Chapter

Introductory Chapter: Overview on Nanomedicine Market

Islam Ahmed Hamed Khalil, Islam A. Arida and Mohamed Ahmed

1. Introduction

Nanomedicine is an emerging field that has caught the interest of many medical scientists and chemists due to its unique characteristics that open the door wide for several unique applications that might lead to solving many problems that were found difficult to tackle in medicine. Nanomedicine has opened a new category of medicines called nanomedicines where the medicine is reduced to the nanoscale size, hoping to enhance its physicochemical properties. The chapter summarizes the nanomedicines that have been approved by the Food and Drug Administration (FDA) or European Medicines Agency (EMA) and the nanomedicines whose clinical trials based on previously published review articles by Anselmo and Mitragotri are ongoing [1, 2].

To gain insight to current trends in nanomedicine research and the most successful types of nanomedicines in the market, the approved nanomedicines are presented in **Figure 1**. The number of approved nanomedicine products is 29 till 2019 [1]. Liposomes represented 44.8% (13 products). Inorganic nanoparticles ranked second with 41.4% (12 products). Other nanoparticles (polymeric and protein) have only 4 products (13.8%). These findings are very interesting as liposomes are one of the oldest nanomedicines. This opens an argument about the challenges in nanomedicine translation as a new platform requires further investigations to prove its activity and safety. On the other hand, cancer nanotherapeutics is ranked first with 10 products in the market, followed by iron-replacement therapies with 8 products. Also, it is worth to mention that imaging agents (six marketed products) are ranked in third place, especially the inorganic nanoparticles (three products).

Moreover, nanomedicines, currently undergoing clinical trials, are presented in **Figure 2**. The number of products under clinical trials is 47 till 2019 [1], where liposomes represented 61.7% (29 products) and micelles ranked second with 19.15% (9 products). Other nanoparticles have only nine products (19.15%). Also, these findings are similar to approved nanomedicine, where liposomes are the most used nanomedicine. On the other hand, 39 products are dedicated for cancer treatment. It is worth to mention that 10 products out of the 39 products are loaded with gene therapy and not chemotherapeutic agents.

Generally, the total number of nanomedicines in the market or in clinical trial are 76 products, where liposome formulations were the most used delivery system with 55.26% (42 products), followed by inorganic nanoparticles with 21% (16 products) as presented in **Figure 3**. According to the World Health Organization in 2015, the first leading cause of death in around 50% of countries is cancer [3]. According to the International Agency for Research on Cancer report that published in 2018 on the global burden of cancer, there are 18.1 million cancer cases and 9.6 million cancer deaths in 2018 [3]. These reports inspired the

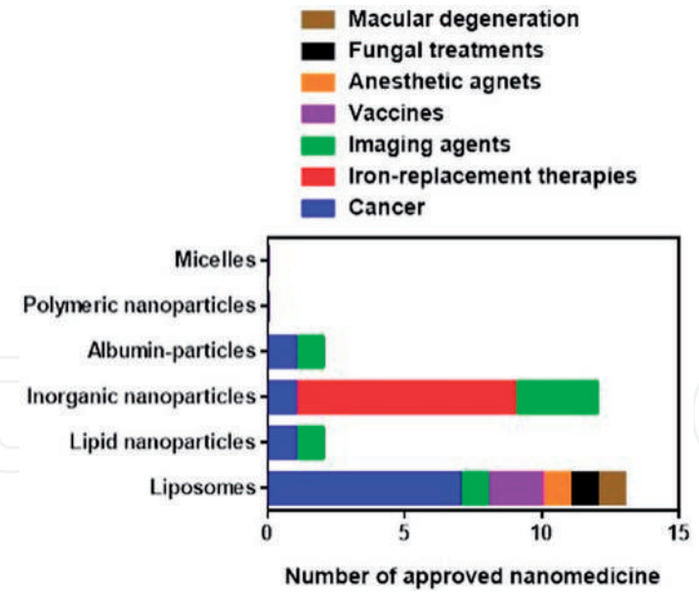

Figure 1.
Clinically approved nanomedicine for therapy and diagnostic.

