delivered the IL-2 immunoactivator together with DOX, but at a much lower dose. *N,N,N*-Trimethyl chitosan-based polymeric nanoparticles loaded with 2 mg/kg of DOX and 1.2  $\mu$ g/animal of IL-2 were injected intravenously into SMMC 7721-bearing mice every two days. When further modified with 8.45% *w/w* folic acid, the polymeric co-delivery system resulted in a five-fold smaller tumor volume than those treated with DOX alone after 14 days [43].

Combination of IL-2 and PCT also has produced a synergistic effect in several reported studies. Wang et al. delivered IL-2 at 5  $\mu$ g in combination with 10  $\mu$ g of PCT per animal using P(MDS)-co-CES polymeric nanoparticles. Co-delivery of IL-2 and PCT resulted in a 2.5-fold lower tumor volume after 17 days of treatment than with individual drug loaded nanoparticles [102]. In another study, Zhao et al. delivered an IL-2 and PCT combination of 2.5  $\mu$ g/kg and 10 mg/kg, respectively, in PLGA/Pluronic

F127-based nanoparticles. The *in vivo* study using a murine melanoma B16-F10 cell line showed significant tumor growth and metastasis inhibition. Additionally, a prolonged overall survival rate was demonstrated in tumor-bearing mice treated by co-delivery of IL-2 and PCT [172].

The release profile of ILs is critical in designing co-delivery systems for IL and chemotherapeutic agents. Systemic administration of IL-2 may initiate an auto-immune response, resulting in side effects from the therapy. Previously, combination of local administration of IL-2 and systemic administration of PCT was used as a combination strategy [179]. IL-2 loaded in polymeric nanoparticles generated a controlled release profile in the systemic circulation. Co-encapsulation of IL-2 with chemotherapeutic agents not only induced a synergistic effect, but also minimized the auto-immune response. Although the co-delivery approach using a single delivery system offers many advantages, pharmacokinetics and cytotoxic side effect still need to be considered. The release profile of IL-2 is critical for the design of co-delivery systems for IL-2 and chemotherapeutic agents.

## 5. Conclusions and Perspectives

In this review, we have discussed the different types of block co-polymers that have been formulated into polymeric co-delivery systems for cancer treatment. We also focused on the different drug combinations used in co-delivery systems and the influence of the combinations on their therapeutic outcomes. As discussed earlier, targeting two multidrug resistance mechanisms simultaneously with a single delivery platform is a promising strategy that can provide synchronized pharmacokinetics and doses to the same cell population. Many co-delivery systems have been developed that exhibit promising anti-tumor efficacy, especially in multidrug resistant tumors. Nevertheless, it has been well-established that cancer cells have the inherent ability to avoid cell death by activation of various anti-apoptotic pathways. These pathways seem to be cell type specific and assault type specific. Therefore, it appears that the effectiveness of decreasing cell viability by double sensitization is cell-specific. That means a certain combination of drugs may work perfectly in one cell line, but may not work at all in another cell line without modification of the combination of drugs. A “one type fit all” formulation seems unlikely for eradication of all types of tumors clinically. Individualized therapy designed specifically for each type of cancer cell with different drug combination may be more likely to be necessary for an effective therapeutic outcome [180].

Polymeric nanoparticles are ideal platforms for co-delivery because of the multicompartments they contain. Amphiphilic co-polymers integrated with different properties have been synthesized to encapsulate hydrophilic agents as well as hydrophobic agents. Cationic moieties in co-polymers also provide the possibility for complexed nucleic acid molecules. Although there is much research focusing on combination delivery using polymeric nanoparticles, little emphasis has been put on the optimization of drug ratios to promote synergistic effects. The synergistic effect of polymeric co-delivery systems depends on multiple factors. First of all, it is important to ensure that the chemistries of delivery, carrier and therapeutic agents do not interact detrimentally with each other [86]. Another challenge is to ensure that the presence of one therapeutic agent does not interfere with the action of others. Additionally, the ratio between two therapeutic agents can directly determine the outcome of a co-delivery system. The release profile of small molecule drug and nucleic acid molecules is another major challenge that requires considerable research.

Overall, a deeper understanding of the optimal ratio between therapeutic agents and the natural heterogeneity of the tumor is necessary for development of polymeric co-delivery systems that maximize therapeutic effects. Additionally, multifunctional polymeric nanoparticles, especially stimuli-sensitive polymeric nanoparticles, allow high drug loading, optimized release profiles, and enhanced *in vivo* stability for the co-delivery of distinctly different classes of therapeutic molecules. There is a need to continue efforts at understanding the relationship between the mechanism of action of encapsulated therapeutic agents and their pharmacological activities. Resolving these challenges should result in multifunctional polymeric nanoparticles with significantly enhanced therapeutic efficacy.

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Review

# Recent Development of pH-Responsive Polymers for Cancer Nanomedicine

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**Abstract:** Cancer remains a leading cause of death worldwide with more than 10 million new cases every year. Tumor-targeted nanomedicines have shown substantial improvements of the therapeutic index of anticancer agents, addressing the deficiencies of conventional chemotherapy, and have had a tremendous growth over past several decades. Due to the pathophysiological characteristics that almost all tumor tissues have lower pH in comparison to normal healthy tissues, among various tumor-targeted nanomaterials, pH-responsive polymeric materials have been one of the most prevalent approaches for cancer diagnosis and treatment. In this review, we summarized the types of pH-responsive polymers, describing their chemical structures and pH-response mechanisms; we illustrated the structure-property relationships of pH-responsive polymers and introduced the approaches to regulating their pH-responsive behaviors; we also highlighted the most representative applications of pH-responsive polymers in cancer imaging and therapy. This review article aims to provide general guidelines for the rational design of more effective pH-responsive nanomaterials for cancer diagnosis and treatment.

**Keywords:** pH responsive polymers; nanomedicine; tumor imaging; drug delivery

## 1. Introduction

Many diseases originate from abnormal biological processes at the molecular level, such as gene mutation, protein misfolding, and cell malfunctions as a result of infections [1]. Advances in genomics, proteomics, and regenerative medicine have inspired development of numerous therapies and technologies for the treatment of various diseases. With over 10 million new cases every year globally, cancer remains a difficult disease to treat and one of the leading causes for death [2]. Cancer is a prevalent disease that involves a series of genome mutations over time and results in both genetic and phenotypic variations in different tumor cells [3,4]. Targeting cancer-specific biomarkers offers great opportunities for precise tumor detection and efficacious drug delivery, and more importantly, causes minimal side effects on normal cells [5]. However, it is impractical to apply a certain cell surface receptor to a broader range of cancers due to their genetic or phenotypic heterogeneity. In contrast, aerobic glycolysis, also known as the Warburg effect, represents a dysregulated energy metabolism in many types of cancer, where glucose is preferentially taken up and converted into lactic acid [6]. As a result, acidosis has occurred concurrently and evolved as a ubiquitous characteristic of cancer [7].

With comparable scales to biologic molecules, nanomaterials have been extensively investigated for biomedical applications over past several decades [8,9]. The nanomaterials are present in small sizes

ranging from 1 to 1000 nanometers with correspondingly large surface-to-volume ratio. A multitude of targeting moieties can be incorporated to the surface of nanostructures via diverse engineering procedures [10,11]. The physiochemical properties such as composition, size, and shape can be sophisticatedly tailored for disease diagnosis, treatment, and prevention [12]. Different physiochemical properties like optical, electrical, and magnetic responsiveness have been introduced to nanostructures to facilitate biological diagnostics [13]. One extraordinary property of nanomaterials is that they are generally multi-component systems. Different components or subunits of the nanostructures can be delicately engineered to address the same specific challenge in medicine, resulting in strong cooperative effect that is absent in monomolecular therapeutics [14]. A recent report revealed that over 250 nanomaterial-based technologies or therapies have been approved by the Food and Drug Administration (FDA) or are currently in different stage of clinical trials [15].

There has been explosive development of numerous nanotechnologies to diagnose and treat cancer [16]. The tumor heterogeneity leads to the growing emphasis on customized nanomedicine [17]. Molecular imaging platforms are used to locate tumor and facilitate image-guided surgery [18]. Due to enhanced permeability and retention (EPR) effect, nanoparticles are more likely to accumulate in tumors and spare the surrounding benign tissues [19]. Since abnormal pH has been recognized as a universally diagnostic hallmark of cancer, the design of pH responsive polymers that are capable of altering their chemical or physical properties are of particular interest for cancer theranostics [20–23]. Moreover, nanoscale polymers with pH responsive segments can strengthen the targeting specificity and hence promote the uptake of nanomedicine into tumor cells [24].

In this perspective, we will review the recent advances in the development of pH responsive nanomaterials for cancer diagnosis and treatment. This article aims to provide general guidelines for the rational design of pH-responsive nanomaterials for the diagnosis and treatment of cancer. Main focus is placed on the polymer designing, mechanistic insights, and specific applications.

## 2. pH-Responsive Polymers

Fabricating pH responsive polymeric nanomaterials has been rapidly developed after the emerging of advanced techniques in polymer synthesis, especially reversible-deactivation radical polymerization such as ATRP and RAFT [25,26]. The technical state of the art allows for precise control over molecular weights and molecular weight distributions, affording polymers with well-defined topology structure [27–29]. The pH responsive polymers can be mainly classified into two categories: (1) polymers with ionizable moieties and (2) polymers that contain acid-labile linkages [30]. The former strategy employs a noncovalent transition to achieve pH responsivity. Typical ionizable moieties include amines and carboxylic acids, which can be protonated or deprotonated at different pH values. The alteration of solubility in aqueous medium serves as an amplified signal to represent the change of environmental pH. For the latter, the backbone of polymers usually comprises acid-labile covalent linkages. The decrease of pH is able to trigger the cleavage of these bonds, causing a degradation of polymer chains or a dissociation of polymer aggregates. Compared to polymers with ionizable moieties, polymers containing acid-labile linkages often present a slower internal structural transition due to the nature of covalent bonding, which facilitate their applications in the drug delivery systems.

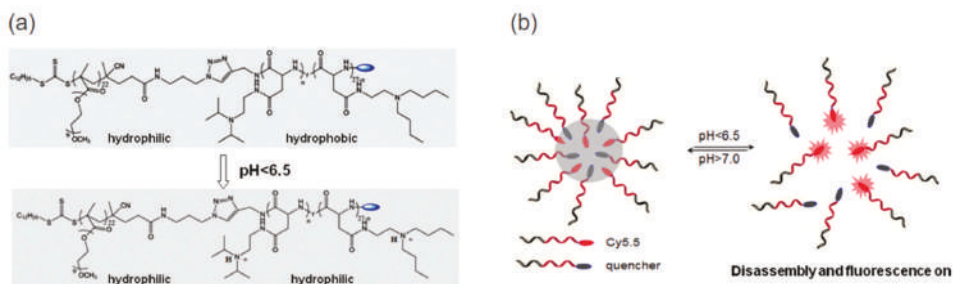
### 2.1. pH Responsive Polymers with Ionizable Groups

The pH response arises from the reversible protonation and deprotonation of ionizable groups at the molecular level. The pKa of a polyelectrolyte, which is defined as the pH with equal concentration of the protonated and deprotonated forms, can serve as a critical benchmark to reflect the polymer ionization behaviors at various pH levels [31]. In general, there are two types of ionizable polymers: basic polymers that accept protons at a relatively low pH and acidic polymers that release protons at a relatively high pH. Consequently, these polybases or polyacids can form positively or negatively charged polymer chains at different pH levels. Common basic moieties include amines, pyridines, morpholines, piperazines; and common acidic groups include carboxylic acids, sulfonic acids,

phosphoric acids, boronic acids, etc. [30]. Among them, amines, especially tertiary amines, have drawn particular attention due to their ease to prepare and the feasibility to finely tune their pKa [32,33]. It was reported that the amines can present a marginally lower pKa when substituted with longer hydrophobic chains [34].

Recently, Gao et al. designed a series of ultra-pH sensitive (UPS) polymers for real-time tumor imaging [35–38]. These nanoprobe can sharply respond to and amplify in vivo pH signals in a very narrow pH span. The UPS nanoparticles are comprised of an amphiphilic block copolymer: PEG-*b*-PMA, where PEG stands for hydrophilic poly(ethylene glycol) and PMA is a hydrophobic segment based on polymethacrylates with tertiary amine substituent. At physiological pH (7.4), the block copolymers stay as core-shell micelles driven by self-assembly. If the environmental pH is lower than the pKa of the pendent tertiary amines, the amines will get protonated and the micelles will dissociate rapidly into unimers. It is worth noting that the process that comprises assembly and dissociation is completely reversible, and fully determined by ambient pH. Acid-triggered dissociation of micelles enable the increase of fluorescence intensity for molecular imaging and release of cargo for drug delivery.

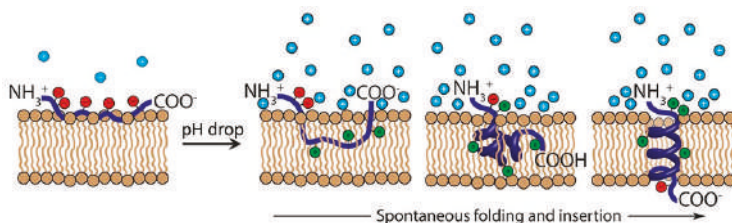
Yan et al. further expand the UPS polymer design based on biodegradable polypeptides (Figure 1) [39]. They modified the peptides with ionizable tertiary amines for pH responsive behavior. Hydrophilic and hydrophobic blocks were synthesized independently and covalently connected by the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) “click” chemistry [40]. The copolymer’s pKa can be readily tuned by altering the ratio of amino substituents. Similar to aforementioned UPS designing, a fluorescent cyanine dye (Cy5.5) was attached at the chain end of the amphiphilic copolymer to convert the subtle pH variation into significant fluorescence intensity change. This study suggested that the ultra-pH sensitivity resulted from the reversible protonation of ionizable amines rather than the peptide backbone. It also inspired the design of degradable ultra-pH sensitive polymers.



**Figure 1.** Design of ultra-pH-sensitive polypeptide micelles. (a) Structure of amphiphilic copolymers with ionizable tertiary amines and their transition at lower pH. (b) pH-triggered disassembly and fluorescence. Reproduced with permission from Liyi Fu, Pan Yuan, Zheng Ruan, Le Liu, Tuanwei Li, and Lifeng Yan, Polymer Chemistry; published by Royal Society of Chemistry, 2017.

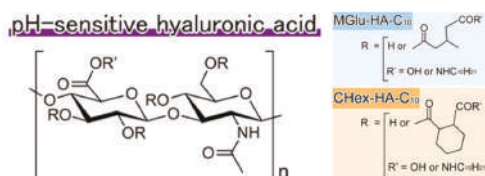
Natural macromolecules with ionizable amino acid residues have also been investigated for pH-triggered delivery of imaging and therapeutic agents [41–45]. Engelman and colleagues have been working on the development of novel pH-responsive transmembrane peptides, pH low insertion peptide (pHLIPs), for basic research and translational applications in membrane biophysics and medicine (Figure 2) [46]. These pH-responsive peptides were derived from the C-helix of the protein bacteriorhodopsin [47]. pHLIPs spontaneously self-assemble into a helix structure and insert across the membrane upon exposure to acidic environment [48]. In physiological condition, where the pH is around 7.4, the ionizable acid residues of the pHLIP (red circles) stay negatively charged and the peptide will be weakly bound to the surface of membrane. Once encountering acidic condition like tumor microenvironment, carboxyl group will be protonated and neutralized. Increased lipophilicity (green circles) as a result of ionization drastically enhances the affinity of pHLIP to the hydrophobic

inner core of the cellular membrane and triggers the formation of a helix and ensuing insertion across the membrane. When the protonatable carboxyl groups are exposed to the normal intracellular pH, pHLIP gets reversibly deprotonated and anchors in the membrane. The pH-responsive behavior of pHLIP can be easily modified by replacing ionizable aspartic acid residues with glutamic acid residues [43,49] or positively-charged lysine residues [41], or other protonatable amino acids [44].



**Figure 2.** Mechanism of pH (Low) Insertion Peptide (pHLIP) Insertion into the Cellular Membrane. In healthy tissue where the pH is around 7.4, the ionizable residues of the pHLIP (red circles) remain deprotonated and negatively charged, and the peptide resides at or near the hydrophilic surface of the cellular membrane. Upon exposure to acidic tumor microenvironment, the ionizable residues and negatively charged C-terminal carboxyl group of the pHLIP become neutrally charged (green circles). The protonation results in an increase in the hydrophobicity of the pHLIP, triggering the pHLIP to spontaneously fold into a helix and insert across the hydrophobic lipid bilayer of cell membrane, resulting in the formation of a transmembrane helix. Following internalization and exposure to cytosol with pH above 7, C-terminal ionizable residues are deprotonated again and anchor the pHLIP into the membrane. Reproduced with permission from Linden C. Wyatt, Jason S. Lewis, Oleg A. Andreev, Yana K. Reshetnyak, and Donald M. Engelman, Trends in Biotechnology; published by Elsevier, 2017.

Polyacids have also been extensively investigated for the design of pH-responsive nanoplateforms. Hyaluronic acid (HA) is a key component of the extracellular matrix and is known to bind to CD44 proteins as a surface receptor on cancer cells [50]. Kono and coworkers reported HA-based pH-sensitive polymer-modified liposomes for tumor-targeted delivery of chemotherapeutics [51]. Instead of simply using HA as targeting moiety, these authors introduced 3-methyl glutarylated (MGlu) units and 2-carboxycyclohexane-1-carboxylated (CHex) units for the design of a new class of pH-responsive polymers with transition pH around 5.4–6.7. Carboxyl group-introduced HA derivatives were prepared by reaction with various dicarboxylic anhydrides. Terminal alkyl chains served as anchor units inserting into the hydrophobic lipid bilayers of liposomes (Figure 3). Upon exposure to acidic endosome pH, both MGlu and CHex modified HA were protonated, enabling lipid membrane disruption and intracellular release of chemotherapeutics. Mechanistic investigation demonstrated that the MGlu/CHex significantly affected the pH-responsive behavior of HA derivatives. Increased hydrophobicity led to a higher transition pH.



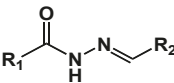
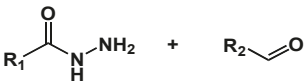
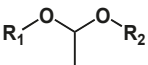
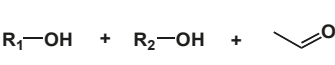
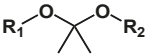
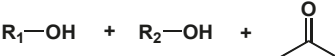
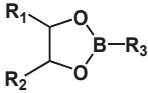
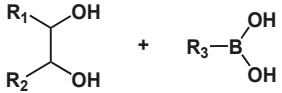
**Figure 3.** Structures of pH-sensitive modified hyaluronic acid derivatives. Reproduced with permission from Maiko Miyazaki, Eiji Yuba, Hiroshi Hayashi, Atsushi Harada, and Kenji Kono, Bioconjugate Chemistry; published by American Chemical Society, 2018.



## 2.2. pH Responsive Dissociation Based on Acid-Labile Linkages

Polymers containing acid-labile or base-labile linkages can respond to the change of pH by degradation. Since the tumor microenvironment is slightly acidic compared to normal physiological environment, polymeric nanoparticles containing base-labile linkages have seldom been used for cancer therapy and will not be covered in the perspective. In contrast, polymers with acid-labile linkages have been extensively utilized for the designing of anticancer drug delivery systems [52]. The most commonly used acid-labile linkages are listed in Table 1. The different degradation mechanisms of the linkages and the products after cleavage are described [53,54].

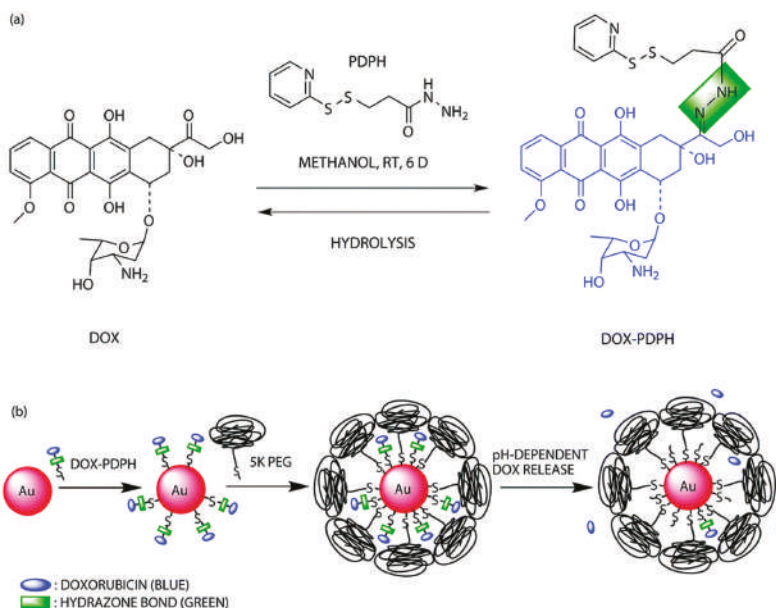
Table 1. Summary of acid-labile linkages.

Type of Linkages	Structure	Product after Acid Cleavage
Hydrazone		
Acetal		
Ketal		
Boronate ester		

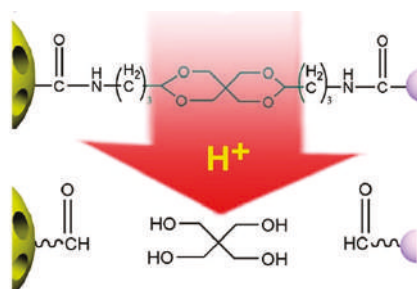
Hydrazone is one of the most explored acid-labile linkages used for acid-responsive dissociation release due to its easy synthesis and moderate sensitivity [55]. Incorporation of hydrazine into the backbone of polymers represents an ideal strategy for the design of tumor-targeted drug delivery systems. Nie group designed a pH-sensitive drug-gold nanoparticle system for tumor chemotherapy and surface enhanced Raman scattering (SERS) imaging (Figure 4) [56]. This multifunctional system comprised of poly(ethylene glycol), doxorubicin (Dox), hydrazone linker, and gold nanoparticles (Au-Dox-PEG). 3-[2-pyridyldithiol]propionyl hydrazide (PDPH) was conjugated with Dox in methanol. PDPH acted as a pH-sensitive linker and introduced thiol groups to ensure the anchoring of drug conjugates onto the surface of gold nanoparticles. Gao and coworkers developed a pH-responsive polypeptide–drug nanoparticles for targeted cancer therapy based on well-defined elastin-like polypeptides and acid-labile hydrazone linker [57]. Wang reported a hydrazone-based multifunctional sericin nanoparticles for pH-sensitive subcellular delivery of anti-tumor chemotherapeutics [58].