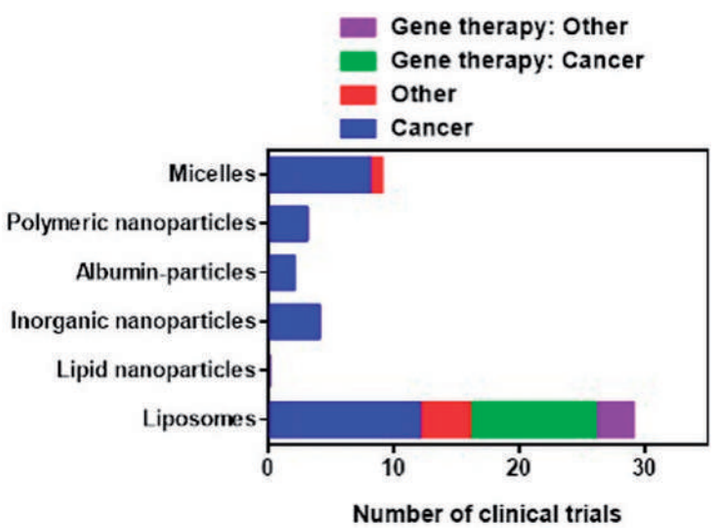

Figure 2.
Nanomedicine currently undergoing clinical trials for therapy and diagnostic.

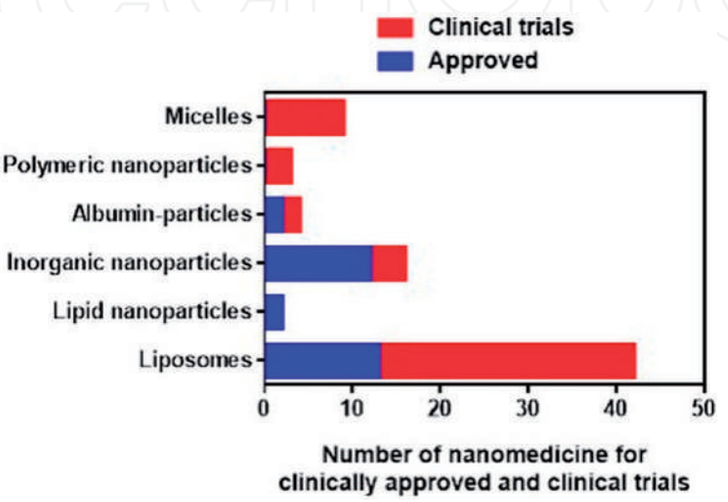

Figure 3.
Total number of nanomedicines approved and under clinical trials (therapeutic and diagnostic).

pharmaceutical industry to invest in this market. As mentioned previously, there are only 10 nanomedicine products out of 29 products available in the market to treat cancer, while there are 39 products out of 47 products for cancer treatment. This number of clinical trials for cancer is mainly derived by the 15 years of support by the US National Cancer Institute through the Centers of Cancer Nanotechnology Excellence (CCNEs) [4].

2. Types of nanomedicines

Nanomedicines are mainly classified into two classes, either inorganic nanoparticles such as gold, silica, and iron oxide or organic nanoparticles such as polymeric, liposomes, and micelles (**Figure 4**). These nanoparticles are mostly used for therapeutic and diagnostic nanoparticles. Inorganic nanoparticles have been used for a variety of applications including lymph node imaging, hyperthermia, and anemia treatment. Some of them have successfully gone through preclinical studies and clinic trials. Along with inorganic nanoparticles, organic-based nanoparticles have successfully reached the clinical phase and currently reached the market for different applications like vaccination, microbial infection, and cancer.

2.1 Liposome-based nanomedicines

Liposome-based nanomedicine is a type of drug formulation where a drug is encapsulated inside the phospholipid bilayer structure to enhance its bioavailability and therapeutic activity. Liposome formulations are one of the oldest nanomedicines with a well-established technique. Many research efforts were focused on using liposomes to encapsulate several cargos like small molecules such as doxorubicin, nucleic acid such as RNAs, and biological molecules such as vaccines for hepatitis A virus. Furthermore, administration of the liposomes without an encapsulated drug is also a possibility if the liposome subunits have a certain therapeutic effect such as sphingomyelin and cholesterol. PEGylation is an option to consider while using liposomes due to its importance in adding stealth to the delivery system. Most of the approved liposome-based nanomedicines are used for the treatment of cancer diseases. They take a large place in research as 10 out of the 29 approved nanomedicines are liposome-based.

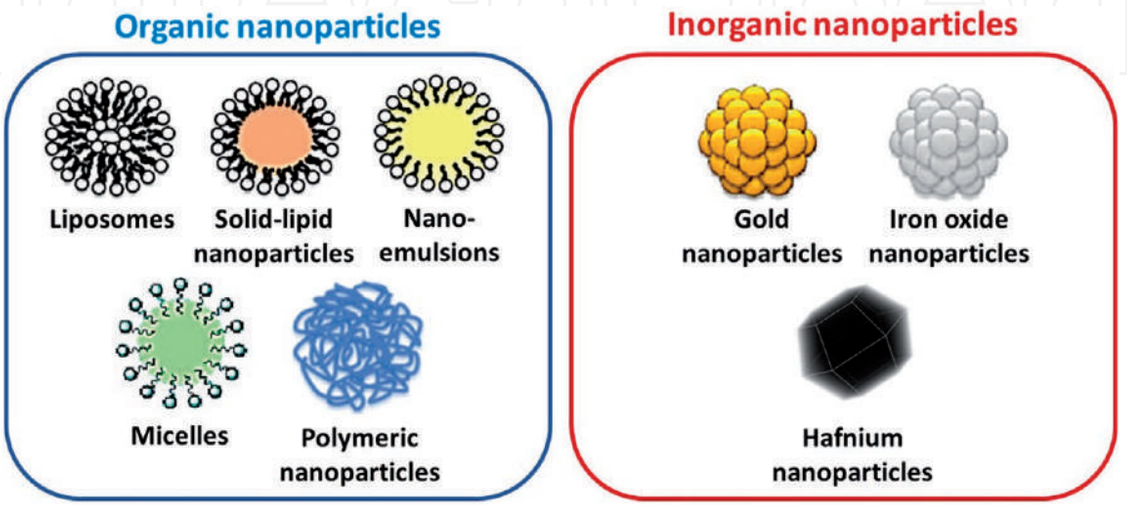

Figure 4.
Clinically approved and investigated nanomedicines including organic nanoparticles and inorganic nanoparticles.

2.2 Lipid-based nanomedicines

Lipid nanosystems including nanoemulsions and solid lipid-based nanoparticles are another form of nanomedicine, which are usually used to encapsulate hydrophobic cargos to improve permeation and control release profile. Usually, a surfactant is used to ensure a uniform dispersion. Lipid nanomedicine can also encapsulate some gene therapeutics such as siRNA or contrast agents used for imaging such as F-butane. Generally, lipid nanomedicine can improve the pharmacological effect by enhancing drug accumulation in targeted tissues beside its biocompatibility. However, there are several drawbacks like rapid clearance due to reticuloendothelial system (RES) uptake and some limitations for administration routes and challenges regarding system stability [5, 6]. Unlike liposomal-based nanomedicines, lipid-based nanomedicines are not limited for cancer diseases only. Some of the diseases that are treated by lipid-based nanomedicines are amyloidosis, hepatitis B, and hepatic fibrosis. Furthermore, several types of nanoemulsion were loaded with drugs like simvastatin, cinnarizine, coenzyme Q10, and cyclosporine, which used as antihyperlipidemia, antihistaminic, antioxidant, and immunosuppressants, respectively.

2.3 Albumin-based nanomedicines

Albumin-based nanomedicines are another form of nanosystems, where albumin, especially human serum albumin (protein), is used as a carrier. Albumin nanosystems can be loaded with different cargos via a simple self-assembly procedure of albumin in aqueous solution with simple crosslinking step. The main advantage of albumin is biocompatibility. Despite that, only 2 out of the 29 listed approved nanomedicines and 2 out of the 65 nanomedicines under clinical trials are albumin-based. It is currently used in imaging and delivering drugs that treat cancer diseases.

2.4 Micelle-based nanomedicines

Micelles are self-assembled nanosystem by amphiphilic molecules that have a hydrophilic part and a hydrophobic one. They have several advantages like high permeability and solubility, which improve drug bioavailability. However, they still have some drawbacks like insufficient control to drug release and cytotoxicity due to amphiphilic molecule use, which interact with cell membrane [5, 7]. Although several reports used block copolymeric micelles to reduce clearance and increase bioavailability of chemotherapeutic agents and other types of drugs, there are no approved micelle-based nanomedicines. However, there are currently nine micelle-based nanomedicines undergoing clinical trials. Majority of them are used for cancer treatment.

2.5 Polymeric-based nanomedicines

Polymeric nanoparticles are one of the most commonly used nanosystems for drug delivery. Several polymers have been used like ethyl cellulose, poly(lactic-co-glycolic acid), polylactic acid, cyclodextrin, alginate, and chitosan. Depending on the nature of the polymer, either hydrophilic or hydrophobic, there are several techniques that have been used to prepare polymeric nanoparticles. Several advantages like relative stability and prolonged duration of action make polymeric nanoparticles a promising platform for the market. However, there are no marketed products based on polymeric nanoparticles. Only three products are currently on clinical trials for cancer.