Acetal and ketal are also commonly used acid-labile linkages, which are very stable under basic conditions, but can be readily hydrolyzed to corresponding carbonyl compound (aldehyde and ketone) and alcohols through a caboxonium ion intermediate upon acidic cleavage [59]. Lu and coworkers reported a novel envelop-like mesoporous silica nanoparticle platform [60]. This system immobilized acetals on the surface of silica (yellow, left) before coupling to gate-keeper nanoparticle (purple, right) (Figure 5). At acidic pH, the acetal was effectively hydrolyzed to remove the gate keepers, allowing the escape of entrapped drug molecules. Liu et al. reported the facile fabrication of acid-sensitive polymersomes for intracellular release of drug over several days [61]. The polymersomes compromising cyclic benzyldiene acetals in the hydrophobic bilayers were stable under neutral pH, whereas were hydrolyzed into hydrophilic diol moieties upon exposure to acidic pH milieu. The pH-triggered hydrolysis can be easily monitored by many experimental methods including UV/Vis spectroscopy and TEM. A novel class of acid degradable poly(acetal urethane) was also

reported for the construction of acid-degradable micelles for delivery of hydrophobic anti-tumor therapeutics [62]. More recently, Wang group designed a new hyperbranched amphiphilic acetal polymers for pH-sensitive drug delivery [63]. Under neutral conditions, the block copolymers self-assembled into well-defined core-shell micelles. This pH-induced acetal cleavage resulted in the drastic decrease of hydrophobicity and dissociation of micelles. De Geest and coworkers synthesized ketal-containing block copolymers as pH-responsive nanocarriers for the hydrophobic anticancer drug paclitaxel (PTX) [64]. The hydrolysis of the ketal groups in the block copolymer side chains at  $\text{pH} < 5$  lead to decomposition of the block copolymer nanoparticles and the PTX release.

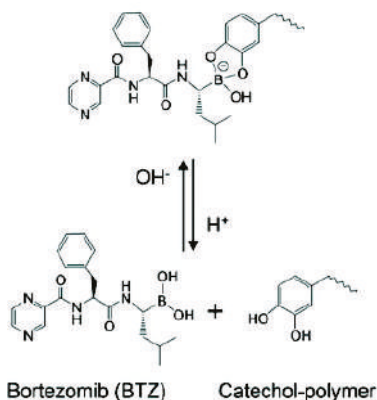


**Figure 4.** A pH-sensitive drug-gold nanoparticle system. (a) Chemical synthesis of the doxorubicin-hydrazone linker conjugate (dox-PDPH). (b) Schematic illustration for the synthesis of the multifunctional drug delivery system and its pH-dependent doxorubicin release. Reproduced with permission from Kate Y. J. Lee, Yiqing Wang, and Shuming Nie, RSC Advances; published by Royal Society of Chemistry, 2015.



**Figure 5.** Schematic illustration of the envelope-type mesoporous silica nanoparticle for pH-responsive drug delivery. Reproduced with permission from Yan Chen, Kelong Ai, Jianhua Liu, Guoying Sun, Qi Yin, and Lehui Lu, Biomaterials; published by Elsevier, 2015.

Boronate esters, formed between a boronic acid and an alcohol, are stable at neutral or alkaline pH and readily dissociate to boronic acid and alcohol groups in a low-pH environment. Boronate esters have been widely employed to exploit pH-sensitive polymeric carriers for anticancer drug delivery. Messersmith et al. conjugated the boronic acid containing anticancer drug bortezomib (BTZ) to catechol-containing polymers via the boronate ester (Figure 6) [65]. Under neutral or basic condition, BTZ and catechol formed stable conjugates via boronate ester linkers, deactivating the cytotoxicity of BTZ. Upon exposure to lower pH, the conjugates readily release free drugs. Levkin and coworkers reported a dextran-based pH-sensitive nanoparticle system by modifying vicinal diol of dextran with boronate esters [66]. pH-triggered hydrolysis of ester linkers resumed the hydrophilic hydroxyl groups of dextran and destabilized the drug-encapsulated nanoparticles. Kim et al. reported a pH-sensitive nanocomplex by grafting phenylboronic acid (PBA) onto the backbone of poly(maleic anhydride) [67]. Dox and PBA readily formed boronate esters following simple mixing.



**Figure 6.** Catechol-containing polymers conjugated the boronic acid containing anticancer drug bortezomib via acid-labile boronate esters. Boronate esters are stable at neutral or alkaline pH and readily dissociate to boronic acid and alcohol groups in acidic environments to release the free active drug. Reproduced with permission from Jing Su, Feng Chen, Vincent L. Cryns, and Phillip B. Messersmith, *Journal of the American Chemical Society*; published by American Chemical Society, 2011.

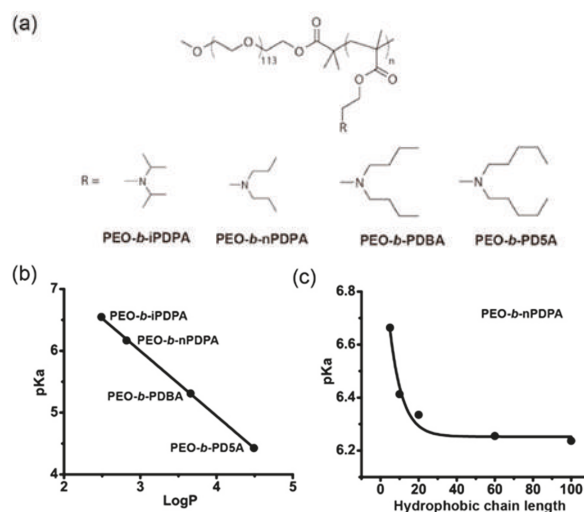
### 3. Tunable pH-Responsive Behavior

A fundamental challenge in nanomedicine is the specific delivery the therapeutic or diagnostic agents to the targeted tissues or cells [68,69]. Targeted delivery has shown promise in reducing off-target effect and lowering toxicity [70]. One major consideration in the design of pH-responsive nanomaterials is choosing polymers with pKa values matching the desired pH range. The acidity of gastrointestinal track is drastically different from that of blood [71]. Intracellular compartments such as mitochondria, endosomes, and lysosomes also have slightly different pH values [72]. Even the level of acidosis shows small variation among different types of tumors [6]. Therefore, a key consideration in the design of tumor-targeted pH-responsive polymers is to ensure the polymers are able to differentiate acidic tumor microenvironment from surrounding normal tissues. The variation in the potency of diverse anti-tumor therapeutics also requires tunable release kinetics of payloads.

Reversible protonation of pH responsive polymers leads to the change of the hydrodynamic volume, chain conformation, water solubility and maybe supramolecular self-assembly. Ionization also allows us to modulate the pH-responsive behavior of these polymers. Mechanistic investigation suggests that the acid-base equilibrium in the ionization process is controlled by the balance between hydrophobic interaction and electrostatic repulsion [73]. The transition pH can be tuned by altering the structural factors that affect the hydrophobic or electrostatic interactions. Environmental factors that shift the transition pH such as ionic strength and species are beyond the scope of this review.

### 3.1. Hydrophobic Modification

Hydrophobic modification is an important approach to modulate the transition pH of ionizable polymers. Incorporating different hydrophobic groups or changing the length of hydrophobic chains can lead to the shift of pKa. Li et al. systematically investigated key structural parameters that affected the transition pH of a series of polymers containing ionizable tertiary amines (Figure 7a) [34]. The hydrophobic interactions can be strengthened by increasing the hydrophobicity of the amine substituents. To accomplish this goal, they synthesized a series of pH sensitive block copolymers with an identical poly(methacrylate) backbone and similar chain length but different linear terminal alkyl groups on the side chain. The polymer with the most hydrophobic pentyl group yielded the lowest pKa at 4.4. Meanwhile, the one with the least hydrophobic isopropyl group as an amine substituent showed the highest pKa, close to 6.6. These results demonstrated that ionizable pH-sensitive copolymers with more hydrophobic amine substituents have a lower pKa. They calculated the octanol–water partition coefficients (LogP) of the repeating unit of hydrophobic segment and used them as a quantitative measure of the strength of hydrophobic interactions. The plot of pKa values as a function of LogP (Figure 7b) showed a linear correlation. To confirm the observation, they synthesized another series of block copolymers with the same backbone and similar chain length, but different cyclic terminal alkyl groups. A plot of transition pH as a function of LogP also showed a similar linear correlation.

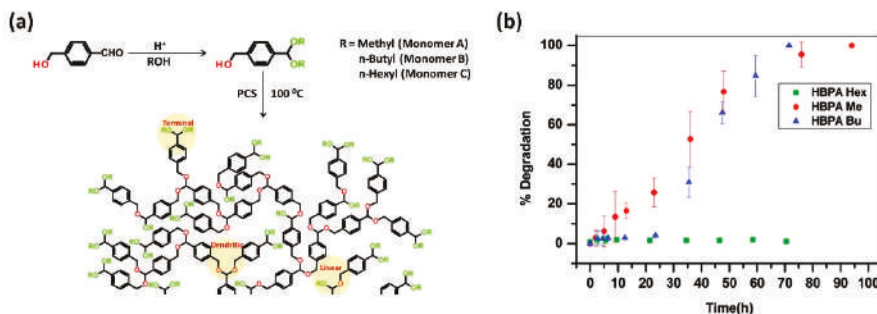


**Figure 7.** Tunable pKa based on hydrophobic modification. (a) Structure of methacrylate-based block copolymers with different alkyl substituents. (b) Increase of the hydrophobicity of the alkyl substituents resulted in the linear decrease of pKa. (c) Increased hydrophobic chain length lead to decreased pKa in a representative poly(ethylene glycol)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PEG-*b*-PDPA) polymer. Reproduced with permission from Yang Li, Zhaohui Wang, Qi Wei, Min Luo, Gang Huang, Baran D. Sumer, and Jinming Gao. Biomaterials; published by Elsevier, 2016.

To prove the concept that hydrophobic chain length has a significant effect on the pKa value, Li et al. synthesized a series of poly(methacrylate)-poly(ethylene oxide) block copolymers with the fixed hydrophilic poly(ethylene oxide) chains but variable chain lengths of the hydrophobic poly(methacrylate) block ( $x = 5, 10, 20, 60$ , and  $100$ ), and evaluated the pKa value shift caused by the change of the hydrophobic chain length. Results indicated that the pKa values were inversely proportional to the length of the hydrophobic chain. The transition pH of block copolymers, with the longest hydrophobic segment, yielded the lowest pKa at 6.2 (Figure 7c). In contrast, polymers with the shortest hydrophobic chain length, displayed the highest transition pH around 6.7. The plot

of pKa values as a function of the hydrophobic chain length showed a dramatic hydrophobic chain length-dependent transition pH shift.

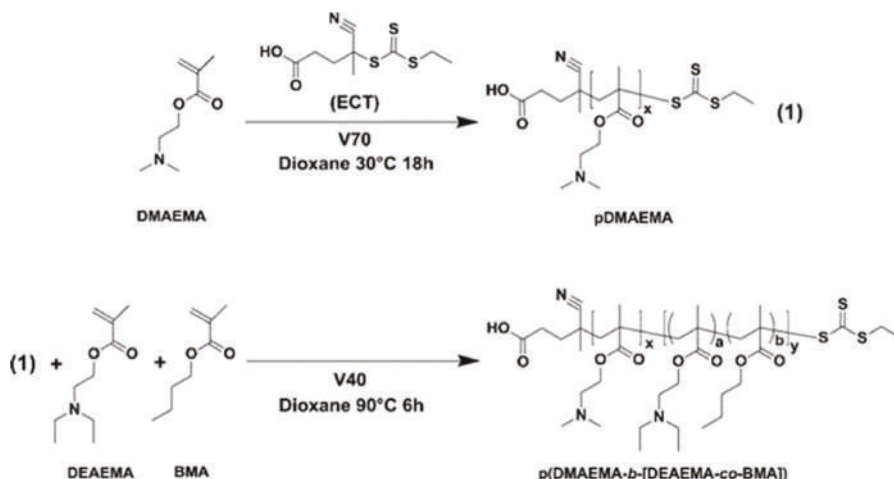
Hydrophobic modification has also been demonstrated to be a practical strategy in tuning the degradation rate of polymeric systems containing acid-labile linkers. Ramakrishnan et al. investigated how the hydrophobic end-groups affected the degradation of hyperbranched polyacetals (Figure 8) [74]. They changed the structure of hyperbranched polymers by altering the pendant alkyl groups of monomers. Results indicated that the degradation rates of the acid-responsive polymers were significantly affected by the hydrophobic nature the terminal alkyl substituents, where more hydrophobic alkyl groups generally contributed to slower degradation and prolonged release of cargos.



**Figure 8.** Hydrophobic modification in tuning the degradation rate of hyperbranched polyacetals. (a) Structure and synthetic route of hyperbranched polyacetals. Monomers with different hydrophobic substituents (methyl, butyl, and hexyl) were used in the preparation of three model branched polyacetals. (b) Branched polyacetal with least hydrophobic methyl groups showed fast degradation within 10 h, whereas polyacetal with most hydrophobic hexyl group showed almost no significant degradation after 4 days. Reproduced with permission from Saptarshi Chatterjee and S. Ramakrishnan. *Macromolecules*; published by American Chemical Society, 2011.

### 3.2. Copolymerization with Non-Ionizable Polymers

Stayton group reported the development of a series of pH-responsive block copolymers for the delivery of siRNA [75]. These polymers were composed of a positively-charged block of dimethylaminoethyl methacrylate (DMAEMA) to mediate siRNA condensation, and a second endosomal releasing block composed of DMAEMA and propylacrylic acid (PAA) in roughly equimolar ratios, together with butyl methacrylate (BMA). The polymers self-organized into micelles at physiological pH while rendered pH-induced disassembly upon exposure to acidic endosomal pH. The transition pH where reversible micellization occurred could be precisely tuned by systematically changing the fraction of non-ionizable hydrophobic segment (Figure 9), with increased BMA ratio exhibited lower pKa values [76]. Moreover, experimental results also showed that higher fraction of BMA also lead to enhanced homolytic activity, indicating that the transition pH was critical for therapeutic effect associated with pH-triggered drug release.

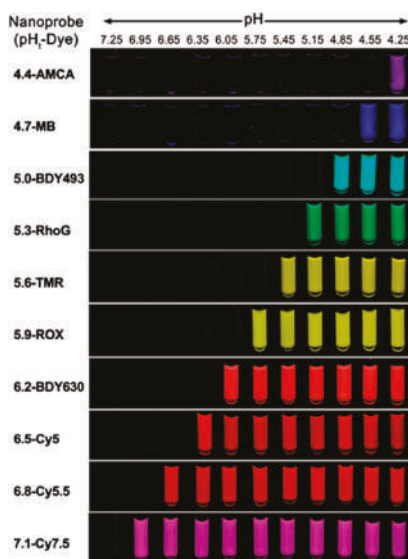


**Figure 9.** Preparation of a representative copolymer consisting of a cationic poly(DMAEMA) (dimethylaminoethyl methacrylate) block and an endosomolytic hydrophobic block incorporating DEAEMA and butyl methacrylate (BMA) at varying molar feed ratios. Reproduced with permission from Matthew J. Manganiello, Connie Cheng, Anthony J. Convertine, James D. Bryers, and Patrick S. Stayton. Biomaterials; published by Elsevier, 2012.

### 3.3. Copolymerization with Ionizable Polymers

Incorporation of additional polyelectrolytes that changes both hydrophobic interactions and electrostatic repulsion has also been reported for the modification of the transition pH of polymers. Gao et al. reported a strategy to design ultra-pH sensitive nanoparticles with broad tunability via a copolymerization method [35]. A series of PEO-*b*-P(R1-*r*-R2) block copolymers were prepared. The hydrophobic block P(R1-*r*-R2) contained two randomly distributed ionizable monomers R1 and R2 with different hydrophobicity. The molar fraction of the two monomers (R1 and R2) can be precisely controlled prior to polymerization. A series of ionizable monomers with different alkyl groups (e.g., ethyl, propyl, butyl, and pentyl) were applied in their study. The pKa values of PEO-*b*-PDPA (propyl) and PEO-*b*-PDBA (butyl) were 6.2 and 5.3, respectively. They were able to get a series of PEO-*b*-P(DPA-*r*-DBA) copolymers with pKa values between 6.2 and 5.3 successfully. A plot of pKa values of the obtained block copolymers as a function of the molar fraction of monomers with butyl group yielded a linear correlation. It is worth noting that matching of the hydrophobicity of the two monomers is critical to maintain the ultra-pH sensitivity. Based on this method, they established a library of nanoprobe with pH transitions cover the entire physiologic range of pH (4.0–7.4) (Figure 10). Compared to simple molecular mixture method, this copolymerization strategy achieves robust and broad tunability in transition pH.



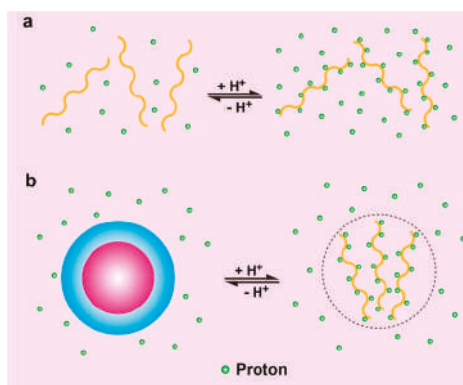


**Figure 10.** Ultra-pH sensitive (UPS) library based on a copolymerization strategy spanning a wide pH range. Polymers prepared from different monomers ionizable tertiary amines were encoded with different fluorophores. UPS polymers with more hydrophobic repeating unit showed lower transition pH as quantified by fluorescence intensity. Reproduced with permission from Xinpeng Ma, Yiguang Wang, Tian Zhao, Yang Li, Lee-Chun Su, Zhaohui Wang, Gang Huang, Baran D. Sumer, and Jinming Gao. *Journal of the American Chemical Society*; published by American Chemical Society, 2014.

#### 3.4. Mechanistic Insights into the Tunable pH-Responsive Behavior

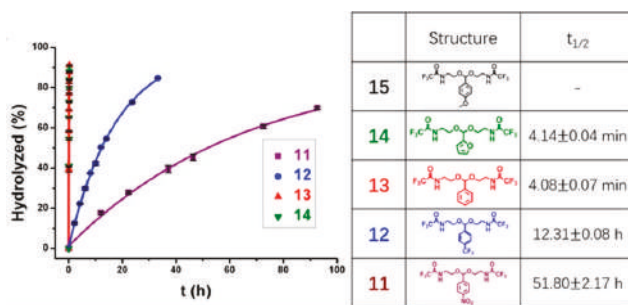
Compared to small molecules, polymerization and supramolecular self-assembly represent powerful strategies to produce high-performance materials at nanoscale [77–80]. Despite the promise in a multitude of biomedical applications, polymeric materials also introduce significantly increased complexity inherent to the multiple interacting components within the systems. The lack of molecular understanding of polymeric systems frequently hampers our ability to rationally design nanomaterials for medicine and healthcare.

In a recent study, Li and coworkers elucidated the molecular pathway of pH-triggered supramolecular self-assembly, which may pave the way for future design of pH-sensitive polymers with easily tunable pKa and pH transition sharpness. They found that hydrophobic phase separation was critical in tuning the pKa values of amphiphilic pH-responsive polymers (Figure 11) [73]. For pH responsive polymers that are not hydrophobic enough at neutral state to self-assemble into nanoparticles, the pH responsive behavior was very similar to that of small molecular pH sensors. The hydrophobic phase separation changes the molecular pathway of protonation in ionizable polymers that can form self-associated nanoparticles. For polyamines, the hydrophobic phase separation significantly shifted the pKa values to lower pH ranges. It is reasonable to hypothesize that the increased hydrophobicity in polyacid systems shifts the transition pH to more basic pH, which is actually validated by hyaluronic acid modified liposomes as described previously [51]. A molecular cooperativity was observed in pH-induced protonation and phase separation, as observed in thermo-responsive polymers and many natural macromolecular systems. This suggests that supramolecular cooperativity may serve as a general strategy in tuning the responsive behavior of stimuli-sensitive nanomaterials [73].



**Figure 11.** Schematic illustration of phase separation induced cooperativity in pH-triggered supramolecular self-assembly. (a) Hydrophilic polymers with ionizable amines were protonated homogeneously, showing no cooperativity. (b) Hydrophobic phase separation (e.g., micellization) drove cooperative protonation of amphiphilic pH-responsive polymers. Phase separation also drove the pKa values of polymers to lower pH because of hydrophobic barrier, which required higher proton concentration (lower pH) to initiate the protonation process. Reproduced with permission from Yang Li, Tian Zhao, Chensu Wang, Zhiqiang Lin, Gang Huang, Baran D. Sumer, and Jinming Gao. Nature Communications; published by Nature Publishing Group, 2016.

Thayumanavan and coworkers systematically investigated the substituent effects on the pH-sensitivity of acetals and ketals [59]. It is well established that the degradation of acetals and ketals operated through a resonance-stabilized cationic intermediate [81]. Their study validated that electron-withdrawing substituents generally decreased the hydrolysis rate due to the cationic nature of the intermediate. Benzylidene acetals that were unsubstituted or substituted with electron-withdrawing groups, exhibited drastically slowed hydrolysis under the pH 5 conditions (Figure 12). For examples, half of phenyl-substituted acetal groups were hydrolyzed after 4 min. Incorporating an electron-withdrawing trifluoromethyl moiety increased the half-life to around 12.3 h. The presence of a resonance-based electron-withdrawing functionality further increased the half-life to 51.8 h. The structural fine-tuning of the linkers offers powerful and practical method in future design of acid-degradable polymeric drug delivery systems.



**Figure 12.** Substituent effects upon the hydrolysis of benzylidene acetals. Electron-withdrawing substituents significantly contributed to decreased hydrolysis rate of acetals. As compared to phenyl substituent with half-life of 4 min, trifluoromethyl increased the half-life of acetal to around 12.3 h. The presence of a resonance-based electron-withdrawing functionality further increased the half-life to 51.8 h. Reproduced with permission from Bin Liu and S. Thayumanavan, Journal of the American Chemical Society; published by American Chemical Society, 2017.

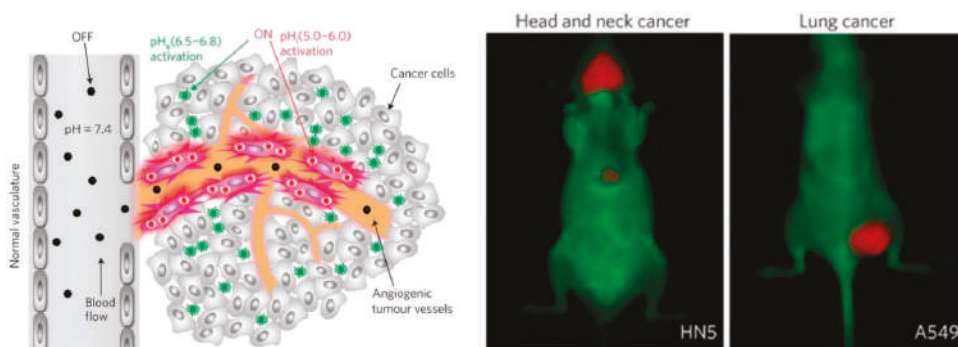
#### 4. Applications of pH-Responsive Nanomaterials in Cancer Diagnosis and Treatment

Cancer remains one of the leading causes of death around the world. Nanotechnology has marked a new dimension in fighting against cancer. pH-responsive nanomaterials that target tumor acidosis offer a new paradigm in addressing the deficiencies of conventional chemotherapy in the diagnosis and treatment of various types of cancer.