2.6 Inorganic-based nanomedicines

Inorganic-based nanomedicines have several subtypes. Due to degradability and biocompatibility issues, few types have been used for therapeutic purpose, while other types for diagnostic purpose like imaging agents. One of these subtypes are metal oxide nanoparticles such as hafnium oxide nanoparticles which enhance tumor cell death via electron production through their stimulation with external radiation. Another subtype is in the form of colloids such as iron dextran colloids, iron gluconate colloid, and other similar derivatives that are usually used for the treatment of iron-deficiency anemia. The last subtype mentioned is iron−/silica−/gold-based nanomedicines, either as nanoparticles with drugs arranged on the surface for the treatment of cancer or as nanoshells/nanoparticles used for thermal ablation of tumors. There are 12 products in the market that belong to this type. Eight products used iron-replacement therapies. On the other hand, four products are currently on clinical trials for treating cancer.

3. Nanomedicines pharmacokinetic and regulations

The pharmacokinetic parameters of nanomedicines are similar to free drugs with addition phase after drug administration, which is the liberation phase beside the standard absorption, distribution, metabolism, and excretion (ADME). This new phase is controlled by particle nature, size, shape, and surface properties. It is worth to mention that particle size is very important for absorption and elimination. Particles with particle size <5 nm is easily excreted from the kidney, while larger particle size could be eliminated by the liver or engulfed by mononuclear-phagocyte system. Moreover, particle size and shape can affect particle accumulation in targeted tissues like ellipsoidal shape that has better distribution and retention in tumor tissue than spherical one. Surface modification of nanoparticles can affect particle uptake and elimination. Many nanoparticles are coated for active and passive targeting. Passive targeting is a non-specific retention in target tissue like solid cancer tissue by enhanced permeability and retention mechanism. Active targeting is the selective uptake of nanomedicine by specific cells. Target moieties could be protein, antibody, or small molecule selective to specific tissues or cells. This mechanism is mainly controlled by homing to overexpressed cell surface receptors.

The Food and Drug Administration classified nanoscale materials to nanomaterials as "materials used in the manufacture of nanomedicine" or nanomedicine as "final products," The FDA approved 51 nanomedicines by the year 2016, 40% of which were in clinical trials between 2014 and 2016. According to the FDA evaluation of nanomedicines, it includes the physicochemical properties, followed by pharmacokinetics evaluation of nanomedicines. The pharmacokinetics evaluation includes (1) rate and amount of absorption, (2) retention in circulation, (3) half-life and complete elimination, (4) bioavailability differences, (5) distribution or accumulation to the body or specific tissue for active targeting, (6) decomposition or metabolism, (7) elimination, and (8) toxicity assessment of nanomedicines. On the other hand, the European Medicines Agency defined nanomedicines as "drugs composed of nanomaterials 1–100 nm in size, and these are classified into liposomes, nanoparticles, magnetic nanoparticles, gold NPs, quantum dots, dendrimers, polymeric micelles, viral and non-viral vectors, carbon nanotubes, and fullerenes." EMA has approved eight commercially available nanomedicines as first-generation nanomedicines. Currently, there are 48 nanomedicines in clinical trials (Phases 1–3) in the EU. EMA evaluates the pharmacokinetics and pharmacodynamics of nanomedicines through investigation of their chemical composition and physicochemical properties [8].

4. Approved application and indication of nanomedicine

4.1 Cancer nanoparticle medicines

Most pharmaceutical industries are focusing on developing new products for cancer as it is the first cause of death in 50% of the countries. Nanomedicine products have a good share in this market with many approved products to treat several types of cancer at various stages. Abraxane® is a famous albumin-particle bound paclitaxel nanomedicine loaded for advanced non-small cell lung cancer, metastatic breast cancer, and metastatic pancreatic cancer. Doxil®, the first approved nanomedicine by the FDA in 1995, is a PEGylated liposome loaded with doxorubicin for ovarian cancer, HIV-associated Kaposi's sarcoma, and multiple myeloma. Marqibo® is a liposomal vincristine for Philadelphia chromosome-negative acute lymphoblastic leukemia. Hensify® is the recently approved nanomedicine for cancer in 2019 by the FDA. It is the hafnium oxide nanoparticles stimulated with external radiation to enhance tumor cell death via electron production for locally advanced squamous cell carcinoma. Most of the approved nanomedicines are non-PEGylated except Doxil and Onivyde, which is interesting as most reports have proven the importance of nanomedicine coating with PEG. Furthermore, all nanomedicine products do not have active target moiety. So, all of these products follow passive targeting approach without even stealth characteristics.