##### 4.1. pH-Sensitive Nanoprobe-Based Fluorescent Imaging and Image-Guided Surgery

Surgery is one of the main options for cancer treatment. When resecting a tumor, a surgeon needs to precisely identify the spread of the cancer. If tumor is not completely removed, there is a great chance of recurrence of cancer. The patients may suffer from crucial organ dysfunction or damage if normal tissues are cut. Thus, intraoperative technologies that can help surgeons visualize the tumor margins during the operations may significantly improve the long-term survival of cancer patients. There is an urgent clinical need for the cheap and practical technology that can universally differentiate tumors from adjacent normal tissues, which could potentially help the surgeons save numerous lives [82–84].

Wang et al. reported a pH-responsive nanoparticle-based strategy for the imaging of a broad range of tumors by nonlinear amplification of tumor acidosis signals [85]. As shown in Figure 13, the ionizable amines were neutralized and the amphiphilic block copolymers stayed at micelles state with conjugated fluorescent dyes quenched due a self-quenching Forster resonance energy transfer (HomoFRET) effect. Upon access to acidic tumor microenvironment or internalized into endocytic organelles in the tumor endothelial cells. This system demonstrated a broad tumor specificity with an extraordinary tumor-to-blood ratio (>300-fold) in a variety of tumor models. Following the exceptional imaging outcome, they continued the study in image-guided surgery with several clinical-compatible fluorescent cameras [86]. The real-time tumor-acidosis-guided detection and resection of tumors significantly improved the long-term survival of tumor-bearing mice.



**Figure 13.** pH-activatable nanoparticles for tumor-specific fluorescent imaging. (Left panel) The nanoparticles stayed in micelle state in normal physiological pH and the fluorescence was quenched as a result of self-quenching Forster resonance energy transfer (HomoFRET) effect. Upon exposure to acidic tumor microenvironment, protonation of tertiary amines lead to the dissociation of micelles and resume of fluorescence. (Right panel) pH-responsive nanoparticles selectively light up tumors instead of surrounding normal tissues in various tumor models. Reproduced with permission from Yiguang Wang, Kejin Zhou, Gang Huang, Christopher Hensley, Xiaonan Huang, Xinpeng Ma, Tian Zhao, Baran D. Sumer, Ralph J. DeBerardinis, and Jinming Gao. *Nature Materials*; published by Nature Publishing Group, 2014.

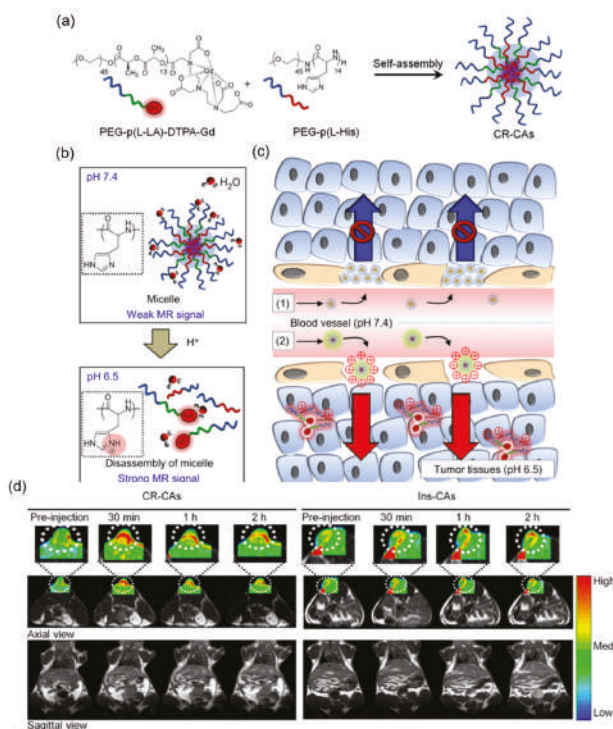
##### 4.2. pH-Sensitive Nanoprobe-Based Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) with a contrast agent has been widely used as a powerful tool for cancer diagnosis [87]. However, most MRI contrast agents are non-targeted for cancer and passively distributed throughout the body, which results in a low efficiency and a need for high doses.

One approach to addressing these problems is to use pH-responsive amphiphilic block copolymers that would work as platforms for the tumor-targeting delivery of MRI contrast agents and MRI signal enhancement in the tumor region.

Kun Na and coworkers developed a cancer-recognizable MRI contrast agents (CR-CAs) using pH-responsive polymeric micelles [88]. The micelles were self-assembled from copolymers of methoxy poly(ethylene glycol)-*b*-poly(L-histidine) (PEG-*p*(L-His)) and methoxy poly(ethylene glycol)-*b*-poly(L-lactic acid)-diethylenetriaminopentaacetic acid dianhydride-gadolinium chelate (PEG-*p*(L-LA)-DTPA-Gd) (Figure 14a–c). In the micelles, *p*(L-His) blocks were the pH-responsive component. The imidazole groups of *p*(L-His) blocks were protonated, causing the broken of the micellar structure in acidic tumoral environment. Paramagnetic gadolinium ( $Gd^{3+}$ ) chelates were the MRI contrast agents that enhance the signal intensity upon the exposure to water molecules as the micelles broken. In addition, the CR-CAs' core was positively charged by protonation of the imidazole groups of *p*(L-His) blocks after extravasation, which rapidly facilitated accumulation of CR-CAs compared with pH-insensitive micelle-based CAs (Ins-CAs) due to the strengthened interaction between the positively charge CR-CAs and the negatively charged cellular membrane. In vivo, the CR-CAs exhibit highly effective MR contrast enhancement in the CT26 murine tumor region of Balb/c mice, while the MR contrast of the tumor treated with Ins-CAs did not show a significant change over time. CR-CAs enabled the detection of small tumors ( $3\text{ mm}^3$ ) in vivo within a few minutes (Figure 14d).

Lee et al. encapsulated  $Fe_3O_4$  nanoparticles, which are frequently used as a contrast agent for MRI, into the pH-responsive polymeric micelle [89]. This technique has been tested on mice and shown an increased signal intensity over a 24-h period compared to a pH-insensitive contrast agent which did not change in signal intensity.



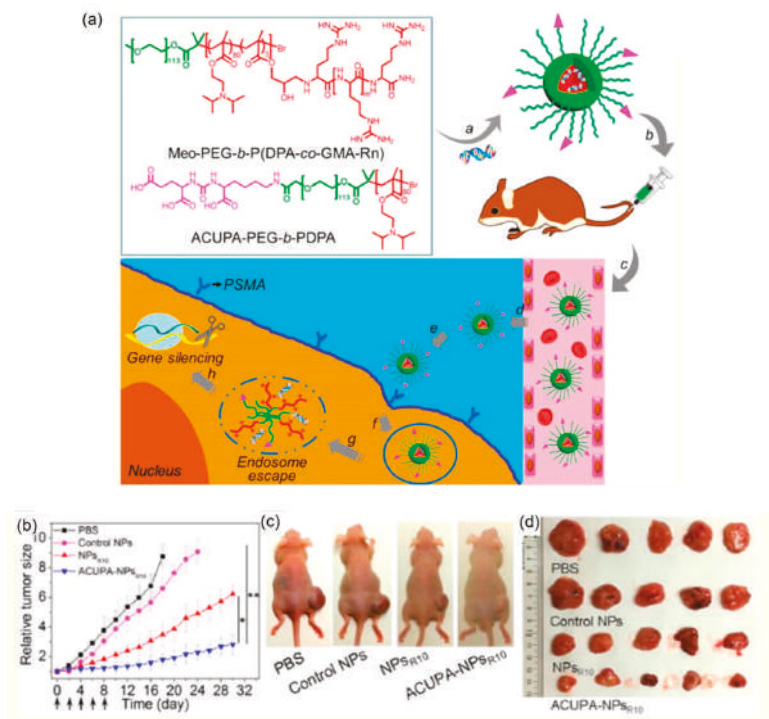
**Figure 14.** A cancer-recognizable MRI contrast agents (CR-CAs) based on pH-responsive polymeric micelles. (a) Schematic representation of the preparation of cancer-recognizable CR-CAs. (b) Schematic representation of the pH-dependent structural transformation and related MR signal change in CR-CAs. Inset: Chemical structural representation of the protonation of imidazole groups in PEG-p(L-His) at acidic pH. (c) Schematic representation of the tumor-accumulation behavior of (1) conventional micelle-based CAs and (2) CR-CAs. (d) Temporal color-coded in vivo longitudinal relaxation time (T1)-weighted MR images of CT26 murine tumor bearing Balb/c mice after the intravenous injection of CR-CAs and pH-insensitive micelle-based CAs (Ins-CAs). Reproduced with permission from Kyoung Sub Kim, Wooram Park, Jun Hu, You Han Bae, and Kun Na. *Biomaterials*; published by Elsevier, 2014.

#### 4.3. pH-Responsive Polymeric siRNA Carriers for Cancer Treatment

The use of RNA interference (RNAi) as a tumor-specific gene therapy has attracted increasing attention, and is considered one of the most promising platforms for cancer therapy [90]. However, naked siRNA is unstable, and the effective intracellular delivery of siRNA into the cytoplasm remains a significant challenge. After cellular uptake by passive or receptor-mediated endocytosis, siRNA predominantly locates in endosomes and is degraded by specific enzymes in the lysosome. Thus, to achieve an effective treatment efficiency, escape of siRNA from endosomes to reach cytoplasm is desired. pH-responsive polymeric carriers that facilitate the endosomal escape have been demonstrated one effective approach to mediate intracellular siRNA delivery.

Shi et al. developed several pH-responsive nanoparticle (NP) platforms, capturing Poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) components, for cancer-specific in vivo siRNA delivery [91,92]. PDPA is a type of polycations containing low pKa amines. Low pKa amine group have been shown to exhibit "proton sponge effect" to induce the endosomal escape [93]. Polycations induce the endosomal escape by binding to the oppositely charged cellular membrane and perturbing membrane integrity. In addition, to synergize the endosomal escape, cationic

membrane-penetrating oligoarginine grafts or cationic lipid-like grafts were also incorporated into the nanoparticles. After cellular uptake, the rapid protonation of the PDPA segment causes the fast disassembly of the nanoparticles, endosomal swelling, and the exposed membrane-penetrating oligoarginine grafts or cationic lipid-like grafts lead to efficient endosomal escape (Figure 15a). In vivo, the siRNA NPs showed efficient gene silencing and significant inhibition of tumor growth (Figure 15b–d).



**Figure 15.** A pH-responsive nanoparticle (NP) platform for cancer-specific in vivo siRNA delivery. (a) Molecular structures of the oligoarginine functionalized sharp pH-responsive polymer methoxyl-polyethylene glycol-*b*-poly(2-(diisopropylamino) ethyl methacrylate-co-glycidyl methacrylate) (Meo-PEG-*b*-P(DPA-co-GMA-R<sub>n</sub>,  $n = 6, 8, 10, 20$ , and  $30$ )) and *S,S*-2-[3-[5-amino-1-carboxypentyl]-ureido]pentanedioic acid functionalized poly(ethylene glycol)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (ACUPA-PEG-*b*-PDPA) and a schematic illustration of the multifunctional envelope-type NP platform for in vivo siRNA delivery and therapy. (b) NPs prepared from Meo-PEG-*b*-P(DPA-co-GMA-R<sub>10</sub>) was denoted as NPs<sub>R10</sub>. Relative tumor size of the Luc-HeLa and PCa cell lines (LNCaP) xenograft tumor-bearing nude mice ( $n = 5$ ) after treatment by Luc siRNA-loaded NPs<sub>R10</sub> (control NPs) and PHB1 siRNA-loaded NPs<sub>R10</sub> (NPs<sub>R10</sub>) and siRNA-loaded ACUPA-NPs<sub>R10</sub> (ACUPA-NPs<sub>R10</sub>). \*  $p < 0.05$ ; \*\*  $p < 0.01$ . (c) Representative photograph of the LNCaP xenograft tumor-bearing nude mice in each group at day 18. (d) Photograph of the harvested LNCaP tumors after a 30-day evaluation. Reproduced with permission from Xiaodong Xu, Jun Wu, Yanlan Liu, Phei Er Saw, Wei Tao, Mikyung Yu, Harshal Zope, Michelle Si, Amanda Victorious, Jonathan Rasmussen, Dana Ayyash, Omid C. Farokhzad, and Jinjun Shi. ACS Nano; published by American Chemical Society, 2017.

Stayton et al. synthesized a diblock polymer composed of PDMAEMA block to condense siRNA and a second block composed of DMAEMA, BMA, and PAA for endosomal-releasing [75].



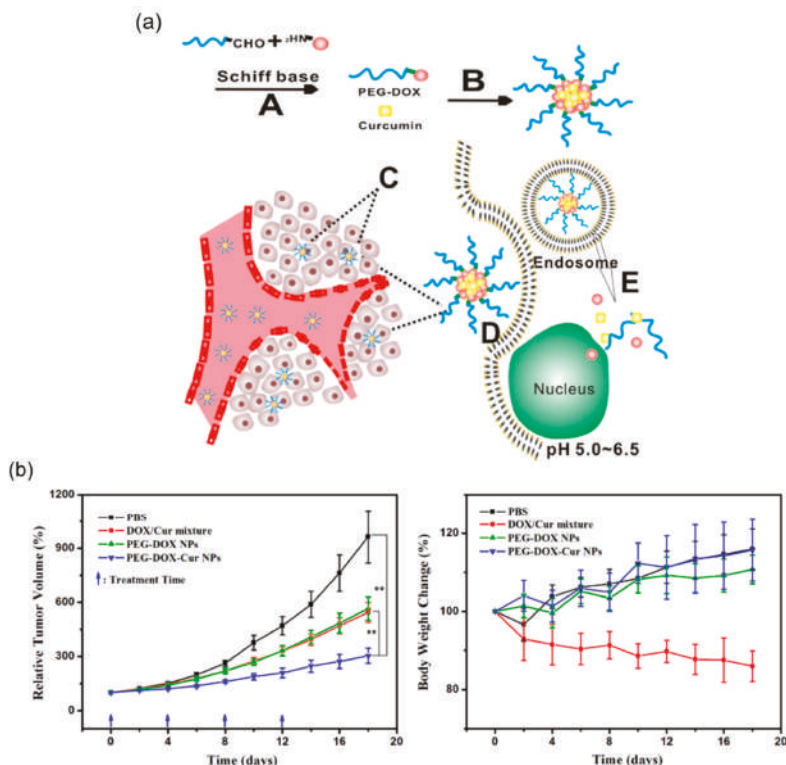
PAA induced the endosomal escape as a pH responsive membrane-destabilizing polymer, which respond to changes in pH by transitioning from an ionized, hydrophilic structure at physiologic pH (~7.4) to a hydrophobic, membrane-destabilizing conformation at endosomal pH values (<6.6). The diblock copolymers condensed siRNA into 80–250 nm particles. In HeLa cells, the siRNA-mediated knockdown of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) increased as the percentage of BMA in the second block increased.

#### 4.4. pH-Responsive Polymeric Anti-Cancer Drug Carriers for Cancer Treatment

Among the various therapeutic strategies for cancer, such as surgery, chemotherapy, radiotherapy, and gene therapy discussed above, chemotherapy is the most often used method in clinical practice [94,95]. However, the side-effects, low therapeutic efficacy and cytotoxicity of the traditional chemical drugs such as Dox, camptothecin and PTX, have hindered the treatment efficiency of cancer chemotherapy [96]. Polymeric nanoparticles have been developed and used as approaches to remove the problems because they can improve pharmacokinetics and biodistribution profiles of anti-cancer drugs via the EPR effect [97]. However, the EPR impact can only improve the accumulation of NPs in tumor tissues, the efficiency of cancer chemotherapy has always still been hindered by the insufficient drug release that induced the concentration of anticancer drugs to the level below the therapeutic window [98]. pH-responsive polymeric drug carriers, which enhance the triggered release of anti-cancer drugs by responding to the tumour acidic microenvironment, have been demonstrated a pathway to address this problem.

For tumor environment triggered drug release, anti-cancer drugs could be either physically encapsulated into pH-responsive nanoparticles or conjugated to polymer through acid-labile bonds. Kim group physically encapsulated Dox into a variety of pHis-based polymeric micelles for the CT26 tumor treatment. In vitro, the destabilized pH-responsive pHis core enhanced the triggered release of Dox into the cancer cell. In vivo, the Dox-loaded micelles showed a higher CT26 tumour suppression than free Dox did [99]. Etrych et al. conjugated Dox onto an amphiphilic *N*-(2-hydroxypropyl) methacrylamide (HPMA)-based polymer by hydrazone bonds [100]. HPMA copolymer–Dox conjugates were stable in a buffer at pH 7.4, Dox was released in a mild acidic conditions of the tumor microenvironment. In vivo, HPMA copolymer–Dox conjugates significantly reduced the toxic side effects of Dox and enhanced the anti-tumor efficacy.

Liu group reported a drug delivery system with the combination of physical encapsulation and covalent conjugation of anti-cancer drugs for cancer treatment [101]. Dox was conjugated to PEG by Schiff's base reaction. PEG-Dox prodrug formed stable nanoparticles (PEG-Dox NPs) in water at physiological pH, and encapsulated curcumin (Cur) into the core through hydrophobic interaction (Figure 16a). When the formed nanoparticles, denoted as PEG-Dox-Cur NPs, are internalized by tumor cells, the Schiff's base linker between PEG and Dox would break in the acidic environment that is often observed in tumors, causing disassembling of the PEG-Dox-Cur NPs and releasing both Dox and Cur into the nuclei and cytoplasm of the tumor cells, respectively. The PEG-Dox-Cur NPs demonstrated enhanced anti-tumor activity in animals compared with free Dox-Cur combination (Figure 16b).



**Figure 16.** pH-responsive nanoparticles with the combination of physical encapsulation and covalent conjugation of anti-cancer drugs for cancer treatment. (a) Schematic illustration of the synthesis and working principle of PEG-Dox-Cur NPs. **A** Synthesis of PEG-Dox NPs via Schiff's base. **B** Preparation of PEG-Dox-Cur NPs by nanoprecipitated technique. **C** Passive tumor targeting was achieved by EPR effect. **D** The PEG-Dox-Cur NPs could be internalized by cancer cells through endocytosis. **E** Dox and Cur were released with the cleavage of the Schiff's base in tumor cells and diffused into nucleus. (b) Relative tumor volume and bodyweight change in Balb/C nude mice after treatment by PBS, free DOX/Car mixture, PEG-Dox NPs and PEG-Dox-Cur NPs. \*  $p < 0.05$ ; \*\*  $p < 0.01$ . Reproduced with permission from Yumin Zhang, Cuihong Yang, Weiwei Wang Jinjian Liu, Qiang Liu, Fan Huang, Liping Chu, Honglin Gao, Chen Li, Deling Kong, Qian Liu, and Jianfeng Liu. Scientific Reports; published by Nature Publishing Group, 2016.

#### 4.5. Challenges and Opportunities of Translating pH-Responsive Nanomaterials

Despite the wide range of success evidences from pre-clinical studies, there remains significant challenges for pH-responsive polymeric nanomaterials to enter clinical path. In addition to the specific cases aforementioned, several general aspects should be taken into account. First of all, the formulation of the polymeric nanomaterial applications is much more complex than conventional technology, such as tablets and injections; this brings difficulties in achieving Good Manufacturing Practice (GMP) standards in large-scale production and quality control. Secondly, developing and standardizing biological assays for human toxicity evaluation and monitoring in clinical trials has to adopt the potential novel biology coming from these materials. Besides toxicity, assays are also needed for clinically relevant biomarker identification and validation. In addition, the pharmacokinetics and pharmacodynamics (PK/PD) study is obligated to grasp a deep understanding of the physiochemical properties of polymeric theranostics, adding layers of complexity to high-throughput data acquisition

and modeling. From a regulatory perspective, the clear guidelines for the nanomaterials to be developed facing forward translation still requires collaborative efforts.

Promising efforts have been invested towards these challenges. The development of reproducible and stable polymeric nanomaterial batch synthesis protocol can potentially benefit from advanced nanoparticle preparation technology [102,103]. Human organs-on-a-chip platform, mimicking key functions in a human body, offers a great opportunity to address the limited predictive value of conventional pre-clinical cell and animal models, which may be readily applied to polymeric nanomaterials for safety and efficacy evaluations [104].

## 5. Summary and Future Perspective

Various physiological abnormalities, including pH, reactive oxygen species, overexpressed proteins or enzymes, associated with cancer offer great opportunities for the design of stimuli-responsive polymeric materials for tumor-targeted delivery of diagnostic and therapeutic agents. There has been rapid growing interest in the design of pH-responsive polymeric materials to target tumor acidosis, a ubiquitous characteristic shared by almost all types of cancers.

Despite obvious promise in pH-responsive nanomaterials, several challenges may warrant further exploration. First, design of pH-sensitive polymeric materials that are safe for medical and pharmaceutical applications remains a major challenge. It is still highly desirable to explore biodegradable pH-responsive polymers with improved biocompatibility and reduced toxicity. Second, additional efforts should also be directed toward improved tumor targeting efficacy. pH-sensitive polymeric materials that can specifically deliver payload to tumor's microenvironment with minimal accumulation in normal tissues or endosomal organelles of tumor cells can drastically improve the therapeutic efficacy and decrease the side effects. However, the pH variation between tumor and surrounding normal tissues is not very significant, and different types of tumors may have slightly different level of acidosis. To meet the requirements of various applications that target tumor microenvironment and intracellular organelles pH-responsive polymers with tunable pKa values are required. Incorporation of multiple modalities into the same polymer or nanoparticle has also shown promise in enhancing tumor targeting efficiency. Intrinsic heterogeneity of tumors may require the design of polymeric nanomaterial system with multiple transition pH values. Moreover, lack of comprehensive mechanistic understanding of pH-triggered responsive behaviors, especially interactions between nanomaterials and in vivo host environment, still hampers our capability in rational design of more effective pH-responsive nanomaterials for cancer treatment. While continuous development of pH-responsive polymers with improved therapeutic efficacy is of great importance, the modification and optimization of existing polymeric materials with validated biocompatibility may represent a more straightforward and efficient strategy.

It's worth noting that nanomedicine is an interdisciplinary field that requires close collaboration between physicists, chemists, biologists, and physicians. Many reviews' proof-of-concept studies need a substantial amount of work before potential successful clinical translation. Nevertheless, recent advances in the polymer design, structure-property correlation, mechanistic understanding, and applications of a multitude of pH-sensitive nanomaterials reviewed here provide general guidelines for future rational design of more effective pH-responsive nanomaterials for cancer diagnosis and treatment.

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Review

# Recent Advances in Phenylboronic Acid-Based Gels with Potential for Self-Regulated Drug Delivery

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**Abstract:** Glucose-sensitive drug platforms are highly attractive in the field of self-regulated drug delivery. Drug carriers based on boronic acid (BA), especially phenylboronic acid (PBA), have been designed for glucose-sensitive self-regulated insulin delivery. The PBA-functionalized gels have attracted more interest in recent years. The cross-linked three-dimensional (3D) structure endows the glucose-sensitive gels with great physicochemical properties. The PBA-based platforms with cross-linked structures have found promising applications in self-regulated drug delivery systems. This article summarizes some recent attempts at the developments of PBA-mediated glucose-sensitive gels for self-regulated drug delivery. The PBA-based glucose-sensitive gels, including hydrogels, microgels, and nanogels, are expected to significantly promote the development of smart self-regulated drug delivery systems for diabetes therapy.

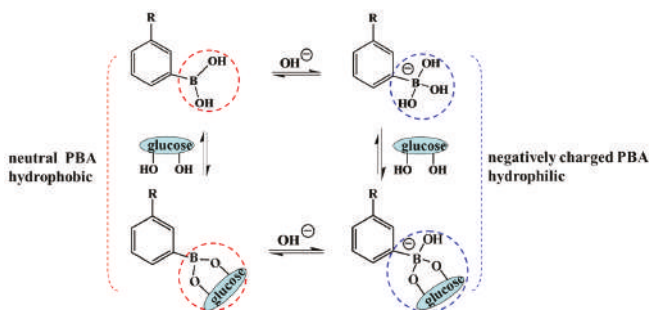
**Keywords:** phenylboronic acid; gel; glucose sensitivity; drug delivery; diabetes therapy

## 1. Introduction

Diabetes mellitus threatens human health seriously with a series of complications, such as cardiovascular disease [1]. The worldwide prevalence of diabetes is predicted to be about 366 million in 2030 with increasing attention for the treatment of diabetes [2]. The frequent injection of exogenous insulin is the major treatment of diabetes. In spite of quickly decreasing the blood glucose level, frequent insulin injection causes inevitable injection suffering and decline of the quality of life of patients. Even though implantable insulin pumps are the current optimal therapy for type 1 diabetic patients, their high cost limits their wide clinical application [3]. Smart insulin modified with an aliphatic domain and a phenylboronic acid (PBA) has been developed to tune the pharmacokinetics of insulin activity for personalized therapy, however, it must interface with insulin pumps, infusion devices, or controlled release materials to further improve performance [4]. An alternative treatment of diabetes with continuous and automatic regulation of drug release triggered by glucose directly is required. Therefore, glucose-sensitive materials, a kind of “intelligent” polymer, have greatly been used in self-regulated systems. Self-regulated systems, which are called an artificial pancreas, control insulin release triggered by elevated level of blood glucose continuously and automatically. By integrating glucose-triggered drug delivery with minimal patient intervention and improved diabetic life quality, glucose-sensitive drug delivery systems may prove valuable in diabetes therapy and replace frequent insulin injection [5].

Three kinds of glucose-sensitive materials have attracted growing scientific attention and are considered novel candidates to serve as self-regulated drug delivery systems. One strategy is based on glucose oxidase (GOD), which converts glucose to gluconic acid upon a pH change of the microenvironment, resulting in physicochemical changes of GOD-incorporated carriers [6]. The physicochemical changes of GOD-incorporated platforms induce glucose-triggered payload release [7–10]. Concanavalin A (Con A) is also be used to design glucose-sensitive drug delivery systems due to the specific binding capacity for glucose, glycopolymers, and polysaccharides [11–14]. However, GOD and Con A are protein-based components, and the instability and biotoxicity of GOD and Con A during fabrication and storage restrict their application in self-regulated drug delivery [15,16].

An alternative mechanism based on totally synthetic components, boronic acid (BA) and especially PBA, and their derivatives, have been largely investigated for self-regulated drug delivery systems [17–20]. Since Okano and coworkers firstly reported PBA-based glucose-triggered drug release, PBA and its derivatives have been greatly employed to design glucose-triggered drug delivery systems [21,22]. PBA-containing platforms are much more stable and suitable for long-term storage than GOD- and Con A-based drug carriers [23]. The principle of the PBA-mediated glucose-sensitive drug delivery system is based on the reversible reaction between PBA and *cis*-diol compounds [24]. There are two states of PBA in aqueous solution. One is the neutral trigonal-planar form and the other is the negatively charged tetrahedral boronate form. Between the two states of PBA moieties there is an equilibrium (Scheme 1). When in aqueous solution with pH above the  $pK_a$  of PBA (i.e., 8.2–8.6), most PBA moieties are negative and relatively hydrophilic. However, the PBA moieties are neutral and hydrophobic when the pH of the solution is below the  $pK_a$  of PBA [25,26]. In the presence of glucose or other 1,2- or 1,3-diols, both kinds of PBA moieties form a 5- or 6-cyclic boronic ester. However, the cyclic boronic ester between neutral trigonal-planar PBA and a diol cannot be formed with easy hydrolysis. The negatively charged PBA state forms a more stable cyclic boronic ester with *cis*-diol compounds, resulting in the increase of the negatively charged PBA content and improved hydrophilicity of PBA-containing materials [27,28]. The increased hydrophilicity of PBA-modified materials induces the swelling, disassembly, and/or destruction of PBA-mediated platforms with subsequent release of the payload. Glucose can also form bis-bidendate complexes with PBA resulting from the reaction between one glucose molecule and two *cis*-diols. Therefore, the bis-bidendate complexes induce the shrinking of PBA-functionalized platforms [29]. All the swelling and shrinking of platforms alter the diffusion behavior of the payload, triggered by glucose-sensitive volume phase transition. PBA-functionalized materials present great potential applications in self-regulated drug delivery [29,30].



**Scheme 1.** Complexation equilibrium between phenylboronic acid (PBA) and glucose.

There are many forms of PBA-functionalized platforms which have been exploited to investigate glucose sensitivity. Polymer micelles, vesicles, and capsules are obtained by the self-assembly of PBA-modified amphiphilic polymers, while layer-by-layer (LbL) films are obtained by the

alternating adsorption of polymers with opposite charges. Even though these devices have good glucose sensitivity, the concentration-dependent disintegration, unstably long-term storability, and weakened mechanical strength restrict their applications in glucose-sensitive drug delivery. PBA-functionalized mesoporous silica nanoparticles (MSN) have attracted improving interests due to their characteristics of high biocompatibility, improved drug loading, and functionalized surface. The glucose-sensitive nanocarriers and LbL films and microcapsules for self-regulated drug delivery have been reviewed [26,31]. Besides, PBA-based hydrogels, microgels, and nanogels with chemically or physically cross-linked structures have great potential applications in self-regulated drug delivery systems. The 3-D structures endow the hydrogels, microgels, and nanogels with reversible physicochemical changes that result in payload release. Usually, hydrogels are macroscopic hydrogels. Microgels are micro-sized hydrogel particles, while nanogels are hydrogel nanoparticles or nanohydrogels with nano-scale dimensions (typically 20–250 nm) [32,33]. The Zhang group and Catargi group have discussed the fabrication and application of PBA-containing macroscopic hydrogels and microgels with discussion of their further development [29,34]. However, studies of PBA-based hydrogels, microgels, and nanogels for self-regulated drug delivery are rare. In summary, this article reviews the recent development of self-regulated drug delivery based on PBA-mediated glucose-sensitive gels.

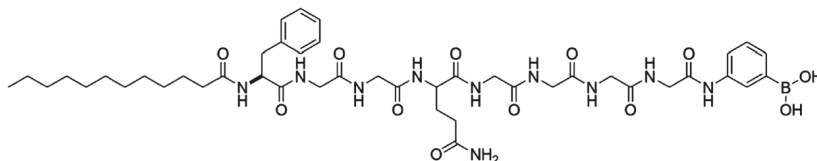
## 2. PBA-Based Hydrogels

Hydrogels have received considerable attention for drug delivery applications. There have been extensive studies on PBA-functionalized hydrogels for self-regulated drug delivery systems.

Low-molecular-weight hydrogels (LMWG) have been largely investigated for controlled drug delivery due to their super-sensitivity to external stimuli. LMWG were formed by the self-assembly of gelators with low-molecular-weight, where the gel-sol transition was easily achieved by changing stimuli. An injectable glucose-sensitive LMWG was prepared by the self-aggregation of the gelator which was synthesized with a pyrene moiety coupled with PBA by L-phenylalanine and a 2,2'-(ethylenedioxy) bis(ethylamine) linker [35]. The pyrene unit and PBA moiety endowed the physical cross-linked LMWG with glucose sensitivity, and the resulting compound was utilized to detect glucose and control insulin release at physiological pH. Differently from chemically cross-linked hydrogels, physically cross-linked networks have transient junctions arising from hydrogen bonding,  $\pi$ - $\pi$  stacking, and van der Waals interactions. The self-aggregation of the gelator provides a new strategy for the design of PBA-based glucose-sensitive hydrogels.

Gao and coworkers investigated the dual-responsiveness of a PBA-based low-molecular-weight organogel to both glucose and pH [36]. In addition, the group studied the gelation properties and glucose sensitivity of a PBA-containing low-molecular-weight organogel based on an alkyl chain (C2–C11), where the glucose sensitivity of the organogel was associated with the molecular structure of the gelator [37]. Integrating an oligopeptide with PBA, the same group demonstrated that the LMWG self-assembled from a gelator exhibited dual-responsive and long-lasting drug delivery [38]. The structure of the gelator is shown in Figure 1. The hydrophilicity of gelator was regulated by the oligopeptide which was made of the natural amino acids L-phenylalanine, glycylglycine, and L-glutamine. The alkyl chain of lauroyl chloride was introduced to adjust the hydrophobicity of the gelator in favor of the sol-gel transition. PBA unit endowed the gelator with glucose sensitivity. When the sol was cooled to body temperature, LMWG was obtained very quickly within two minutes. The sol-gel transition resulted from the enhanced  $\pi$ - $\pi$  stacking owing to the benzene units as well as hydrogen bonding and van der Waals force within the gelators. The LMWG was very stable during the drug release even for three months or with severe shaking, indicating its long-lasting drug delivery. The pH-sensitivity of the hydrogels, a result of the ionization of the PBA and amide moieties in acid or alkali solutions, was used for doxorubicin delivery for cancer therapy. Importantly, PBA endowed the hydrogels with glucose sensitivity. The release of phenformin, an antidiabetic drug, was regulated in response to changes in glucose concentration. The fact that increasing

glucose concentration induced much more drug release indicated its promising application in a self-regulated drug delivery system. Integrating the pH-sensitivity with glucose-triggered drug release, the long-lasting glucose/pH-responsive hydrogels will have notable importance in the development of self-tuning controlled-release systems for diabetes and cancer therapy.



**Figure 1.** The structure of the gelator used for self-regulated drug delivery. (Reprinted from ref. [38]).

Even though LMWG used as a drug carrier possesses good glucose-sensitive drug delivery, the stability of LMWG is not sustainable enough for long-term drug delivery compared to the supramolecular hydrogels with chemical cross-linked structure. A multiresponsive hydrogel was synthesized by copolymerization of (2-dimethylamino) ethyl methacrylate (DMAEMA) and 3-acrylamidophenylboronic acid (AAPBA) using *N,N'*-methylenebisacrylamide (NNMBA) as a cross-linker [39]. The P(DMAEMA-*co*-AAPBA) interpenetrating (IPN) hydrogels possessed glucose-triggered drug release under physiological pH and temperature. The increased Lewis acidity of the boron center lowered the  $pK_a$  of the PBA residues. The Lewis acid–base interactions between the electron-poor boron atom and the electron-rich nitrogen atom on poly(DMAEMA) endowed the hydrogel with glucose sensitivity under physiological pH. These multiresponsive hydrogels have important application in self-regulated drug delivery.

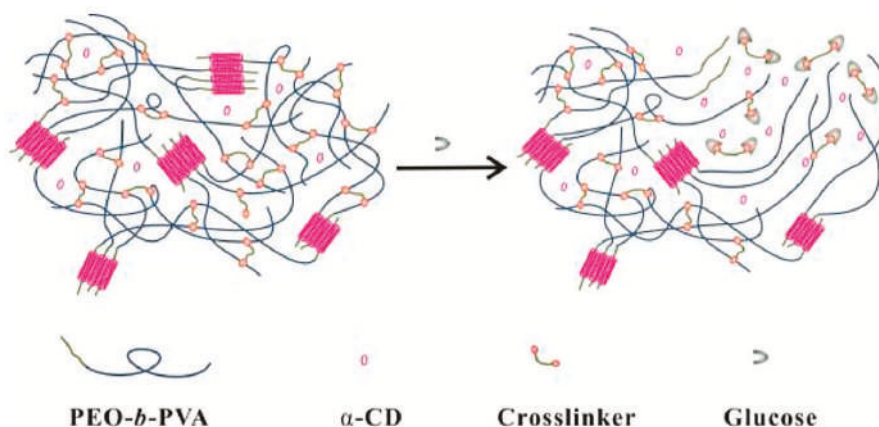
Also based on poly(DMAEMA), a novel triple-responsive semi-interpenetrating (semi-IPN) hydrogel was exploited [40]. Poly(3-acrylamidophenylboronic acid-*co*-(2-dimethylamino) ethyl methacrylate) (P(AAPBA-*co*-DMAEMA)) has a cross-linked structure incorporated with interpenetrating  $\beta$ -cyclodextrin-epichlorohydrin ( $\beta$ -CD-EPI)—P(AAPBA-*co*-DMAEMA)/( $\beta$ -CD-EPI) semi-IPN hydrogel. The higher content of  $\beta$ -CD decreased the equilibrium swelling ratios (ESRs) of semi-IPN hydrogels due to the complexation between PBA groups and the dihydroxyl of  $\beta$ -CD. Besides, pH, temperature, ionic strength, and glucose concentration of the media significantly affected the ESRs of the hydrogels. Using ibuprofen and aminophylline as hydrophobic and hydrophilic model drugs, the drug loading and release profiles were investigated. The structure of the hydrogels affected the drug loading ratio. Increased  $\beta$ -CD content led to of ibuprofen and a lower drug loading ratio of aminophylline. The lower drug loading ratio of hydrophilic aminophylline in hydrogels with high  $\beta$ -CD content resulted from the lower ESRs, which was induced by the increased complexation between PBA and  $\beta$ -CD. However, the loading ratio of hydrophobic ibuprofen increased with the increase of  $\beta$ -CD content in hydrogels. The reason was that hydrophobic ibuprofen formed inclusion complexes with  $\beta$ -CD via host–guest interactions. The release profiles of drug from the hydrogels could be adjusted by pH, temperature, glucose concentration, and type release medium. The semi IPN hydrogels may be useful as a guideline for the optimal design of glucose-sensitive drug delivery systems.

To enhance the response of PBA-based hydrogel to blood glucose concentration, a comb-type grafted poly(*N*-isopropylacrylamide-*co*-3-acrylamidophenylboronic acid) (poly(NIPAM-*co*-AAPBA)) hydrogel was exploited [41]. The hydrogel was introduced by grafting poly(NIPAM-*co*-AAPBA) side chains onto cross-linked poly(NIPAM-*co*-AAPBA) networks. The comb-type hydrogel presented a rapid response to the blood glucose concentration owing to the effects of the freely mobile ends of the grafted poly(NIPAM-*co*-AAPBA). Grafted poly(NIPAM-*co*-AAPBA) side chains formed complexes with glucose quickly without any restrictions than that between cross-linked poly(NIPAM-*co*-AAPBA) hydrogel and glucose. The rapid response rate endowed the grafted hydrogel with attractive applications in self-regulated drug delivery systems.



Besides the structure of hydrogels, the composition of hydrogels also affects the glucose sensitivity. Magda and coworkers studied the effect of chemical composition on the response of zwitterionic glucose-sensitive hydrogels using DOE (design of experiments) methods [42]. Zwitterionic glucose-sensitive hydrogels were prepared by the copolymerization of *N*-(3-(dimethylamino)propyl) acrylamide (DMA PAA) and AAPBA. The molar ratio of AAPBA/DMA PAA and the wt % of the monomer in the pregel solution were the primary factor for determining the value of the inverse of the 1st order rate constant, where a decreasing amount of cross-linker obtained faster glucose responses.

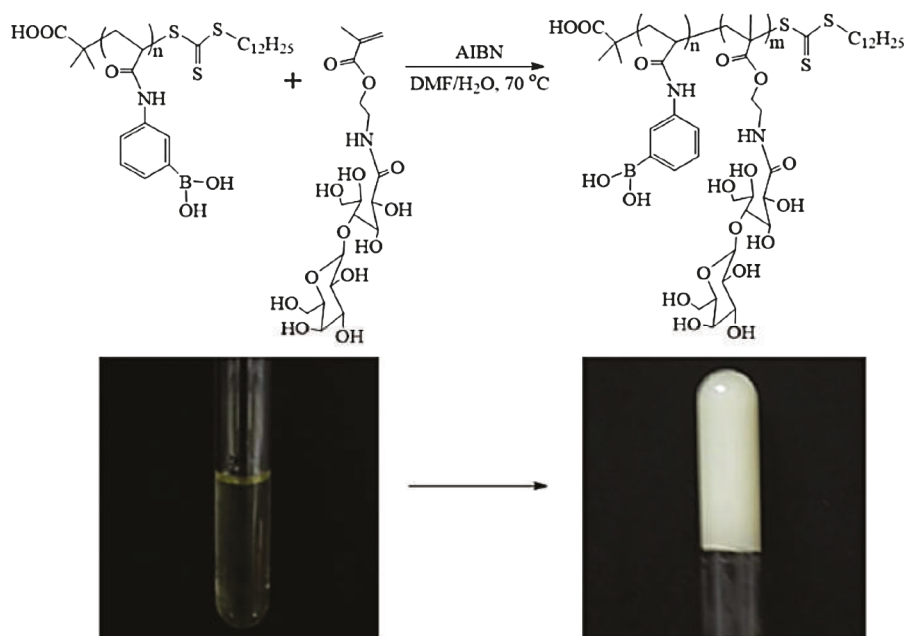
In addition to stable chemical cross-linked hydrogels using a small molecular as the cross-linker, reversible dynamic cross-linked hydrogels have also been designed as glucose-sensitive drug carriers. Hydrogels were obtained by the reversible covalent complexation of PBA with *cis*-1,2 or *cis*-1,3-diol compounds as a dynamic link [43,44]. Glucose-sensitive solid-like hydrogels based on dynamic covalent chemistry and inclusion complexation were also designed [45]. By simply mixing the solutions of poly (ethylene oxide)-*b*-poly vinyl alcohol diblock polymer (PEO-*b*-PVA),  $\alpha$ -cyclodextrin ( $\alpha$ -CD), and a double PBA-terminated PEO cross-linker, hydrogels were easily obtained. As shown in Figure 2, the inclusion complexation between PEO and  $\alpha$ -CD, and the dynamic covalent bonds between PBA and PVA, strengthened the hydrogel network. In addition, the formation of hydrogels was the cooperative interaction of the inclusion complexation and dynamic covalent chemistry. The increase of  $\alpha$ -CD content shortened the gelation time and enhanced the structural recovery ability with the significant contribution of increased dynamic covalent chemistry to the cross-linking density of hydrogel network. The release of FITC-labeled BSA from the hydrogels had no burst release property at physiological pH, indicating that the FITC-labeled BSA was loaded inside the hydrogels instead of being adsorbed on the surfaces of the hydrogels. These hydrogels have great potential as self-regulated drug delivery vehicles with tunable glucose sensitivity.



**Figure 2.** Schematic and the glucose-sensitive mechanism of the hydrogel composed of PEO-*b*-PVA,  $\alpha$ -CD, and double PBA-terminated poly (ethylene oxide) (PEO) cross-linker. (Reprinted from ref. [45].)

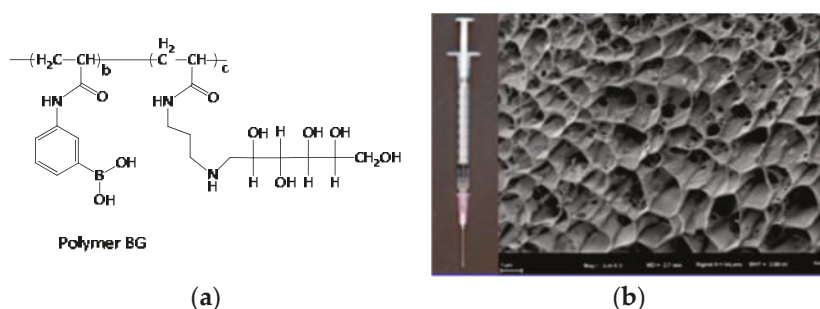
Via dynamic boronic ester bonds, glycopolymer hydrogels were exploited to control insulin release triggered by glucose [46]. The copolymerization of AAPBA and 2-lactobionamidoethyl methacrylate (LAMA) was conducted by the reversible addition–fragmentation chain transfer (RAFT) method and the obtained block glycopolymer formed hydrogels by phenylboronate-diol crosslinked binding (Figure 3). The LAMA content played an important role in the swelling of the hydrogels. For the hydrogels with the highest LAMA content, the equilibrium swelling ratio was up to 1856%. In addition, with high LAMA content, the insulin loading capacity of the hydrogels increased. Higher content of LAMA enhanced the charged phenylborates, which resulted in enhanced hydrophilicity of the hydrogels and subsequently promoted drug permeation and adsorption. The introduction

of carbohydrate moieties improved the cytocompatibility of the glycopolymer hydrogels. The insulin release from the glycopolymer hydrogels exhibited glucose sensitivity due to glucose-induced dissociation of boronic ester linkages of hydrogels. Importantly, the released insulin possessed an original conformation compared with standard insulin. PBA-based glycopolymer hydrogels are an alternative design of glucose-sensitive drug delivery systems.