4.2 Iron-replacement nanoparticle therapies

Iron-replacement therapy to treat anemia is surprisingly another area for nanomedicine due to the significance of nanoscale iron-oxide colloid system in improving iron absorption to the body. The main advantage of iron-oxide nanomedicine is replacing the injection of free iron with its associated toxicity. Most of these nanosystems are coated with either polysaccharide or polymer to reduce iron toxicity. CosmoFer® is the first approved iron dextran colloid by the FDA in 1996. Injectafer® is the most recent one in 2013 by the FDA, which is iron carboxymaltose colloid.

4.3 Nanoparticle/microparticle imaging agents

Another area for nanomedicine, especially the inorganic ones, is diagnostics, mainly imaging agents. Iron-oxide nanomedicines are also approved as contrasting agents for magnetic resonance imaging, which is used to generate contrasted images for different types of cancers. The magnetic property and small particle size allow the distribution of iron-oxide nanomedicine in tumor tissue, which provide a precise imaging of cancer borders. Additionally, perflutren is also used as ultrasound contrast agent in either lipid- or albumin-based nanomedicines. Phospholipid-stabilized microbubble is another form of nanomedicine as ultrasound contrast agent, which is approved in 2001 by the EMA. Its main mechanism is encapsulating air bubbles, which act as reflectors for ultrasound.

4.4 Nanoparticles for vaccines, anesthetics, fungal treatments, and macular degeneration

Several clinical applications have been studied using nanomedicine. Diprivan® is the first FDA-approved nanomedicine in 1989 for anesthesia. Another field for nanomachine is vaccination with two products, which are Epaxal® for hepatitis A and Inflexal V® for influenza. Both vaccines are liposome-based nanomedicine due

to the similarity of liposome structure to cell structure. Another famous liposome product is AmBisome®, which is a liposome loaded with amphotericin B for treating systemic fungal infections with reduced toxicity. Abelcet® is another approved lipid-based nanomedicine loaded with amphotericin B. Finally, Visudyne® is a liposomal verteporfin for treatment of subfoveal choroidal neovascularization from age-related macular degeneration, pathologic, or ocular histoplasmosis.

5. Conclusion

Nanomedicines are currently in the middle of the road with great potentials but require many development considerations regarding assessment of physicochemical properties, pharmacokinetic properties, and pharmacodynamic applications. Based on the recent trends with 47 products in clinical trial phases, it is expected that within the next few years, more products will be available for several applications, especially cancer.

Conflict of interest

The authors declare no conflict of interest.

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Chapter

Phage Capsids as Gated, Long-Persistence, Uniform Drug Delivery Vehicles

Philip Serwer, Elena T. Wright and Cara B. Gonzales

Abstract

Over the last 25 years, cancer therapies have improved survivorship. Yet, metastatic cancers remain deadly. Therapies are limited by inadequate targeting. Our goal is to develop a new drug delivery vehicle (DDV)-based strategy that improves targeting of drug delivery to solid tumors. We begin with a capsid nanoparticle derived from bacteriophage (phage) T3, a phage that naturally has high persistence in murine blood. This capsid has gating capacity. For rapidly detecting loading in this capsid, here, we describe procedures of native agarose gel electrophoresis, coupled with fluorescence-based detection of loaded molecules. We observe the loading of two fluorescent compounds: the dye, GelStar, and the anticancer drug, bleomycin. The optimal emission filters were found to be orange and green, respectively. The results constitute a first milestone in developing a drug-loaded DDV that does not leak when in blood, but unloads its cargo when in a tumor.

Keywords: agarose gel electrophoresis, bacteriophage T3, bleomycin, buoyant density centrifugation, capsid impermeability, GelStar

1. Introduction

Current therapies for cancerous tumors suffer from both toxic secondary effects and the development by the tumor of drug resistance. These effects usually block therapy for metastatic cancers, the cause of 90% of cancer deaths [1–5]. For solving these problems, our first thesis is that the best strategy is to increase tumor specificity of anticancer drug delivery in several, *independent* stages. If, for example, three stages are used and each stage is 80% efficient (20% nonefficient) in increasing specificity, then overall efficiency is 99% [$100 \times (1.0 - 0.2^3)$]. In this case, (1) drug dosages to tumors can be raised 100× without changing toxicity and, therefore, (2) tumor cell evolution of drug resistance is minimized.

The primary alternative is to continue testing chemotherapies [6–8], immunotherapies [9–11] and radiotherapies [12–14] that have tumor-specificity determined at one independent stage. This one stage is often cellular DNA replication, which is more rapid and, therefore, more drug- and radiation-sensitive, in cancerous cells than it is in healthy cells. One-stage strategies are >100 years old for immunotherapy and radiotherapy. Chemotherapeutic agents typically used are over 50 years old [8]. Even major effort has not produced systematic therapies for metastatic cancer.