**Figure 3.** The formation of PBA-containing glycopolymer hydrogel before and after. (Reprinted from ref. [46]).

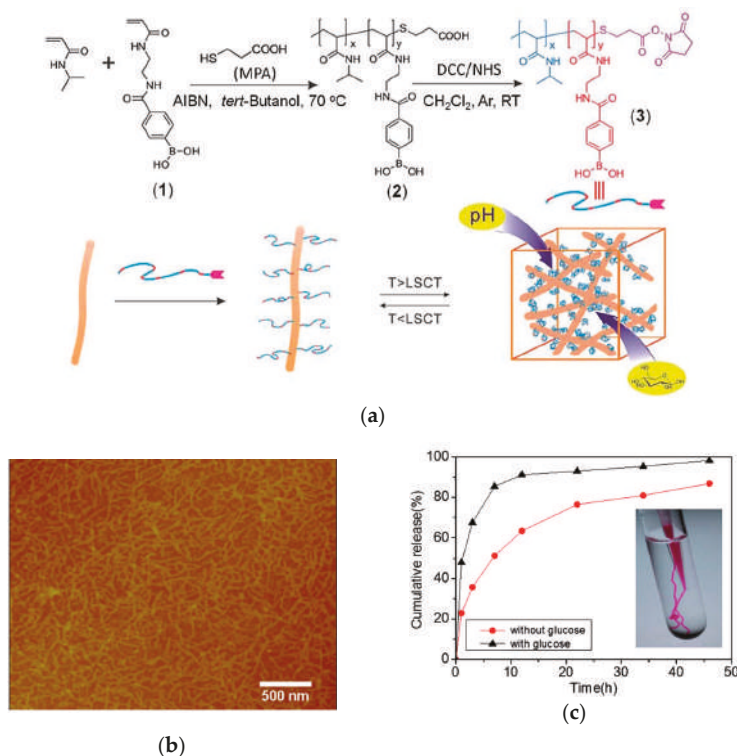
Another series of glucose-sensitive block glycopolymer hydrogels based on dynamic boronic ester bonds were exploited for glucose-sensitive drug delivery [47]. As shown in Figure 4a, block glycopolymer, (3-propionamidophenyl)boronic acid (*N*-(3-((2,3,4,5,6-pentahydroxyhexyl) amino)propyl)propionamide) (noted polymer BG), was cross-linked through PBA–glucose complexation within the glycopolymer and injectable self-healing hydrogels were obtained. The glycopolymer with 10–60% content of PBA formed self-supporting hydrogels at 5% weight in water, which could be easily loaded and extruded through a needle with the reformation of hydrogels (Figure 4b). The hydrogels possessed rapid recovery with shear-thinning and self-healing behaviors which were consistent with the injectable hydrogel. In addition, the glycopolymer hydrogels exhibited glucose-triggered rhodamine B release. Integrating injectable and self-healing properties with glucose-induced drug release, glycopolymer hydrogels have potential application in the treatment of diabetes.



**Figure 4.** (a) Structure of PBA-containing glycopolymer BG; (b) the soft and injectable hydrogel formed by polymer BG with 50% PBA, and the SEM image of the obtained hydrogel. Scale bar: 1  $\mu\text{m}$ . (Reprinted from ref. [47]).

Hydrogels based on PBA-containing polymers for self-regulated drug delivery system have been studied widely, while the bioconjugates of PBA-functionalized polymer and protein or peptide have been rarely used for glucose-sensitive drug delivery. Multiresponsive hydrogels were prepared from the bioconjugates of end-functionalized PBA containing copolymers and rod-like M13 viruses [48]. The end-functionalized PBA containing copolymer poly(NIPAM-*co*-PBA)-NHS was synthesized by two steps (Figure 5a). Firstly, poly(NIPAM-*co*-PBA)-COOH was synthesized by chain transfer free radical copolymerization of NIPAM and 4-(1,6-dioxo-2,5-diaza-7-oxamyl) phenylboronic acid (DDOPBA) using 3-mercaptopropionic acid (MPA) as the chain transfer agent. Then, the  $-\text{COOH}$  of the obtained poly(NIPAM-*co*-PBA)-COOH was transferred into *N*-hydroxysuccinidic ester. The hybrid virus-polymer bioconjugate was prepared by the conjugation of poly(NIPAM-*co*-PBA)-NHS to rod-like M13 virus, which was a natural protein assembly with a large amount of functional groups. The gelation behavior of virus-polymer bioconjugates was multiresponsive and reversible. The gelation of virus-polymer bioconjugates was owing to the collapsed hydrophobic state of poly(NIPAM-*co*-PBA), which conferred the attractive interactions between the viruses and drove the viruses into the hydrogels. AFM revealed that the rod-like virus was interconnected with itself inside the hydrogel with large pores (Figure 5b). The virus-polymer bioconjugates were in sol state when the temperature was below the critical gelation temperature ( $T_g$ ), and in the gel state when the temperature was above  $T_g$ . The  $T_g$  of virus-polymer bioconjugates was sensitive to pH. At pH 7.10, the  $T_g$  was 15  $^{\circ}\text{C}$ , and increased when the pH increased. Furthermore, the  $T_g$  of the hybrid virus-polymer bioconjugates was 18  $^{\circ}\text{C}$  in the absence of glucose, while it was 23  $^{\circ}\text{C}$  in the presence of glucose at pH 7.65. In addition, the negligible hysteresis for the sol-to-gel and opposite gel-to-sol transition cycles indicated the reversible gelation behavior of the virus-polymer bioconjugates. Besides, the temperature-sensitive gelation behavior was in favor of the encapsulation of bioactive species inside the hydrogels with high loading capacity. Insulin-loaded hydrogels were obtained by the injection of a mixture of insulin and polymer-grafted virus at 4  $^{\circ}\text{C}$  into PBS buffer at 37  $^{\circ}\text{C}$  (inset of Figure 5c). Glucose-sensitive insulin release was also observed (Figure 5c). Glucose-triggered insulin release, combined with reversible temperature-, pH-, and glucose-regulated gelation behavior, endowed the PBA-containing polymer-virus bioconjugates with great potential in self-regulated drug delivery.

Cross-linking glucose-sensitive hydrogels based on PBA provides suitable semiwetting with reversible swelling/shrinking changes which are suitable for self-regulated drug delivery. However, the glucose sensitivity of bulk hydrogels hysteretically results from the slow permeation of glucose into the hydrogel network. In addition, the practical application of bulk hydrogels for the regulation of blood glucose levels is restricted by the implantation or other surgical approach. Even though injectable hydrogels are convenient to administer, a lot of effort is needed to promote the practical application of glucose-sensitive hydrogels for diabetes therapy.



**Figure 5.** (a) Schematic representation of the preparation of the hybrid virus–polymer bioconjugate of end-functionalized PBA- and *N*-isopropylacrylamide (NIPAM)-containing copolymer to the rod-like M13 virus, and the multiresponsiveness of the hybrid virus–polymer bioconjugate; (b) Internal structure of the gel as revealed by AFM; (c) insulin release behavior of the virus based hydrogel in the presence or absence of glucose. Inset in (c) hydrogel forms instantly when the polymer grafted virus in the sol state was injected into the aqueous solution at 37 °C. (Reprinted from ref. [48]).

### 3. PBA-Functionalized Microgels

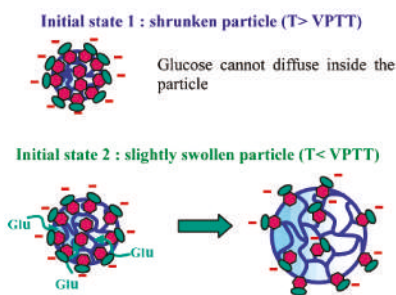
Even though PBA-based hydrogels are glucose-sensitive, the implantation administration of bulk hydrogels and the inconvenient injection of injectable hydrogels restrict the practical application of glucose-sensitive hydrogels in self-regulated drug delivery. Compared with hydrogels, PBA-based microgels have promising application in glucose-sensitive drug delivery [49]. Microgels are gel particle dispersions with average diameters ranging between 50 nm and 5 mm [50]. The small size endows the microgels with rapid swelling or shrinking in response to environmental changes. The glucose-response time of PBA-containing microgels are much faster than that of hydrogels. Kataoka and coworkers reported that gel beads containing AAPBA and NIPAM need a very long time (as long as 400 min) for glucose sensitivity [51]. However, microgels with the same components need only  $10^2$  s for glucose-induced swelling [52]. Besides faster glucose-response time, the administration of microgels using a minimally invasive method is another advantage of microgels for self-regulated drug delivery. In addition, microgels display high stability, which is promising for glucose-sensitive drug delivery vehicles.

A range of PNIPAM-based and PBA-functionalized microgels have been synthesized via the copolymerization of NIPAM and PBA-containing comonomers or the postpolymerization of functional PBA derivatives to PNIPAM microgels [53–57]. Poly(*N*-isopropylacrylamide-*co*-acrylic acid)

P(NIPAM-*co*-AA) was modified by 3-aminophenylboronic acid (3-APBA), resulting in PBA-mediated glucose-sensitive microgels [58,59].

Glucose-induced swelling of the poly(*N*-isopropylacrylamide-*co*-3-acrylamidophenylboronic acid) (P(NIPAM-3-AAPBA)) microgel was explained by the formation of a 1:1 glucose–phenylboronate complex, which increased the degree of ionization and created a Donnan potential between the gel phase and the liquid phase [52,60].

For P(NIPAM-PBA) microgels, the swelling response of microgels to glucose relates to the microgel composition and glucose concentration. Ravaine studied the effects of PBA content and glucose concentration on the swelling of monodispersed PNIPAM submicrometric microgels modified with AAPBA at different temperatures [61]. Compared to native PNIPAM particles, the incorporation of AAPBA decreased the volume phase transition temperature (VPTT) of the P(NIPAM-PBA) microgels. With the increase of PBA content, the swelling response of the microgels increased. However, the swollen state of the microgels that was achieved with same PBA content was strongly dependent on the initial temperature of the suspension with a constant glucose concentration. As shown in Figure 6, when the initial temperature was above VPTT, the microgels were smaller and the swelling was slight. In this case, glucose was unable to diffuse inside the microgel particle when it was initially collapsed. Only a small part of glucose linked to the PBA which was incorporated on the surface of microgels. However, when the initial temperature was below VPTT, a slight swelling of the microgels was aided the permeation and diffusion of glucose to the inside of the microgel particle. As a result, all the PBA was linked by glucose to form more hydrophilic boronic ester, inducing a much higher swelling degree of the microgels.

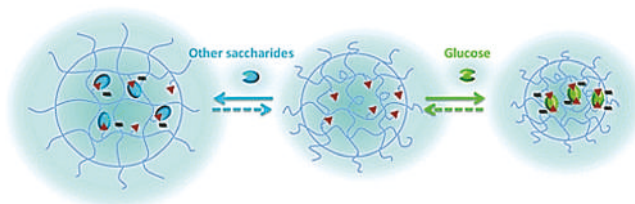


**Figure 6.** Schematic representation of microgel swelling upon glucose addition, showing the importance of the initial state. (Reprinted from ref. [61]).

Zhang and coworkers also studied the volume phase transitions of glucose-sensitive PBA-functionalized P(NIPAM-3-AAPBA) microgels which were synthesized by the coupling of 3-APBA to P(NIPAM-*co*-AA) microgels [62]. The microgels presented a two-stage thermosensitive volume phase transition in the presence of glucose. When temperature increased, the microgels underwent a small degree of collapse and a large volume change followed. In addition, the glucose sensitivity of the microgels was affected by pH. At pH = 7.5, which is below the  $pK_a$  of PBA, the glucose-induced size changes of the microgels were negligible. In this case, most PBA groups were hydrophobic with few boronate esters produced. However, when the pH increased, the glucose sensitivity of the microgels was enhanced as a result of the improved hydrophilicity of the microgels with resultant boronate ester. The glucose-induced size expansion of the microgels was depressed by high ionic strength due to the weakening of Donnan potential. At room temperature, remarkable glucose-induced swelling was observed in the microgels with high content of PBA.

Most PBA-based microgels exhibit glucose-induced expansion due to the production of glucose–mono(boronate) complexes [57,63]. The glucose–mono(boronate) complexes make the ionization equilibrium of PBA shift from uncharged and hydrophobic state to the charged and

hydrophilic state with the formation of tetrahedral boronate esters. The tetrahedral boronate esters further increase the hydrophilicity of the microgels and induce the swelling of microgels due to the glucose-induced increase in the Donnan potential. Besides glucose-induced swelling of microgels, glucose-induced shrinking of microgels also has been reported. As shown in Figure 7, glucose exhibits the unique property to form glucose-bis(boronate) complexes through its furanose form [64–66]. However, most physiological relevant saccharides, such as galactose, mannose, and fructose, can only bind to boronic acid as monodentate complexes [67]. Zhang designed a poly(*N*-isopropylacrylamide-*co*-2-acrylamidophenylboronic acid) (P(NIPAM-2-AAPBA)) microgel, which was synthesized by modification of the poly(*N*-isopropylacrylamide-*co*-acrylic acid) (P(NIPAM-AAc)) microgel with 2-aminophenylboronic acid (2-AAPBA) [17]. In the presence of glucose, the size of the P(NIPAM-2-AAPBA) microgels decreased, showing contraction-type glucose sensitivity. The glucose-induced shrinking of the microgels was related with the glucose-bis(boronate) complexes in which one glucose molecule complexed with two PBA groups in a 1:2 binding model. The resultant glucose-bis(boronate) complexes increased the cross-linking density and reduced the degree of swelling of the microgels. The contraction-type glucose-sensitive microgels offer a new strategy for the design of glucose-sensitive microgels.



**Figure 7.** Schematic representation of glucose-induced microgels shrinking with glucose-bis(boronate) complexation (Reprinted from ref. [66]).

Except for contraction-type glucose-sensitive microgels, glucose-induced shrinking/swelling microgels were also designed. Ravaine and coworkers firstly designed the PBA-containing microgels cross-linked with bis-boronate complexes which either swelled or shrank selectively depending on glucose concentration under physiological conditions [66]. The microgels were obtained by copolymerization of DDOPBA with an alkylacrylamide (NIPAM, *N*-isopropylmethacrylamide (NIPMAM), or *N*-ethylmethacrylamide (NEMAM)) and a cross-linking agent (*N,N'*-methylenebis(acrylamide) (BIS), or ethylene glycol dimethacrylate (EGDMA)). The effect of monomer composition on the swelling/shrinking behavior of the microgels was studied. For microgels based on NIPAM and PBA (P(NIPAM-PBA) microgels), the VPTT was associated with the PBA content. More PBA content lowered the VPTT of P(NIPAM-PBA) microgels, which was conformed in the author's earlier work [61]. However, high glucose concentration increased the VPTT of P(NIPAM-PBA) microgels. In addition, at constant PBA content, the VPTT of NIPAM microgels was lower than that of NIPMAM microgels, which was lower than that of NEMAM microgels. All kinds of microgels displayed glucose-sensitive behaviors. When at a pH above the  $pK_a$  of PBA, EGDMA cross-linked NEMAM and NIPMAM microgels exhibited a two-step behavior wherein low glucose concentration induced microgel shrinkage and high glucose concentration induced microgel swelling. The shrinking behavior was associated with the additional cross-linking junctions due to glucose-bis(boronate) complexes at low glucose concentration. When the glucose concentration increased, the glucose-bis(boronate) complexes were converted to glucose-mono(boronate) complexes owing to the glucose-induced cross-link disruption. As a result, the microgels swelled under high glucose concentration due to the increased polymer charges and hydrophilicity. The glucose-response behavior of the microgels was related to the nature of the cross-linker and the modification of the PBA. The shrinking/swelling behavior was selective for glucose because the saccharide-bis(boronate)



complexes were highly selective for glucose and could not occur with other sugars, such as fructose, which was also demonstrated by Asher and coworkers [67].

As mentioned above, polymers based on NIPAM, NEMAM, and EGDMA are thermosensitive and the VPTT of P(NIPAM-PBA) microgels are adjusted by glucose concentration and PBA content. Some other kinds of PBA-based microgels also possess volume phase transition behavior. The glucose-responsive volume phase transition behavior of poly(phenylboronic acid) (pPBA) microgels was switchable [68]. The preparation of the pPBA microgels was comprised 3-VAPBA covalently bonded onto the microgels of oligo(ethylene glycol)-based polymers (Figure 8). The stability of the pPBA-2 microgels was improved by further addition of a poly(acrylamide) (poly(AAm)) gel layer onto pPBA-1 microgels. In the presence of glucose, the glucose-response behaviors of pPBA microgels were different under different temperatures. At lower temperatures below 29.0 °C, the microgels shrunk when glucose was added, while at higher temperatures above 33.0 °C, the microgels swelled when glucose was added. Around 31.0 °C, negligible volume change of the microgels was recorded upon adding glucose. The pPBA microgels with switchable glucose-responsive volume phase transition behavior provide guidelines for the design of glucose-sensitive microgels.

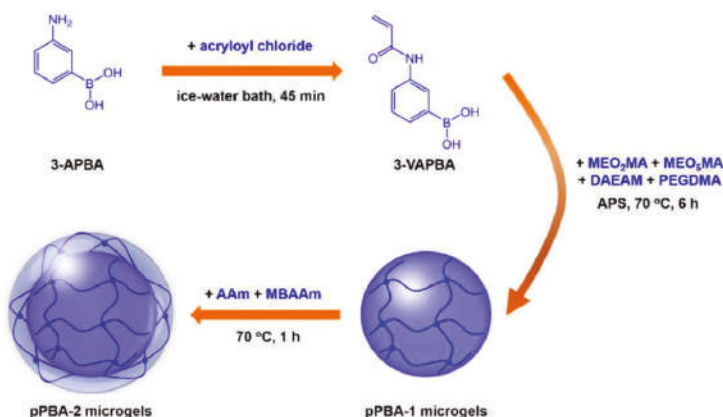
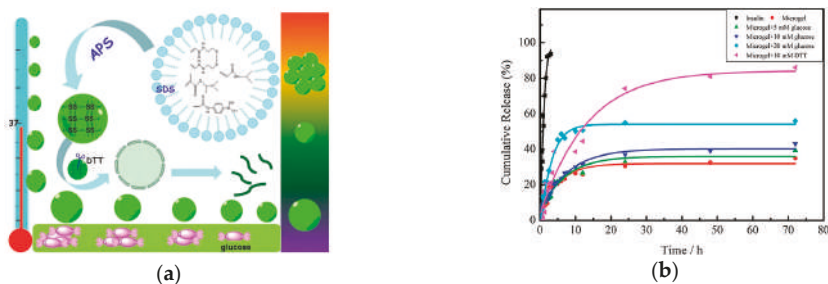


Figure 8. Illustration of the synthesis of the proposed pPBA microgels. (Reprinted from ref. [68]).

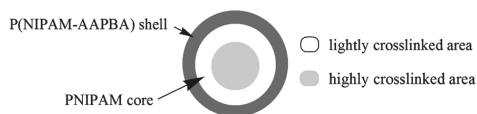
Another multifunctional microgel with high stability and degradability was designed [69]. As shown in Figure 9a, the microgels were prepared by free radical polymerization of NIPAM, DMAEMA, and AAPBA through a precipitation emulsion method using reductive degradable *N,N'*-bis(acryloyl)cystamine (BAC) as the cross-linker. The as-synthesized microgels exhibited pH-, temperature-, and glucose sensitivity at physiological conditions and gradual degradation. The porous network structure of the microgels was in favor of the trapping of insulin. Higher glucose concentration triggered the faster release of a larger amount of insulin. The microgels were degraded in the presence of dithiothreitol (DTT), which has similar function to glutathione tripeptide (GSH). As a result, a higher amount of insulin was released (90%) from the multiresponsive microgels in the medium (Figure 9b). The degradable multifunctional microgels may provide a new strategy for the design of PBA-functionalized self-regulated drug delivery systems.

Additionally, core-shell microgels based on NIPAM and PBA were exploited to investigate their glucose sensitivity. PNIPAM (core)/P(NIPAM-AAPBA) (shell) microgels were prepared by the modification of PNIPAM (core)/P(NIPAM-AA) (shell) microgels with 3-APBA [70]. The core-shell microgels exhibited three structure-related phase transitions when heating (Figure 10). The first phase transition was assigned to the P(NIPAM-AAPBA) shell, while the second and the third phase transitions were related to the PNIPAM core. The structure of the PNIPAM core was heterogeneous. The cross-linking density decreased gradually from the core towards the periphery due to the higher polymerization rate of cross-linking monomer BIS than that of the PNIPAM monomer. As a result, the

PNIPAM core had a quasi-core-shell-like structure with a “quasi-core” with a higher cross-linking density and a “shell-like” with a lower cross-linking density. The phase transition temperature of the “shell-like” structure was lower than that of the “quasi-core”, providing the two phase transitions of the PNIPAM core. Besides, the core-shell microgels exhibited glucose-triggered swelling due to the PBA moieties on the P(NIPAM-AAPBA) shell.



**Figure 9.** (a) Schematic illustration of smart multifunctional microgels with pH-, temperature-, and glucose sensitivity at physiological conditions and gradual degradation; (b) Release profiles of microgels in the presence of glucose in PBS of 7.4 at 37 °C. (Reprinted from ref. [69]).



**Figure 10.** Schematic illustration of the structure of PNIPAM (core)/P(NIPAM-AAPBA) (shell) microgel particles, where the PNIPAM core can be further divided as highly cross-linked “core” with BIS-rich and lightly cross-linked “shell” that is BIS-poor. (Reprinted from ref. [70]).

The glucose-induced shell permeability of core-shell microgels with the same components of NIPAM and PBA was also demonstrated [71]. Differently, the core of P(NIPAM-AAPBA) microgels was cross-linked by degradable cross-linker *N,N'*-(1,2-dihydroxyethylene)bisacrylamide (DHEA), while the shell was cross-linked by BIS. The cross-linked core of the microgels could be degraded by addition of stoichiometric amount of NaIO<sub>4</sub>. The permeability of the P(NIPAM-PBA) shell was controlled by temperature and pH change. More importantly, glucose concentration also could tune the shell permeability. The shell permeability increased with increased glucose concentration, which resulted from the increased borate ester. The fundamental understanding of the glucose-induced permeability control is very important for the design and potential application of PBA-based glucose-sensitive microgels.

PBA-functionalized cross-linking microgels exhibit glucose-induced swelling or shrinking depending on the structure of borate ester between PBA and glucose. At lower glucose concentration, the glucose-bis(boronate) complexes induce the shrinking of microgels with a swollen state. In this condition, there is contraction-type glucose sensitivity. At higher glucose concentration, glucose-mono(boronate) complexes lead to glucose-induced expansion of microgels. Even though the investigations were performed with in vitro studies, it is still worth emphasizing that the reversible glucose-triggered swelling/shrinking changes of PBA-based microgels have potential application in self-regulated drug delivery.

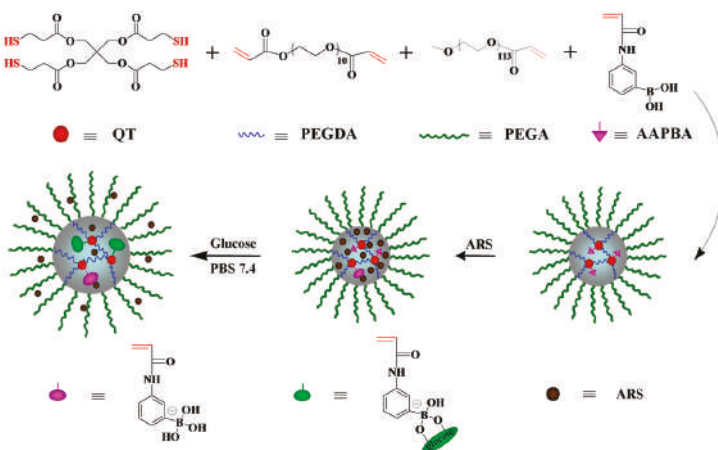
#### 4. PBA-Functionalized Nanogels

Even though microgels are most commonly used as the glucose-sensitive drug delivery carrier, the current studies are achieved with the VPTT and in vitro drug delivery. In contrast, nanogels with cross-linking structure are more stable and have received much more attention in drug delivery

systems [72]. Nanogels, nanosized particles with cross-linking polymer networks, combine the properties of both hydrogels and nanomaterials [73]. Nanogels have enhanced stability with a long blood circulation time because the particle size and surface properties can be manipulated to avoid rapid clearance by phagocytic cells [74,75]. In addition, a larger surface area of the nanogel is convenient for functionalization of the nanogel [76].