Apparently, new, possibly more biology-based, strategy is needed to counter risk that the above one-stage-based strategies, in general, are not realistic for reaching objectives (reviewed in [1, 15–17]).

In theory, one implementation of multi-stage strategy starts with drug delivery in a drug delivery vehicle (DDV) that is gated. The gate is opened to load drug, closed in circulation to deliver drug and opened again in tumors to administer drug. Stages of DDV-derived toxicity reduction are the following tumor-specific events: (1) DDV delivery, (2) DDV opening and, (3) drug activation, for masked drugs. We address stages (1) and (2) here.

In this implementation, tumor-specific delivery is achieved via the EPR effect. The EPR effect is the spontaneous accumulation of nanoparticles in tumors, observed for an uncharacterized phage in 1940 [18] (reviewed in [19]). The causes of the EPR effect are (1) porosity of tumor blood vessels and relative tightness of healthy blood vessels, so that nanoparticles enter tumors, but usually not healthy tissue and (2) poor tumor lymphatic drainage, so that nanoparticles remain [20–25]. The EPR effect is the basis for the use of several FDA-approved, drug-loaded, liposomal DDVs [23, 24, 26].

However, circulating, drug-loaded, liposomal DDVs undergo drug leakage that causes significant toxicity [22, 23, 25, 27]. Also, liposomes are removed from circulation by the macrophage-phagocyte innate immune system. That is to say, liposomes are not very persistent. Chemical solutions to the leakage problem do not exist to our knowledge. Chemical solutions to the persistence problem (e.g., polyethylene glycol derivatization [28, 29]) introduce quality control problems and are not adaptable to future improvements, for example, achieving of tumor-specific unloading. The second thesis is that the optimal solution is linked to finding an appropriate, biologically produced, microbial DDV. Unlike some biology-based anticancer strategies [1, 15, 16, 19], use of a DDV is implementable with nononcolytic viruses and, therefore, avoids the dangers [19] of using oncolytic viruses.

In practice, we have discovered a phage T3 capsid that appears to have DDV-favorable characteristics needed for implementation of our strategy. First, phage T3 (and presumably its capsid) has recently been found to have exceptionally high persistence in mouse blood (3–4 h), unlike the T3 relative, T7 [30]. Second, one empty, but otherwise phage-like T3 capsid, is impermeable (for over 20 years) to the compound, Nycodenz (821 Da molecular weight) (reviewed in [31]). But, when the temperature is raised to 45°C, Nycodenz enters this capsid [32], presumably through a gate that opened. The concept is that, if we adapt this capsid to use as gated DDV, some of the needed engineering has already been done by natural selection.

Phages T3 and T7 are illustrated at the left in **Figure 1**. The gated capsid is illustrated at the right in **Figure 1**. This capsid is generated during DNA packaging that had been initiated by a DNA-free procapsid called capsid I (not shown). During packaging, capsid I expands and becomes the more angular and stable capsid (capsid II) illustrated in **Figure 1**. In nature, the capsid-gate is a ring of 12 gp8 molecules (**Figure 1**) that acts as entry portal for DNA during DNA packaging [31–33]. Most T3 and T7 capsid II particles are purified after having detached from DNA during infected cell lysis. The amount is 5–10 mg capsid II per liter of culture. The last purification step is buoyant density centrifugation in a Nycodenz or Metrizamide density gradient. Nycodenz low density (NLD) capsid II has the gp8 "gate" and is impermeable to Nycodenz and Metrizamide. The low density (1.08 g/ml) is caused by high internal hydration, which is caused by Nycodenz impermeability.