There are several methods for the preparation of glucose-sensitive nanogels based on PBA. Li prepared glucose-sensitive nanogels by one-pot copolymerization of poly(ethylene glycol) methylacrylate (PEGMEM) and AAPBA with BIS as a cross-linking agent [77]. Hollow nanogels composed of PNIPAM and poly(*N*-phenylboronic acid acrylamide) were obtained by two-step colloidal template polymerization [78]. The nanogels had interpenetrating polymer network structure which provided the stability of the nanogels. The morphological structure of inner cavity increased the drug loading content of the nanogels. More importantly, the nanogels were both temperature- and glucose-responsive.

By one-pot copolymerization of pentaerythritoltetra (3-mercaptopropionate) (QT), poly(ethylene glycol) diacrylate (PEGDA), poly(ethylene glycol) acrylate (mPEGA), and AAPBA, a nanogel with a disulfide cross-linked core and PEG shell was obtained [79]. The copolymerization of the monomers was performed via thiol-ene click reaction between thiol and double bond. The obtained nanogels had a core-shell structure. The core was performed by tetrathiol functional QT and bifunctional PEGDA with the free mercapto groups in the nanogel, and the free mercapto groups were terminated by AAPBA and mPEGA (Figure 11). The introduction of PBA group endowed the nanogels with remarkable glucose sensitivity, which was confirmed by fluorescence spectrometry using Alizarin red S (ARS) as a fluorescent probe. Insulin, a model drug, was released from the nanogels with highly glucose concentration dependence. Additionally, methyl thiazolyl tetrazolium, lactate dehydrogenase, and hemolysis assays confirmed the nontoxicity and biocompatibility of the nanogels. The glucose-sensitive nanogel with good biocompatibility has promising potential for application in self-regulated drug delivery.

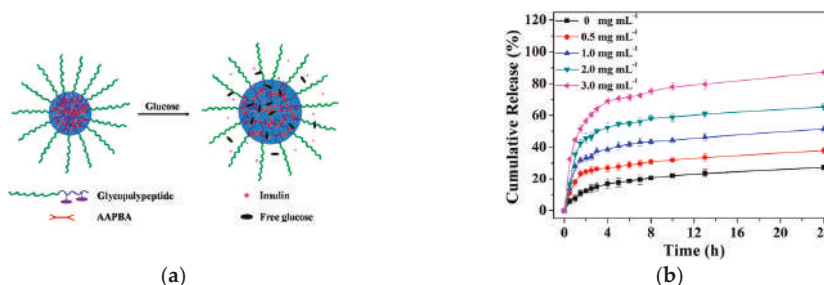


**Figure 11.** Structure of the nanogel and glucose-sensitive behavior of ARS-loaded nanogel in PBS at pH 7.4. (Reprinted from ref. [79]).

Nondegradable materials are unfavorable for blood clearance after drug delivery, which limits their clinical application in self-regulated drug delivery. The glucose-sensitive nanogels with improved biocompatibility and degradability have attracted more interest. A novel multifunctional chitosan and PBA-based nanohydrogel with enhanced glucose sensitivity was designed, which was prepared by the modification of chitosan-poly (acrylamide-*co*-methacrylic acid) nanohydrogel with 3-APBA [80]. The

glucose-triggered volume phase transition and release profile of the model drug ARS (comparative to insulin as a drug as well as a dye for bioseparation) were studied at various glucose concentrations, pH, and ionic strengths. The nanohydrogel may find applications in bioseparation and glucose-induced drug delivery with enhanced sensitivity toward glucose.

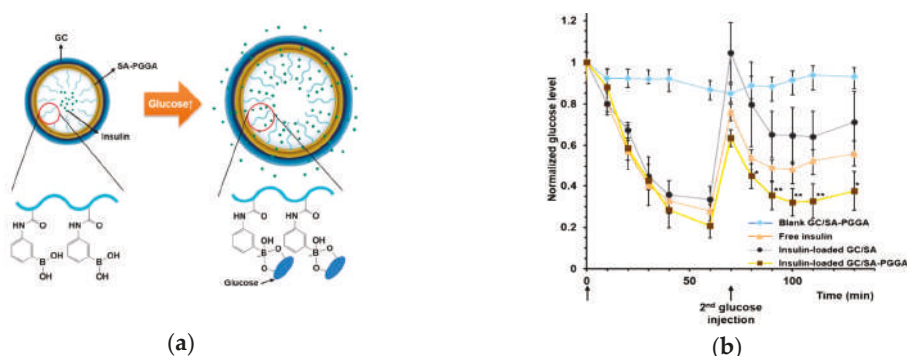
Besides chitosan, polypeptides are greatly used in drug delivery systems. Chen and coworkers exploited a novel kind of glucose-sensitive polypeptide nanogels via a two-step procedure. The glycopolypeptide, methoxy poly(ethylene glycol)-*block*-poly( $\gamma$ -benzyl-L-glutamate-*co*-( $\gamma$ -propargyl-L-glutamate-graft-glucose) (mPEG-*b*-P(BLG-*co*-(PLG-g-Glu))) was fabricated by clicking 2'-azidoethyl-O- $\alpha$ -D-glucopyranoside to the PLG unit in mPEG-*b*-P(BLG-*co*-PLG). Then, the mPEG-*b*-P(BLG-*co*-(PLG-g-Glu)) was cross-linked through boronate esters between the glucose moieties on glycopolypeptide with adipoylamidophenylboronic acid resulting in the glucose-sensitive polypeptide nanogels [81]. Insulin, a model drug, was loaded in the nanogels and the insulin release from the nanogels possessed excellent glucose sensitivity (Figure 12). There was a competitive binding mechanism for glucose-triggered insulin release. When free glucose was added, the complexes between PBA and glucose moieties on glycopolypeptide were destroyed due to the formed complexes between PBA and free glucose. Therefore, more free glucose entered into the nanogel core and the cross-linking density of the nanogels decreased, endowing the nanogels with more hydrophilicity and swelling. As a result, the preloaded insulin was released, induced by glucose, and high glucose concentration triggered more insulin release with a higher release rate. In addition, the polypeptide nanogels exhibited good cytocompatibility and hemocompatibility. The biocompatible nanogels with intelligent glucose-induced insulin release ability may have potential applications in diabetes therapy.



**Figure 12.** (a) Schematic illustration of glucose-triggered insulin release from PBA-functionalized polypeptide nanogel; (b) Cumulative insulin release from the insulin-loaded nanogel in PBS with various glucose concentrations at pH 7.4, 37 °C. (Reprinted from ref. [81]).

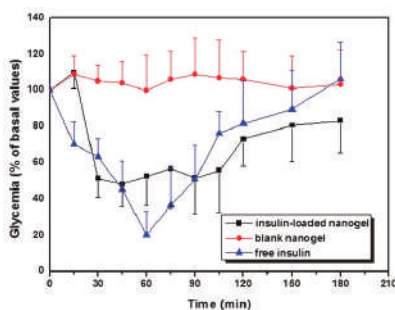
Also using polypeptide, glycol chitosan (GC)/sodium alginate(SA)-poly(L-glutamate-*co*-N-3-L-glutamylphenylboronic acid) (PGGA) graft polymer (GC/SA-PGGA) double-layered nanogels were prepared by an isotropic gelation method and electrostatic interactions between GC and SA-PGGA (Figure 13a) [82]. In glucose solution, the binding between glucose molecules and PBA moieties on PGGA converted the PGGA chains to hydrophilic structures. Therefore, the hydrophilicity of nanogels was enhanced, resulting in the swelling of nanogels and consequent insulin release triggered by glucose. Furthermore, a mouse study was conducted to demonstrate the controlled insulin release capability of GC/SA-PGGA double-layered nanogel *in vivo*. Glucose was administrated to mice by retro-orbital injection to raise the blood glucose levels to diabetic glucose ranges at 0 min. Excluding the blank GC/SA-PGGA group, other groups were administrated glucose again at 70 min (Figure 13b). The blood glucose levels of the groups (excluding blank GC/SA-PGGA group) possessed a similar behavior with progressively lowered glucose levels before the second glucose injection. However, after the second glucose injection, the blood glucose levels of the free insulin and insulin-loaded GC/SA groups no longer had significant decrease. In contrast, the blood glucose level of insulin-loaded GC/SA-PGGA nanogels group was kept relatively low for a longer time, indicating that the nanogels

exhibited controlled insulin release with high pharmacological activity to decrease the blood glucose levels. The glucose-triggered insulin release makes the GC/SA-PGGA double-layered nanogels a promising approach for diabetes treatment.



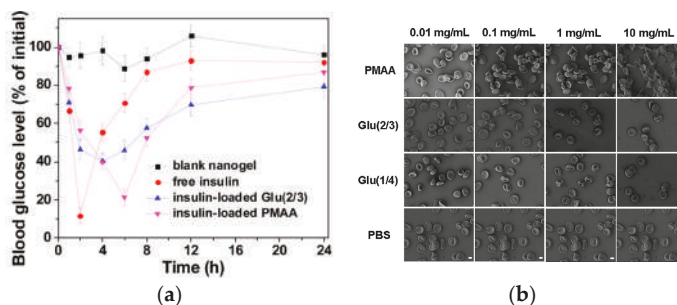
**Figure 13.** (a) Schematic illustration of glucose-sensitive GC/SA-PGGA double-layered nanogel controlled insulin release by complexation between PBA derivatives and glucose; (b) Normalized blood glucose levels in mice by retro-orbital administration of blank GC/SA-PGGA nanogels, free insulin ( $0.5 \text{ IU kg}^{-1}$ ), insulin-loaded GC/SA ( $0.5 \text{ IU kg}^{-1}$ ), and insulin-loaded GC/SA-PGGA nanogels ( $0.5 \text{ IU kg}^{-1}$ ). (Reprinted from ref. [82]).

An injectable nanogel with interpenetrating polymer networks of PNIPAM, dextran, and poly(3-acrylamidophenylboronic acid) (P(NIPAM-Dex-PBA)) was exploited using maleic acid-dextran as a cross-linker [83]. The nanogels presented reversible glucose sensitivity under physiological conditions, which was related to dextran content. The preloaded insulin was released from the nanogels with high dextran content triggered by glucose. More importantly, the considerable hypoglycemic effect of the insulin-loaded nanogels was also confirmed. As shown in Figure 14, *in vivo* experiments demonstrated that the blood glucose level of diabetic rats treated with insulin-loaded nanogels was maintained in a low state for almost two hours. The reduction of blood glucose level for insulin-loaded nanogels treated diabetic rats was 51% of the baseline level, which was associated with long-term controlled insulin release triggered by glucose. In addition, insulin-loaded nanogels maintained stable blood glucose levels without remarkable fluctuations of blood sugar. The insulin-loaded nanogels with prolonged and stable blood glucose reduction effect may have potential applications for diabetes treatment.



**Figure 14.** Profiles of glycemia after a subcutaneous administration of free insulin ( $2.0 \text{ IU kg}^{-1}$ ), insulin-loaded nanogels ( $4 \text{ IU kg}^{-1}$ ), and blank nanogels in fed diabetic rats. Before the injections, glycemia was  $426 \pm 13 \text{ mg mL}^{-1}$ . Results are expressed as mean  $\pm$  SD ( $n = 8$ ). (Reprinted from ref. [83]).

Even though most PBA-functionalized nanogels are exploited to achieve the glucose sensitivity and glucose-triggered hypoglycemic effect, the particular and comprehensive studies on the blood compatibility of the nanogels have been reported rarely. Zhou and coworkers explored the glucose-sensitive insulin controlled release and blood compatibility of PBA-based nanogels [84]. The nanogels were prepared through a thermally initiated precipitation copolymerization of methacrylic acid (MAA) and AAPBA monomers using ethylene glycol dimethacrylate (EGDMA) as a cross-linker. By changing the molar ratio of MAA/AAPBA, nanogels with different PBA contents were obtained and noted as Glu(a/b), in which a/b represent the molar ratio of MAA/AAPBA. The release of FITC-insulin from Glu(2/3) was dependent on the glucose concentrations without initial burst release. In particular, the blood glucose levels of diabetic rats treated with insulin-loaded Glu(2/3) nanogels were maintained at a low concentration (below the 70% of initial blood glucose level) with a long-term stable hypoglycemic effect compared with the free insulin group (Figure 15a). Moreover, a series of blood assays and in vitro/vivo assays confirmed the good blood compatibility of PBA-based nanogels. The nanogels did not cause aggregation and morphological change of the red blood cells (Figure 15b). The activated partial thromboplastin time (APPT) is used to evaluate the intrinsic and common coagulation pathways. The glucose-sensitive nanogels had no interactions with the coagulation factors and/or partial thromboplastin reagent which was confirmed by APTT. In addition, the nanogels had little effect on the extrinsic pathway of blood coagulation examined by prothrombin time (PT). Thromboelastography (TEG) assay also indicated the anticoagulant effects which were attributed to the removal of thrombin from the blood by the nanogels via electrostatic interactions. Toxicity studies further confirmed the blood compatibility of the nanogels. This work provides a strategy for the study of biocompatibility of glucose-sensitive drug carriers. Thus, compatible nanogels with good hypoglycemic effect have potential application in diabetes therapy.



**Figure 15.** (a) The profiles of rats' blood glucose concentrations after injection with different samples; (b) Effect of the different nanogels on the aggregation and morphology of red blood cells. (Reprinted from ref. [84]).

## 5. Conclusions

PBA-functionalized hydrogels, microgels, and nanogels provide glucose-triggered drug release due to reversible swelling and shrinking induced by glucose. PBA-based glucose-sensitive hydrogels can be prepared by physical or chemical cross-linking, such as the self-aggregation of gelators, cross-linkage by cross-linkers, or reversible covalent complexation of PBA with *cis*-1,2 or *cis*-1,3-diol compounds. Even though the PBA-mediated hydrogels possess great glucose sensitivity, the poor ease of administration, i.e., they cannot be administrated by injection but probably as an implant, limit their application in diabetes therapy. In contrast, PBA-based microgels and nanogels with micro- or nano-scale dimensions exhibit excellent biocompatibility, tunable sizes, large bioconjugated surfaces, as well as convenient injection administration. For PBA-based microgels, studies are focused on the VPTT and the mechanism of glucose sensitivity. The development of PBA-based nanogels has seen significant progress in terms of glucose sensitivity, biocompatibility, and pharmacokinetic and



hypoglycemic effect. PBA-functionalized nanogels with convenient administration via injection have practical application for diabetes therapy.

Even though there is considerable progress of PBA-based hydrogels, microgels, and nanogels for glucose-sensitive drug delivery, some challenges still have to be overcome to promote the clinical application of PBA-mediated platforms in diabetes therapy.

Firstly, the specificity and selectivity of glucose sensitivity are important for the design and application of glucose-sensitive platforms. Besides glucose, there are physiologically relevant saccharides, such as fructose, mannose, and galactose, which can bind to PBA and form mono(boronate) complexes. PBA and derivatives are able to form mono(boronate) complexes as well as bis(boronate) complexes with glucose. The influence of sugars on glucose sensitivity of PBA-based platforms must be considered. Additionally, the glucose sensitivity of drug carriers must be selective, that is, the platforms must timely and rapidly adjust the insulin release on-demand. The ideal drug carrier should release insulin quickly with precise dosage in response to hyperglycemic state, while not releasing glucose during normoglycemic states to keep the blood glucose levels in the normal range.

Secondly, adopting simplified preparation of PBA platforms is conducive to maintain the repeatability and controllability of the structure and property of glucose-sensitive carriers for different preparations. In addition, the glucose-sensitive platforms with high bioactivity of preloaded insulin should be administrated easily. Injection is an ideal approach because it can reach blood circulation with enhanced glucose sensitivity. The drug delivery system must be designed ingeniously with easy and repeatable preparation and convenient administration to promote its clinical application.

Thirdly, biocompatibility without long-term side effects of the platforms is a challenge for the design of PBA-based glucose-sensitive drug delivery systems. Diabetes is a lifelong chronic disease and the treatment of diabetes is a long-term process. Therefore, glucose-sensitive platforms must be nontoxic and friendly to the body without inducing inflammation. By adopting biodegradable and biocompatible materials, such as poly(acrylic acids) and polypeptides, the biocompatibility of PBA-based matrices can be enhanced.

Although some issues still need to be overcome, self-regulated drug delivery systems have promising application in diabetes treatment.

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Review

# Hydrogels and Their Applications in Targeted Drug Delivery

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**Abstract:** Conventional drug delivery approaches are plagued by issues pertaining to systemic toxicity and repeated dosing. Hydrogels offer convenient drug delivery vehicles to ensure these disadvantages are minimized and the therapeutic benefits from the drug are optimized. With exquisitely tunable physical properties that confer them great controlled drug release features and the merits they offer for labile drug protection from degradation, hydrogels emerge as very efficient drug delivery systems. The versatility and diversity of the hydrogels extend their applications beyond targeted drug delivery also to wound dressings, contact lenses and tissue engineering to name but a few. They are 90% water, and highly porous to accommodate drugs for delivery and facilitate controlled release. Herein we discuss hydrogels and how they could be manipulated for targeted drug delivery applications. Suitable examples from the literature are provided that support the recent advancements of hydrogels in targeted drug delivery in diverse disease areas and how they could be suitably modified in very different ways for achieving significant impact in targeted drug delivery. With their enormous amenability to modification, hydrogels serve as promising delivery vehicles of therapeutic molecules in several disease conditions, including cancer and diabetes.

**Keywords:** hydrogels; applications; targeted drug delivery; drug release; hydrophobic drug delivery; clinical translation; versatile platform; administration routes; diverse therapeutic areas

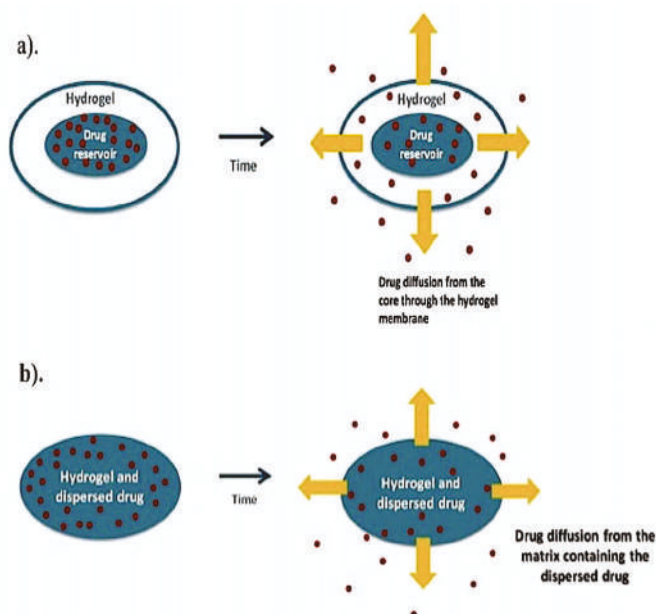
## 1. Introduction

Hydrophilic polymeric networks that are capable of imbibing huge volumes of water and undergoing swelling and shrinkage suitably to facilitate controlled drug-release are called hydrogels. Their porosity and compatibility with aqueous environments make them highly attractive bio-compatible drug delivery vehicles. Their applications are manifold and for several biomedical needs as they are moldable into varied physical forms such as nanoparticles, microparticles, slabs, films and coatings. USA has been the largest producer of hydrogels and is expected to remain so for a few more years [1]. Hydrogels are promising, trendy, intelligent and ‘smart’ drug delivery vehicles that cater to the specific requirements for targeting drugs to the specific sites and controlling drug release. Enzymatic, hydrolytic or environmental stimuli often suffice to manipulate the hydrogels for the drug release at the desirable site [2]. Like the two sides of a coin, there are also the disadvantages associated with their use. The primary disadvantage in drug delivery would be the hydrophobicity of most drugs. The water-loving polymeric core is probably not very ideal to hold incompatible hydrophobic drugs, which is a challenge since many that are currently used and effective in disease therapy are hydrophobic. The tensile strength of these hydrogels is weak and this sometimes causes early release of the drug before arrival at the target site. The following review discusses on how hydrogels are being manipulated presently for improved targeted drug delivery. The modern trend in which the hydrogels are exploited for drug delivery are covered.



The attractive physical properties of hydrogels, especially their porosity, offer tremendous advantages in drug delivery applications such as sustained release of the loaded drug. A high local concentration of the active pharmaceutical ingredient is retained over a long period of time via a suitable release mechanism controlled by diffusion, swelling, chemical or based on some environmental stimuli.

Diffusion-controlled drug delivery with hydrogels uses reservoir or matrix devices that allow diffusion-based drug release through a hydrogel mesh or pores filled with water. In the reservoir delivery system, the hydrogel membrane is coated on a drug-containing core producing capsules, spheres or slabs that have a high drug concentration in the very center of the system to facilitate a constant drug-release rate. While the reservoir delivery system produces time-independent and constant drug release, the matrix system works via the macromolecular pores or mesh. This type of release is time-dependent drug release wherein the initial release rate is proportional to the square root of time, rather than being constant (Figure 1).



**Figure 1.** (a). Drug-containing core is coated with hydrogel membrane and the drug concentration is higher in the center of the system to allow constant release rate of the same in reservoir delivery system. (b). Uniform dissolution or dispersion of the drug throughout the 3D structure of the hydrogel is achieved using matrix delivery (reprinted (adapted) with permission from [3]; the article is open access and the content reusable).

The swelling-controlled drug release from hydrogels uses drugs dispersed within a glassy polymer which when in contact with a bio-fluid begins swelling. The expansion during swelling occurs beyond its boundary facilitating the drug diffusion along with the polymer chain relaxation. The process, otherwise referred to as Case II transport, supports time-independent, constant drug release kinetics. Since the gradient between the dispersed drug in the hydrogel and its surrounding environment allows the active ingredient diffusion from a region of higher concentration within the hydrogel to a lower one, the process is also referred to as anomalous transport as it combines both the processes of diffusion and swelling for enabling drug release.

Ocular drug delivery carriers have been developed using hydrogels that are covalently crosslinked. These soft, biodegradable hydrogels with high swelling capacity remain in-situ in the lacrimal

canal offering greater comfort for the patient. Collagen or silicone that may be used decides if the punctal-plug system could be used temporarily or permanently, respectively. Poly(ethylene glycol) hydrogels are commonly used for producing ophthalmic drug delivery systems.

Drug release in response to environmental changes would be an ideal delivery system as the release becomes very controlled and non-specific side effects at off-target sites are alleviated. Thus, sensitive drug delivery devices responsive to changes in pH, temperature, ionic strength or glucose concentration have been developed that are advantageous in the therapy of diseases such as cancer, and diabetes, characterized by local physiological changes specific to the various disease stages. The polymer composition of the hydrogel responsive to the environmental stimuli is manipulated to make it responsive to the environment [3].