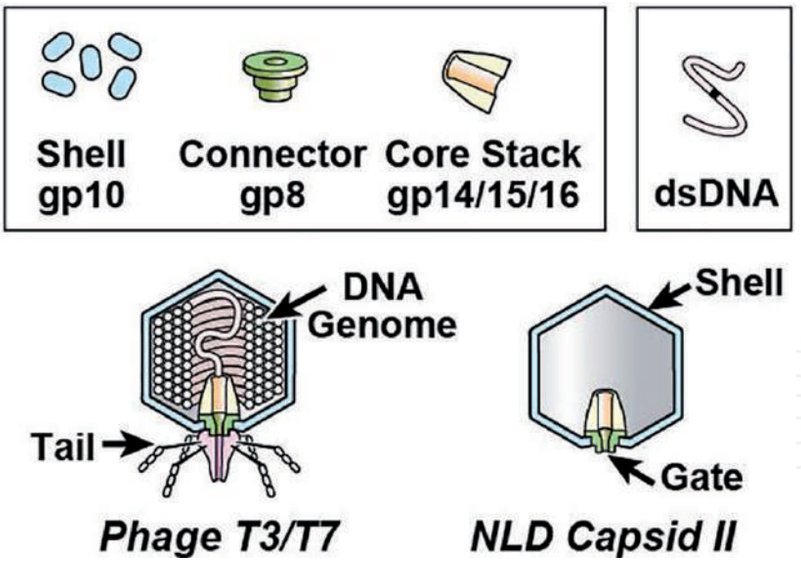

Figure 1.
Phages T3 and T7 (left); and T3 and T7 NLD capsid II (right). The graphic legend at the top indicates the various capsid components. A protein is labeled by gp, followed by the number of the encoding gene. Analogous T3 and T7 genes have the same numbers. If NLD capsid II is used as DDV, the perimeter of the DDV is defined by the gp10 shell; the gate is the gp8 portal. All structures are the same for phage T3 as they are for phage T7 (reviewed in [31, 32]).

Nycodenz high density (NHD) capsid II is Nycodenz-permeable (1.28 g/ml) and is completely separated from NLD capsid II during buoyant density centrifugation in a Nycodenz or Metrizamide density gradient [31, 33–35]. We use NHD capsid II as a control during loading experiments.

In the case of T3 NLD capsid II, gated entry of Nycodenz has been observed via raising of temperature. The likely entry channel was the axial hole of the gp8 ring. This conclusion was drawn, in the case of T7 NLD capsid II, from (1) entry kinetics of the fluorescent dye, bis-ANS [1,1′-bi(4-anilino)naphthalene-5,5′-di-sulfonic acid; 673 Da], and (2) covalent cross-linking of bis-ANS to channel proteins [33]. Asymmetric reconstruction-cryo-EM [36] revealed that obstruction of the T7 gp8 channel (and presumably the T3 channel) varies.

In support of working to implement the above gating-based strategy, the following quote from 2005 presents an expert opinion of what is needed for the next generation of anticancer DDVs [27]. "An ideal liposomal anticancer drug would exhibit little or no drug release while in the plasma compartment, thus ensuring limited exposure of the drug to healthy tissue. This feature would also maximize drug delivery to disease sites, as mediated by the movement of the drug-loaded liposomes from the plasma compartment to the extravascular space at disease sites, such as a region of tumor growth. Following localization, however, the drug-loaded liposome must transform itself from a stable carrier to an unstable carrier. This would ensure that the drug, which has localized in the diseased site, is bioavailable." To our knowledge, no details for such "controlled release" via a liposomal DDV have been published. The system described here is designed to accomplish what is described in the above quote. However, implementation uses gating of a DDV, not programmed instability of a DDV.

To proceed further, we need a procedure for rapidly determining whether a drug is loaded in a capsid. In the current study, we have developed native agarose gel electrophoresis (AGE) for this purpose. The detection is performed via capsid band fluorescence produced by the compound loaded.

2. Results

2.1 Detection of test compounds: GelStar

We tested the loading of two fluorescent compounds. The first was GelStar, a fluorescent nucleic acid stain typically used after AGE. In contrast, we incubated GelStar with our capsid and then performed AGE without further use of GelStar. The second compound was bleomycin, an anticancer drug [37, 38] that is also fluorescent [38]. Neither the manufacturer nor the vendor provided either the structure of GelStar or the concentration of commercial GelStar solutions. GelStar is sold in solution only.

The dominant fluorescence emission of nucleic acid-bound GelStar is in the green range. Apparently not previously documented is that the dominant fluorescence emission of free GelStar is in the orange range, at least when the GelStar is in an agarose gel. Ultraviolet light stimulated GelStar fluorescence emission vs. GelStar dilution is shown in **Figure 2**. Free GelStar, at several dilutions, had been pipetted in 5 μl amounts onto an agarose gel before ultraviolet light illumination and photography through an orange filter (spots labeled G in **Figure 2**). The effective volume in μl (dilution, multiplied by 5) of the stock GelStar solution is also indicated. In **Figure 2**, the color of GelStar spots is orange for all dilutions, as it also is found to be (not shown) with yellow and green emission filters. The orange color is real and is not produced by the emission filter because green Alexa 488 dye fluorescence retains its green color (spots labeled A in **Figure 2**). The number next to the Alexa 488 spots is the total amount (μg) of Alexa 488, also applied in 5 μl amounts.

DNA-bound GelStar had the expected green emission at all dilutions, when viewed through the same orange emission filter used for **Figure 2** (right side of the right panel of **Figure 3**). Green emission was also dominant when yellow and green emission filters were used for DNA-bound GelStar (not shown).