Hydrogels considerably enhance the therapeutic outcome of drug delivery and have found enormous clinical use. The temporal and spatial delivery of macromolecular drugs, small molecules, and cells have greatly improved through hydrogel use for drug delivery [2]. Drug delivery using hydrogels, however, has not been free of challenges, but constant improvements are being made to identify the hydrogel design best suited for specific drug delivery purposes. Therefore, this paper discusses the recent trends in drug delivery applications using hydrogels, including their translation to the clinic and their applications to successfully deliver hydrophobic drugs.

## 2. Current Trend in Hydrogel Based Targeted Drug Delivery

### 2.1. Supramolecular Hydrogels

The supramolecular hydrogel system is composed of intermolecular interactions that are non-covalent and has two or more molecular entities held together. The non-covalent cross-linking is a very attractive aspect of these hydrogels as it helps circumvent the problems of limited drug loading potential and drug incorporation for use only as implantables which would be the only possibility with a covalently cross-linked network. Apart from offering the right physical stability for the hydrogels, these achieve drug loading and gelation simultaneously in an aqueous environment without the need for a covalent cross-linking. Recent progress has been made with supramolecular hydrogels using self-assembled inclusion complexes between cyclodextrins and bio-degradable block copolymers that provide sustained and controlled release of macromolecular drugs [4].

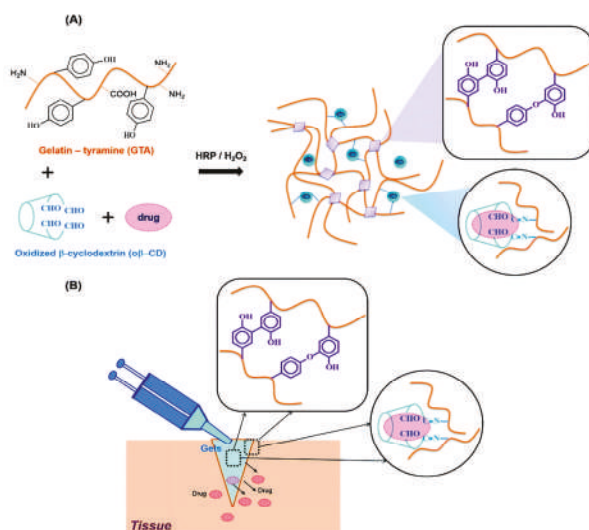
Natural cyclic oligosaccharides composed of six, seven or eight D-(+)-glucose units linked by D-(+)-1,4-linkages (termed  $\alpha$ -,  $\beta$ - and  $\gamma$ -CD, respectively), are called cyclodextrins and are well-suited for use in supramolecular systems. They offer hydrophobic internal cavities with a suitable diameter and their ability to generate supramolecular inclusion complexes with various polymers make them ideal drug delivery vehicles.

A recent study involved development of a glycoconjugate prepared by amidation of homopoly-L-guluronic acid block obtained from *D. antarctica* sodium alginate with mono-6-amino- $\beta$ -CD. The study was aimed at treating Chagas disease caused by *Trypanosoma cruzi*. Lipophilic non-hydroxylated coumarins were loaded into the hydrophobic core of the  $\beta$ -cyclodextrin to render them with trypanocidal activity. Interaction between the carboxylate groups of unconjugated  $\alpha$ -L- glucuronate residues with calcium ions was used to produce supramolecular hydrogels of glycoconjugate of homopoly-L-guluronic block fraction (GG) with 6-NH<sub>2</sub>- $\beta$ -CD. As the *T. cruzi* parasites have only one mitochondrion, it is an ideal target for drugs to manipulate its energy process and apoptosis. Mitochondrial membrane potential studies revealed that the cyclodextrin complex with the drugs produced significant oxidative stress to destroy the parasites. The drug in the complex had increased solubility, showed improved bio-availability, controlled drug release and improved trypanocidal activity in comparison to the corresponding free amidocoumarins [5].

Cyclodextrin-functionalized polyhydrazines were used to prepare hydrogels in-situ via hydrazine bond formation with aldehyde groups on dextran aldehyde. No toxicity was observed *in vitro* with these hydrogels and they could accommodate nicardipine as hydrophobic drug into the cyclodextrin

cavities. Steady release of nicardipine over 6 days was observed with the hydrogel preparation having higher hydrazine linkages. Thus, a gel capable of hydrophobic drug release in an in-situ formed device over extended periods was generated [6].

Bleeding control and wound healing by bio-adhesive hydrogels find enormous biomedical applications. In situ forming hydrogels are used to heal injured tissues based on their ability to accumulate and produce a fibrin bridge that permit fibroblast migration and collagen secretion for healing tissue injury.  $\beta$ -Cyclodextrins are a non-toxic adjuvant for pharmaceutical and mucoadhesive applications. Partly oxidized  $\beta$ -cyclodextrin was used in a recent study to exploit aldehyde groups on a hydrogel matrix for favorable reaction with amines in the tissue to result in an imine bond (Schiff's base reaction) in order to adhere to the skin and to provide improved cyclodextrin solubility in order to improve loading efficiency. Blending gelatin (the common extracellular component) with the  $\beta$ -cyclodextrin partly oxidized with oxidation in the presence of  $\text{H}_2\text{O}_2$ /horseradish peroxidase, resulted in very rapid formation of gelatin- $\beta$ -cyclodextrin hydrogels (Figure 2). Hydrophobic drugs such as dexamethasone could be released with 2.7 fold higher efficacy when delivered in presence of the cyclodextrin relative to the gelatin-only hydrogels [7].



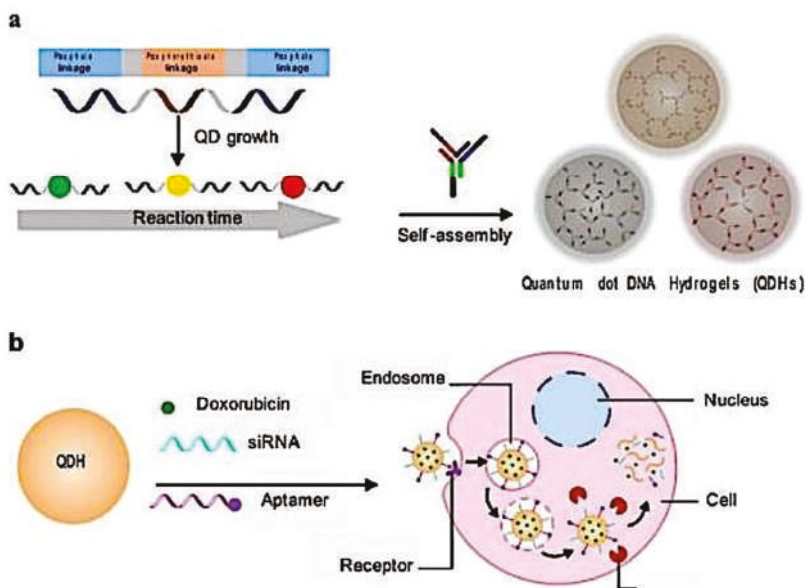
**Figure 2.** (A). Graphical representation of the methods for cross-linking to obtain gelatin- $\beta$ -cyclodextrin (GTA-ob-CD) hydrogels to load hydrophobic drugs. (B). Schematic representation of adhesive GTA-ob-CD hydrogels in situ formed by combining HRP catalysis and the Schiff base reaction with therapeutic release (reprinted [7] with permission from the The Royal Society of Chemistry. The article is licensed by Creative Commons and the link to the license is <https://creativecommons.org/licenses/by/3.0/>).

Curcumin has been shown to have several therapeutic benefits and found enormous applications in conventional therapy. The challenging aspect of its delivery is the extremely low aqueous solubility. However, a glycyrrhetic acid (GA) molecule-modified curcumin-based hydrogel has been developed to address the problem of delivery of the insoluble drug for hepatocellular carcinoma. The GA molecule-modified curcumin supplied in the pro-gelator form could produce a supramolecular hydrogel *in vitro* due to disulphide reduction by glutathione (GSH) and increase curcumin bioavailability and solubility as reported in HepG2 cells. Higher cellular uptake and potent anti-cancer activity were observed with the hydrogel *in vitro* relative to an already known curcumin-targeting compound that was tested [8].

## 2.2. DNA-Hydrogels

Hybrid bionanomaterials could be developed using DNA as the building block. Predictable two- or three-dimensional structures are formed from DNA molecules. Highly structured networks are formed by hybridizing complementary DNA molecules and the resultant hydrogel structures expand upon encounter with an aqueous environment that result in swelling. Not only do these materials append to any other type of nucleic acid molecules (such as siRNA, miRNA), but they can also load DNA binding drugs. High solubility, bio-compatibility, versatility and responsiveness are key features of such hydrogels. Apart from these features they can also be tagged with suitable fluorescent molecules for tracking biological studies *in vitro* [9].

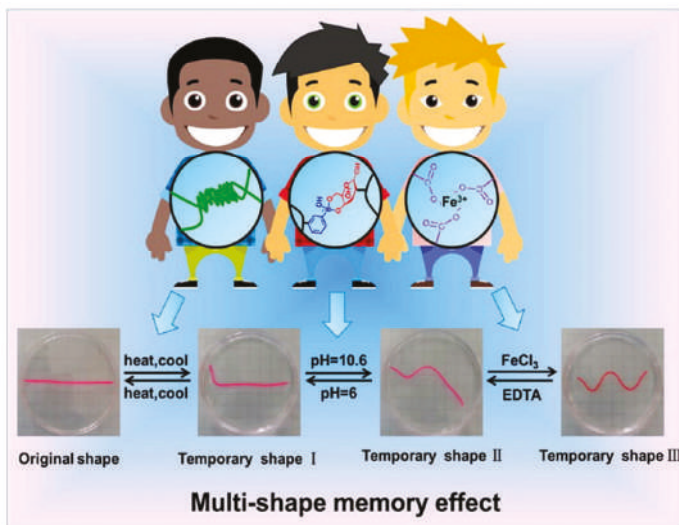
An interesting application of hydrogels has been made with the development of multi-functional quantum dot (QD) DNA hydrogels. DNA hydrogels are composed of complementary strands of DNA hybridized to form a crosslinked network that swells in an aqueous environment. However, in order for biological studies to be more easily and effectively performed there has to be a tracer or a fluorescent molecule attached to the hydrogel which would then be a superlative option offering both targeted delivery and imaging. With this aspect in mind Zhang et al. recently developed a QD-based DNA hydrogel that had highly tunable size, spectral and delivery properties and bound to the DNA binding drug doxorubicin (Figure 3). The drug targeted cancer cells and the QD DNA hydrogel increased the potency of the drug *in vitro*. The single-step assembling zinc sulphide QD Doxorubicin DNA hydrogels showed increased tumor accumulation *in vitro*, high bio-compatibility, was threefold more efficacious than free DOX and served as an excellent tool for *in vivo* bio-imaging in monitoring tumor growth over time. Aptamers such as siRNA were used to target specific cell types to deliver drug specifically and modulate protein expression in various cell types [9] (Figure 3).



**Figure 3.** (a). Schematic view of the synthesis process of the DNA functionalized QDs followed by the formation of the QD hydrogel through hybridization with DNA. (b). Schematic view of the modification process on the QD hydrogel for specific targeting of the cell using aptamer for drug delivery. Release of Doxorubicin and siRNA happens after uptake into the cell via endocytosis (reprinted (adapted) with permission from [9]; the article is open access and the content reusable. Creative Commons International License 4.0 for the article is available from <http://creativecommons.org/licenses/by/4.0/>) [9].

Drug-loaded cytosine-phosphate-guanine (CpG)-DNA hydrogels have been used for cancer immunotherapy. These CpG sequences containing hydrogels elicit immune responses. As an example, these DNA hydrogels containing CpG nucleotides stimulated innate immunity through Toll-like receptor 9 and promoted immune responses of ovalbumin (OVA) incorporated into the gel by acting as an adjuvant. Adverse reactions were considerably reduced with the use of the CpG-DNA hydrogel in comparison to the OVA injected with alum or complete Freund's adjuvant [10,11].

There has been significant advancement in hydrogel modifications for enhancing the shape memory and the reversibility of the hydrogel shape after any prolonged stress on them. (example: external stimuli such as temperature). This advances the thermal responsivity behavior of the hydrogels and an example is the construction of supramolecular hydrogel with tunable mechanical properties and multi-shape memory effects. These hydrogels make use of agar that is physically cross-linked to form the hydrogel network and a supramolecular network that is cross-linked by suitable chemical bonds that fix shapes temporarily and produces a multi-shape memory effect made possible by reversible interactions (Figure 4). The supramolecular hydrogel possesses enormous bio-compatibility and bio-degradability characteristics and rely on non-covalent interactions to drive the self-assembly of small molecules in water. The formed structures have supramolecular architectures and encapsulate water [12,13].



**Figure 4.** Schematic depiction of a novel  $\text{Fe}^{3+}$ -, pH-, thermoresponsive hydrogel with tunable mechanical properties easily changed by adjusting cross-linking densities of polymers. Moreover, several co-ordination interactions along with supporting stimuli could be exploited to stabilize temporary shapes in order to realize shape memory behavior. Programmable multi-shape memory effect can be realized by combining the 3 reversible switches (reprinted (adapted) with permission from [12]; copyright (2017) American Chemical Society).

A programmed temporary shape can turn into the memorized original shape when placed in an appropriate environment or exposed to a trigger. Such shape-memory hybrid hydrogels could also be synthesized using DNA cross-linkers. These hydrogels not only undergo phase transitions in the trigger of the stimulus, but also possess memory code to recover to the original matrix shape. Guo et al. developed a pH-controlled shape memory DNA hydrogel that was formed by co-polymerization of acrylamide residues with acrydite modified with (1) cytosine-rich sequences (forming i-motif subunits) and (2) nucleic acids exhibiting self-complementarity. Self-assembly of cytosine-rich nucleic acid strands into an i-motif structure occurred at pH 5.0 and the disassembly to a random coil form

happened at pH 8.0, leading to a quasi-liquid state for the hydrogel. Re-acidification to pH 5.0 restored the original structure of the gel [14,15].

### 2.3. Bio-Inspired Hydrogels

A newer variety of hydrogels used for drug delivery applications are the bio-inspired hydrogels. These 3D materials recapitulate the biological micro-environment relevant to the disease condition and support studies on how the targeted drug delivery process could be optimized, how the therapy behaved *in vivo*, how the disease progressed, and so on. These are particularly useful in cancer therapy as the disease is particularly complex and normally associated with intricate cellular and physiological changes that require progressive monitoring. Engineering such microenvironments would thus be a very useful approach to promote research and study the disease condition and therapeutic process better. The stiffness of the 3D model used for studying liver cancer is a critical attribute to regulate molecular diffusivity and malignancy. The elastic moduli of the collagen gels were increased by stiffening interconnected collagen fibers with varied amounts of poly(ethylene glycol) di(succinic acid *N*-hydroxysuccinimidyl ester). The softer gels produced malignant cancer spheroids while the stiffer ones showed suppressed malignancy. The model provided better understanding and regulation of the emergent behaviors of cancer cells [16].

Contact lenses that are bio-inspired have been developed recently with improved drug delivery properties suited to perfectly match the eye condition in the diseased state, especially of the anterior-eye segment.

Inner layer of ethyl cellulose and Eudragit S 100 hydrogels encapsulated with diclofenac sodium salt showed sustained drug release in simulated tear film. Sustained drug levels in tear fluid for a prolonged period of time in relation to eye drops was observed. The drug timolol was released in response to lysozyme in the medium and not in PBS. Enzyme cleavable polymers in hydrogels enabled this.

Ergosterol-liposome grafted silicone materials loaded with nystatin (an anti-fungal agent) resembled a fungal infection and triggered nystatin release through a competitive mechanism. In the absence of the ergosterol (important sterol in the cell membranes of fungi) in the medium, the drug release was very negligible [17–20].

With recent progress in cancer therapy using immunology, the research has progressed in the area with the use of cells such as erythrocytes, macrophages, stem cells, dendritic cells and bacteria as building blocks to create targeted delivery systems. Stem cell membrane coated gelatin nanogels that were high tumor targeting and bio-compatible drug delivery systems were developed recently.

Those gelatin nanogels had excellent stability and tumor-targeting ability *in vitro* and *in vivo*. Targeting doxorubicin (DOX) enhanced anti-tumor therapeutic efficacy significantly higher than the gelatin-DOX of free DOX nanogels. Evident side effects were absent in the tissues of heart, liver, spleen, lung and kidney as was revealed with histopathology staining of treated mice [21].

A similar bio-mimetic drug delivery carrier is the endosomal membrane-coated nanogel extracted from the source cancer cells for specific delivery of the small molecule drug. Hyaluronic acid nanogel composed of SiO<sub>2</sub>/Fe<sub>3</sub>O<sub>4</sub> nanoparticles formed the inner core of the hydrogel and the endosome membrane outer shell was used. The hyaluronic acid could target CD44 receptors overexpressed in a variety of tumor types. Core-shell mesoporous silica nanoparticles with Fe<sub>3</sub>O<sub>4</sub> nanocrystals were used as the core to encapsulate photo-initiators and cross-linkers, followed by hyaluronic acid coating on the surface via electrostatic interaction. Photo-polymerization upon UV irradiation resulted in the *in situ* formed nanogel in endosomes following incubation of resultant nanoparticles with the source cells. In the presence of the loaded anti-cancer drug doxorubicin, the endosomal membrane nanogels could specifically interact with the target source cells followed by internalization to release doxorubicin. High targeting specificity, cytotoxicity and uptake of the prepared endosomal membrane coated nanogels with DOX over the bare DOX-nanogels was observed *in vitro* [22].



Detoxification of pore-forming toxins resulting from animal bites/stings using a 3D bio-inspired hydrogel matrix resembling the 3D structure of liver is a recent achievement. The 3D matrix resembling the liver is created by the hydrogel while the polydiacetylene nanoparticles installed in the hydrogel matrix serve to attract, capture and sense toxins. The 3D printed biomimetic detoxification device with installed nanoparticles would be a breakthrough innovation replacing vaccines, monoclonal antibodies, antisera to eliminate toxins from blood. Commonly used conventional antidote molecules target specific epitope structures on the pore forming toxins warranting specific treatments for the various toxins. IV-nanoparticle administration for binding and removal of toxins may cause secondary risk by poisoning from nanoparticle accumulation in liver. While challenges do not limit nanoparticle use for toxin clearance, the 3D printed *in vitro* devices have been clinically approved for toxin removal [23].

Adhesive bio-inspired gels comprising of sodium alginate, gum arabic, and calcium ions have been developed in order to mimic properties of natural sundew-derived adhesive hydrogels. Mouse adipose-derived stem cells were used in combination with the sundew-inspired hydrogel to confer interesting properties such as improved wound healing, enhanced wound closure, less noticeable toxicity and inflammation. Sundew plant's natural adhesive hydrogels were quite promising, but the researchers were quite intrigued about collecting the desirable quantity from the natural source. This inspired development of the above described hydrogel type with improved properties [24].

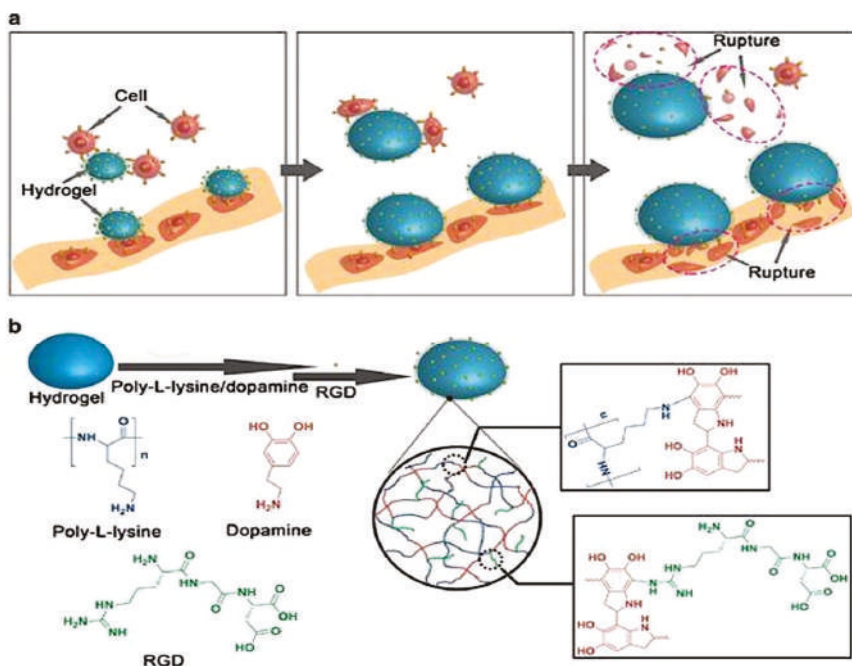
Magnetically actuated gel-bot (Mag-bot) is yet another recent discovery that facilitates remote control of the motion of magnetically actuated hydrogels. These hydrogels adopt a crawling motion resembling that of a maggot and move in a confined space of 3D porous media including, foams, tissues, fibres and so on, opening up broad options for targeted drug delivery. Pattern structures and lubrication effects on hydrogel mobility in the confined 3D space have been experimented in the paper [25].

It is not just the maggot that has inspired development of a new generation of hydrogels with applications in targeted drug delivery but also the drosera. The drosera-inspired model of hydrogel for targeted drug delivery with a bifunctional attribute to it has been developed recently. These hydrogels use the 'catch and kill prey' mechanism as a drosera would. They have a bottom layer functionalized with double stranded DNA that can bind the anti-cancer drug doxorubicin and there are aptamers on top that offer targeting advantages to these particles. Though there is requirement for *in vivo* studies, the sustained expression of specific aptamer on surface and the continuous killing of cancer cells by sustained drug release make these hydrogels an ideal targeted drug delivery vehicle for killing cancer cells [26].

#### 2.4. Multi-Functional and Stimuli-Responsive Hydrogels

Hydrogels that were multi-functional and carriers of anti-cancer drugs are a typical example of the versatility of these delivery vehicles and their amenability to chemical modifications to enhance their therapeutic effects. Magnetite nanoparticles that supported increased intracellular uptake by HeLa cells also had folate ligand on them to enable targeted delivery. Moreover, the hydrogel polymers were thermally responsive and DOX loaded. Those modified hydrogels offered advantages such as increased cellular uptake and apoptotic activity *in vitro* [27].

Another stimuli-responsive hydrogel developed very recently made use of biocompatible thermally responsive polymers that facilitated rupture of cancer cells. The study was conducted *in vitro* with an external source of heat and showed successful cell rupture. Those hydrogel particles had RGD (Arginine, Glycine, Aspartate) peptides attached to their surface that could bind to cells (Figure 5). The paper discussed experiments performed to confirm firmness of the RGD peptides with the cells (ex: MDAMB21) and their rupture using external heat stimulation.

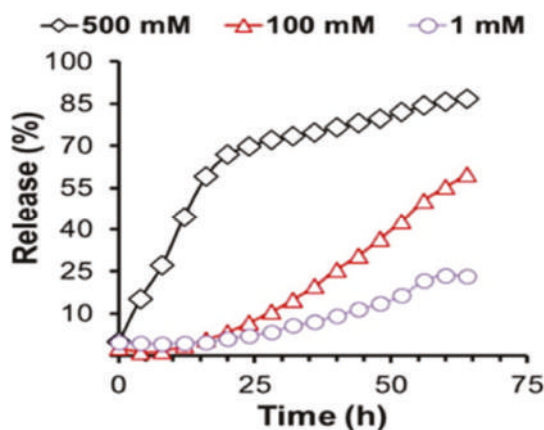


**Figure 5.** (a). Schematic illustration of the cancer cell attachment on stimuli-responsive hydrogel surface. External stimulus such as temperature causes expansion of the hydrogels and rupture of the cancer cells. (b). Schematic depiction of the surface modification of hydrogel. The first layer of coating was polydopamine (PDA) and poly-L-lysine (PLL) followed by a layer of RGD peptides for binding cancer cells (reprinted (adapted) from [28]; the article is open access and the content reusable. Creative Commons International License 4.0 for the article is available in <https://creativecommons.org/licenses/by/4.0/>) [28].

Since *in vivo* conditions in tumors normally produce a higher temperature than the surrounding areas, and also that it has been proved that the physical force generated from the expansion of the cells when they were heated was sufficient to kill them, that type of thermally responsive hydrogels proved to be highly advantageous options for achieving targeted cell killing [28].

Hydrogen peroxide trigger-mediated release of drugs could be achieved using hydrogels. This has been demonstrated previously with the use of ABC type triblock copolymer poly [(propylenesulfide)-*b*-(*N,N*-dimethylacrylamide)-*b*-(*N*-isopropylacrylamide)] [29,30] (PPS-*b*-PDMA-*b*-PNIPAM). A model hydrophobic drug, the dye red Nile was encapsulated in the hydrogel in order to demonstrate this. The gelation to form the cross-linked hydrogel happened above 37 °C and the ambient 25 °C temperature was sufficient to encapsulate the hydrophobic drug through the formation of micelles. Thus, the temperature-responsive polymer could cross-link to be a stable hydrogel carrying the hydrophobic drug based on the temperature modification and also showed a release of the red colored dye based on the H<sub>2</sub>O<sub>2</sub> release in the environment (Figure 6).

Apart from the polymer solubility switch mechanism that helped release based on the presence of the reactive oxygen species in the environment, cleavable units of polymer that underwent scission in presence of ROS have also been developed. These are also bio-degradable and help avoid any toxic build up or unwanted immune response in the system from the presence of the hydrogels [31,32].



**Figure 6.** Drug release dependent on Reactive Oxygen Species concentration as displayed by PPS<sub>60</sub>-b-PDMA 150-b-PNIPAAm150 triblock polymer-based thermos-responsive Nile red-loaded hydrogels. Nile red-loaded hydrogels (5 wt% triblock copolymer concentration) in PBS (pH 7.4) have been used to demonstrate Hydrogen Peroxide dependent drug release kinetics *in vitro* at 37 °C. 1, 100 and 500 mM concentrations of H<sub>2</sub>O<sub>2</sub> over a 64 h time course were incubated with the hydrogel samples to study ROS dependent drug release (reprinted (adapted) with permission from [31]; copyright (2014) American Chemical Society).

Diselenide-containing block copolymer and a peptide amphiphile were used as building blocks for the construction of a UV responsive hydrogel. The gel sol transition of the hydrogel occurred in presence of gamma irradiation. The peptide amphiphile was composed of nalproxen, the drug and a hexapeptide sensitive to UV irradiation, the radiation trigger was sufficient to cause release of the drug from hydrogel. The stimuli responsive hydrogel system was thus capable of providing both chemo- and radiotherapy benefits on application [13].

Covalently cross-linked hydrogels are typically stable and elastic while the physically cross-linked hydrogels produced through ionic interactions are less stable and exhibit reduced mechanical properties. The dynamic covalent chemistry is an option to acquire stability, elasticity and also shear-thinning and self-healing characteristics for the hydrogel. Based on this, Volkan et al. reported the synthesis of hydrogel networks from reversible interactions between phenylboronic acid and *cis*-diols. The gel strength was dependent on pH and the hydrogel was evaluated for protein delivery *in vitro*. The pKa of phenylboronic acid and the pH of the environment were the determining factors for establishing the extent of hydrogel cross-linking. Soft, moldable hydrogels were produced that could be injected using standard syringe needles and were demonstrated to exhibit shear thinning and healing properties in addition to their injectable nature. Protein encapsulation was performed with the hydrogel, and the release effect monitored. Size-dependent protein release could be attributed to the mesh size of the hydrogel network. Insulin and IgG release from the hydrogel network was monitored in presence of glucose. Glucose-responsiveness of the hydrogel was confirmed, and the release kinetics could be controlled by the mesh size of the hydrogel. 3T3 fibroblast cells showed no significant toxicity as quantified using MTT assay for 24 hr. No chronic inflammation was observed *in vivo* and the materials were quite bio-compatible [33].

The temperature sensitive hydrogels respond by undergoing sol-gel transitions with changes in temperature from room ones to the physiological ones. Recently developed hydrogels were that were thermo-responsive and accommodated hydrophobic drugs efficiently were built from amphiphilic triblock co-polymers, poly(*N*-isopropylacrylamide)-*b*-poly(4-acryloylmorpholine)-*b*-poly(2-(((2-nitrobenzyl)oxy)carbonyl)amino)ethylmethacrylate)(PNIPAM-*b*-PNAM-*b*-PNBOC). The hydrogel carried the hydrophilic drug gemcitabine and the hydrophobic drug doxorubicin. The triblock

co-polymers first assembled into micelles that had the hydrophobic and temperature responsive components formed below the lower critical solution temperature, while higher polymer concentration and temperature above the critical gelation temperature were used to form the hydrogels of physically cross-linked micellar nanoparticles. The study demonstrated the synthesis, characterization and the temperature and UV irradiation triggered synergistic release of both the hydrophobic and hydrophilic drugs *in vitro* [34,35].

Hydrogels formed by covalent cross-linking of polymers to facilitate targeted drug delivery have been achieved in recent years. The nanoparticle-hydrogel is a hybrid system that is formed in three different ways. The first method is to entrap a hydrogel within a nanoparticle, the second one is to form a 3D hydrogel network with the nanoparticles by crosslinking of the latter using hydrophobic interactions or mixing up nanoparticles of opposite charges and the final one is to covalently couple the nanoparticle with the hydrogel [36]. A liposome cross-linked hybrid hydrogel has been developed recently. Glutathione-triggered release from the stimuli responsive polymer favored drug release. Arylthioether succinimide cross-links were introduced between the Peg polymers and the liposome nanoparticles to produce the 3D hydrogel network. In presence of glutathione, the matrix was degraded, and the encapsulated drug molecules were released. Maleimide-functionalized liposomes crosslinked using peg polymers were constructed for co-delivery of doxorubicin and cytochrome-C (apoptotic cascade initiator) and their release testing *in vitro* was monitored in presence of glutathione. The presence of glutathione is a trigger for release from the hydrogel in a reducing microenvironment such as a tumor [37].

### 3. Diverse Physical Attributes of Hydrogels for Drug Delivery

Structural-modification amenability of the hydrogels renders them in various shapes and sizes. This feature is particularly interesting for drug delivery applications in order to design the hydrogels as per the target sites to which the drugs have to be delivered. The hydrogel-based dosage forms can have different designs and shapes depending on the route of drug administration (Table 1).

**Table 1.** Different types of hydrogel products administered via different routes of administration [38–50].

Routes of Administration	Shape	Typical Dimension
Peroral	Spherical beads	1 $\mu$ m to 1mm
	Discs	Diameter of 0.8 cm and thickness of 1 mm
	Nanoparticles	10–1000 nm
Rectal	Suppositories	Conventional adult suppositories dimensions (length $\approx$ 32 mm) with a central cavity of 7 mm and wall thickness of 1.5 mm
Vaginal	Vaginal tablets	Height of 2.3 cm, which of 1.3 cm and thickness of 0.9 cm
	Torpedo-shaped pessaries	Length of 30 mm and thickness of 10 mm
Ocular	Contact lenses	Conventional dimensions (typical diameter $\approx$ 12 mm)
	Drops	Hydrogel particles present in the eye drops must be smaller than 10 $\mu$ m
	Suspensions Ointments	N/A
Transdermal	Circular inserts	Diameter of 2 mm and total weight of 1 mg (round shaped)
	Dressings	Variable
Implants	Discs	Diameter of 14 mm and thickness of 0.8 mm
	Cylinders	Diameter of 3 mm and length of 3.5 cm

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#### 4. Specific Therapeutic Areas Using Hydrogels for Drug Delivery at Present

##### 4.1. Ophthalmic

Conventional eye drops have problems with sustained drug delivery and there is huge wastage of drug immediately following application through eye drainage. Dextenza is a very recently FDA approved (3 December 2018) ocular therapeutic hydrogel formulation for human use. This is used for ocular pain following ophthalmic surgery and is the first intracanalicular implant developed for drug delivery and developed by the company Ocular Therapeutix (Bedford, MA, USA) [51].

Thermo-responsive polymer developed by mixing poly(acrylic acid-graft-*N*-isopropylacrylamide) (PAAc-graft-PNIPAAm) with PAAc-co-PNIPAAm gel and incorporating epinephrine was used in the *in vitro* evaluation of ophthalmic drug release. The approach augmented the effect of intraocular pressure reduction from 8 h with the traditional drops to 36 h. The cross-linking density of the hydrogel affected the capillary network formation and offered a convenient controlled drug release method for ophthalmic drug delivery [52].

Intra-ocular pressure (IOP) elevates during glaucoma and alleviating this pressure has been quite challenging. Hydrogels could be used to resolve this problem by using them to prepare soft contact lenses composed of polymers to form networks. The highly hydrated polymer networks of hydrogels cause the drug to elute out very rapidly and this is not favorable for glaucoma therapy, which mainly uses hydrophilic drugs. However, with suitable modifications, soft contact lenses have been developed using polymers of *N,N*-diethylacrylamide and methacrylic acid, which delivered the hydrophilic drug timolol for about 24 h, thereby opening up ways to allow sustained hydrophilic drug delivery using hydrogels. Storing the contact lenses in a hydrated state can leach out drug and to wear them all the time are the limitations though [53].

Inner layer-embedded contact lenses have been investigated for the sustained release of highly water-soluble drug betaxolol hydrochloride on the ocular surface. Cellulose acetate and Eudragit S-100 were selected as the inner layer of the contact lenses which showed a promising sustained drug release for over 240 h in tear fluid of rabbits *in vivo* to create a controlled-release carrier of the drug in ophthalmic drug delivery [19].

Controlled drug release behavior from hydrogels was also evaluated using nepafenac as the model drug. 3D cross-linked thermos and pH sensitive hydrogel was designed that was composed of carboxymethylchitosan (CMC) and poloxamer with glutaraldehyde as the cross-linking agent. The hydrogel was found to undergo reversible sol-gel transition at temperature and/or pH alteration at a very low concentration. Sustained release of the drug nepafenac was observed in the *in vitro* model and maximum release was observed at 35 °C and pH 7.4. Cytocompatibility of the hydrogel with human corneal epithelial cells was high [54].

##### 4.2. Oral, Intestinal

Gastroretentive drug dosage forms (GRDDFs) are particularly attractive for drugs that are absorbed in the proximal part of gastrointestinal tract. Enhancing the retention time of the drugs in the GI tract is very important in order to improve their bioavailability and enhance their therapeutic effects. These dosage forms could be exploited for their muco-adhesion to the gastric mucosa, modified to float or sink in order to prevent leaving the stomach or increase their swelling behavior and make them as large to prevent passage through pylorus for prolonged periods. Based on these ideas, polyionic complex hydrogels of chitosan with ring-opened PVP have been developed for Osteoporosis therapy. The formulation was used to release alendronate in the upper GI tract. Enhanced muco-adhesion, delayed clearance from swelling, minimal localized irritation, improved bioavailability and slower release of the active ingredients are the interesting aspects of the preparation. Also, *in vivo* experimentation showed that these hydrogels could provide optimized PK properties that maintained the drug in the therapeutic levels for a sustained period of time, minimizing fluctuations in therapeutic levels, hence also the possible side effects [55].

Inflammatory diseases such as irritable bowel syndrome have been recently treated using hydrogels. These provided safer alternatives to delivery methods that may cause systemic toxicity. Zhang et al. developed negatively charged hydrogels that preferentially accumulated in the positively charged inflamed colon and acted as carriers of the corticosteroid drug dexamethasone (Dex). The hydrogel was prepared from ascorbyl palmitate which had labile bonds responsive to inflammatory conditions and was Generally Regarded as Safe (GRAS) for administration. Enema administration to the colon of inflammation targeting (IT) hydrogel microfibers not only reached the target site but also stayed there owing to charge interaction. The formulation was therapeutically very efficacious and revealed lesser systemic drug exposure than with free Dex in the IBS mice model *in vivo* [56].

Complexation hydrogel prepared from poly (methacrylic acid-g-ethylene glycol) [P(MAA-g-EG)] has been described. The targeting ligand used is the octarginine cell-penetrating peptide that causes specific delivery of insulin to the intestine. This method facilitated ideal targeting, absorption at target and allowed immediate release of insulin from absorption site. Great hypoglycemic responses were achievable and increased insulin absorption was noted from diabetic rat models used for testing. 18% glucose reduction was observed immediately on administration of the hydrogel containing insulin [57].

#### 4.3. Cardiac Illness and Cancer

Myocardial infarction is a leading cause of death and disability in the world. Intramyocardial administration of biomaterials such as hydrogels along the perimeter region of myocardial infarction has proven to be beneficial.

Chen et al. proposed the use of a combination of curcumin (known for its anti-oxidant, anti-inflammatory and anti-oxidation properties) and nitric oxide (known as an anti-angiogenesis agent) in a hydrogel to treat myocardial infarction. The mixed component hydrogel created with the combination drugs improved therapeutic efficacy synergistically. Protective effects such as myocytic apoptotic death alleviation, reduced collagen deposition, increased vessel density (attributable to NO in the combination) and upregulated Silent Information Regulator 1 (SIRT-1), a histone deacetylase that confers resistance to the heart from ischemic injury were observed in diseased mice models *in vivo*. The hydrogel was prepared using peptide derivatives of curcumin and NO in a ratio of 4:1 and showed sustained curcumin release at a low concentration of 2.5 µg per ml per 24 h. NO was released in presence of the enzyme  $\beta$ -galactosidase that could break glucosidic bonds to release NO [58].

Growth factors and cytokines (paracrine factors) secreted by stem cells have been proven to be effective in repairing damaged myocardial tissue. The whole cocktail of the paracrine factors is referred to as a secretome and is isolated *in vitro*. The biomolecular composition of the secretome can be manipulated suitably by varying stem cell culture conditions. An injectable hydrogel to deliver to peri-infarct myocardium has been recently developed using secretome from human adipose derived stem cell secretome. Nano-composite hydrogel was formed from a combination of gelatin and laponite carrying the secretome and tested both *in vitro* and *in vivo* for their therapeutic effects via monitoring angiogenesis, scar formation and heart function. Significantly reduced scar area and improved cardiac function were observed *in vivo* in the secretome loaded hydrogel group in relation to the control [59].

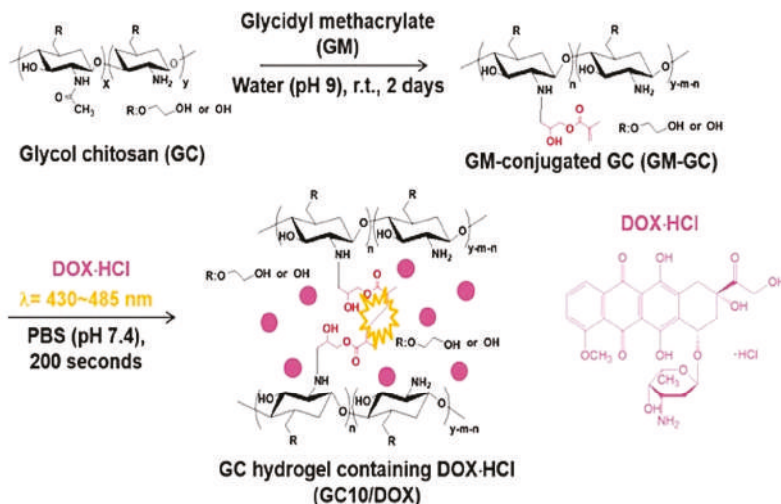
The very recent development of a paintable hydrogel to serve as cardiac patch for treating myocardial infarction is worth mentioning in this context. The hydrogel eliminates the damage to tissue through suture or light triggered reactions as it is paintable. It has been constructed by a  $\text{Fe}^{3+}$  triggered polymerization reaction wherein the covalently linked pyrrole and dopamine undergo simultaneous polymerization with the trigger and the conductive polypyrrole produced also uniquely cross-links the network further. The functional patch is both adhesive and conductive and forms a suture-free alternative for reconstruction of cardiac function and revascularization. Bonding within 4 weeks to the beating heart boosts the transmission of electrophysiological signals with conductivity profiles equivalent to that of the normal myocardium [60].

Bio-material based immunotherapy platforms for targeted drug delivery to cancers are the latest trend observable in cancer therapy. Based on this idea, novel STINGels have been developed by Leach



et al, that are peptide hydrogels to show controlled delivery of cyclic dinucleotides (CDNs). Dramatic improvement in survival was observed in murine models of head and neck cancer in comparison to CDN alone or CDN delivered from a collagen hydrogel [61].

Thyroid cancer treatment using local drug delivery system formed of glycol chitosan (GC) hydrogel and doxorubicin hydrochloride (DOX·HCl) called GC10/dox has been recently developed (Figure 7). Visible light regulated the storage and swelling aspects of the hydrogel and a controlled sustained release followed the initial burst release within 18 hours. Potent antitumor effects were observed *in vivo* and *in vitro* in comparison to free DOX·HCl and this is a promising research direction for thyroid cancer therapy [62].



**Figure 7.** To glycol chitosan solution glycidyl methacrylate (GM) was added in water (adjusted to pH 9) and maintained for 2 days at room temperature. The white solid conjugate of GM was dissolved in water and riboflavin added. DOX·HCl was added and the mixture was irradiated using blue visible light (430–485 nm, 2100 mW/cm<sup>2</sup>) for 10 minutes in order to promote hydrogelation (reprinted with permission from [62]; the article is open access and the content reusable. Creative Commons International License 4.0 for the article is available in <https://creativecommons.org/licenses/by/4.0/>).

Injectable hydrogels responsive to Reactive Oxygen Species that degrade in the presence of ROS and promote immunogenic tumor phenotype via local gemcitabine delivery is a recent discovery. The PVA cross-linked hydrogel with ROS-labile linkers enhance anti-tumor response with a localized release of immune checkpoint blocking antibody (anti-PD-L1 blocking antibody (aPDL-1) in *in vitro* and immunogenic *in vivo* mouse models. Tumor recurrence prevention after primary resection is the therapeutic advantage of this chemo-immunotherapy [63].

## 5. Translation to the Clinic

With enormous potential for therapeutic applications, several hydrogel formulations have crossed the barriers of *in vitro*/pre-clinical studies and found their way into the market. Some of them are still in the clinical study phases. Hydrogels have evolved over time to one of the best and the most versatile drug delivery platforms. Table 2 lists the widespread practical applications of the hydrogel concept that have been translated to the clinical level.

**Table 2.** Examples of hydrogels translated to clinical use [64–68].

Product	Type of Hydrogel	Therapeutic Application	Drug Delivered
Sericin	Dextran	Optically trackable drug delivery system for malignant melanoma	Doxorubicin
Hyalofemme/ Hyalogyn	Carbomer propylene glycol, Hyaluronic acid derivative	Vaginal dryness, estrogen alternative	Hyaluronic acid derivative
Dextenza	Polyethylene glycol	Intra-canalicular delivery for post-operative ophthalmic care	Dexamethasone
Regranex	Carboxymethyl cellulose	Diabetic foot ulcer	Recombinant human platelet derived growth factor
muGard	Mucoadhesive	Oral lichen planus	
	2% Poloxamer	Cervical cancer recurrence	Carboplatin

## 6. Conclusions

Hydrogels offer a versatile platform for the therapy of several diseases including cancer and diabetes. The water-loving nature of hydrogels and the ability to shrink and swell depending on several environmental cues or the mere presence of water is attractive for drug delivery applications. They have a high degree of porosity and the polymers building them could be cross-linked to varying degrees by adjusting their densities. With a physical structure highly amenable to modification in several ways, the hydrogel applications are not just limited to targeted drug delivery. They also find applications in hygiene products, wound dressings, contact lenses and tissue engineering.

Recent developments of hydrogels in the field of targeted drug delivery have been tremendous. They are modified with targeting ligands and diverse polymer types that confer very interesting properties on them for drug delivery. Ophthalmic drug delivery is an area seeing significant impact in therapy from hydrogels. From comfortable contact lenses to biodegradable drug delivery the applications in eye care have been enormous. They are 90% water, provide steady state drug release over days or months, deliver small molecules or large proteins, are fully absorbed in delivery and remain visible during monitoring [51].

Noteworthy is the application of pH responsive hydrogels for cancer therapy and glucose responsive hydrogels for diabetes. The use of modified stem cell membranes for targeted delivery is a very recent and attractive strategy for drug delivery. These membranes coated on hydrogels (nanogels) loaded with drugs are highly specific to the disease site in cancer and are highly bio-compatible.

Immunotherapy platforms using hydrogels are very significant in cancer therapy. Hydrogels enabling localized delivery of antibodies and other immune-regulatory molecules at cancer sites are promising drug delivery vehicles for cancer therapy. Gastro-retentive drug dosage forms (GRDDFs) are versatile drug delivery platforms for intestine and they offer the advantages of adjusting the nanoparticle size to facilitate retention of the active ingredient in the GI tract for as long as required.

Though the hydrogel-based drug delivery was originally influenced by the hydrophobicity of the drugs, several improvements have been made recently including development of cyclodextrins modified to accommodate the hydrophobic drug sufficiently. Adhesive and conductive patches developed using hydrogels are useful in cardiac repair and vascularization. Remotely controlled motility of hydrogel (mimicking motion of a magbot) and the QD DNA hydrogels are novel ideas to facilitate targeted drug delivery.

As discussed in the paper, though there are several hydrogel formulations in clinical use, there is always scope for improvement and modification of hydrogels to enhance their applications. With subtle modifications to the existing ones, the hydrogels could become superlative drug delivery vehicles surpassing the disadvantages and current limitations with the use of several conventional delivery forms and provide promising results for therapy of several illnesses.

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