2.2 Detection of test compounds: bleomycin

Without fluorescent compound, the background of an agarose gel was blue when emission was photographed without an emission filter (not shown). Thus, not surprising was that optimal detection of bleomycin was not obtained with a blue filter, even though the blue range was where peak emission was previously found for bleomycin [38]. Among the blue, green, yellow and orange filters, optimal detection was obtained with the green filter.

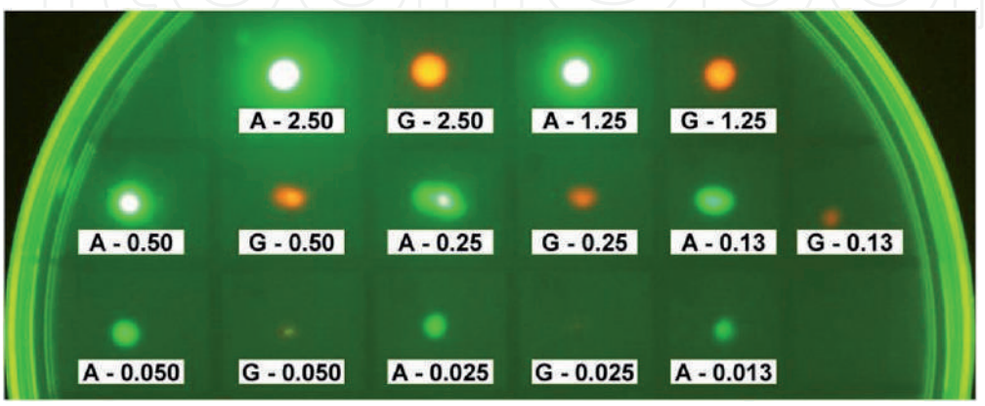

Figure 2.
Fluorescence of free GelStar. GelStar and Alexa Fluor 488 were diluted and, then, pipetted onto the surface of an agarose gel. The fluorescence was photographed through the orange filter. The GelStar samples are indicated by G, followed by the effective volume (μl) of the original, undiluted GelStar solution. The Alexa Fluor 488 samples are indicated by A, followed by the amount (μg) of Alexa Fluor 488.

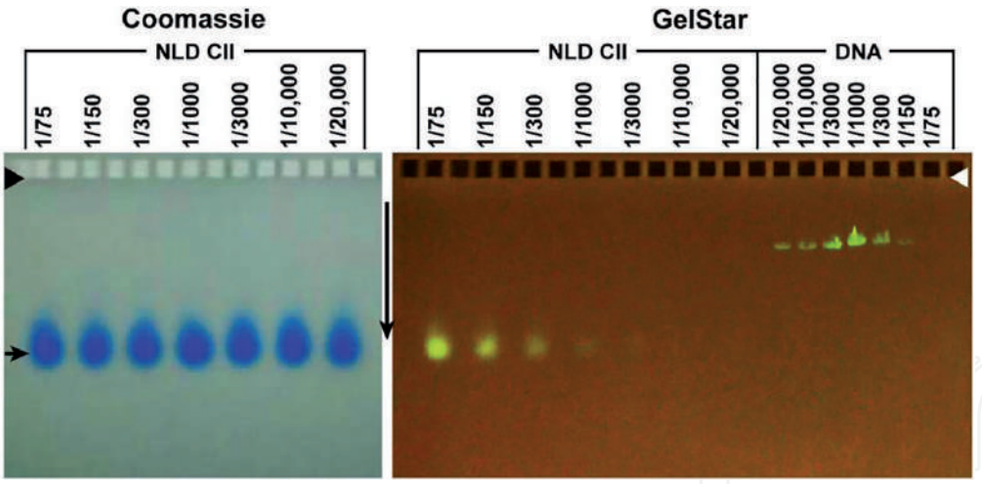

Figure 3.
Association of GelStar with T3 NLD capsid II. A commercial GelStar solution was diluted to the extent indicated above a lane and incubated with T3 NLD capsid II. Association of GelStar with the capsid was determined by AGE, followed by, first, photography of fluorescence (right panel, lanes labeled NLD CII) and, then, staining of protein with Coomassie blue (left panel). Also analyzed was purified T3 DNA (right panel, lanes labeled DNA), which does not stain with Coomassie blue. The arrow indicates the direction of electrophoresis; the arrowheads indicate the origins.

With the green emission filter, the minimal detected bleomycin amount was 0.2–0.4 ng when a bleomycin dilution series like the GelStar dilution series in **Figure 2** was photographed (not shown). Contrast enhancement of images was used at these lower amounts.

2.3 Loading of GelStar in NLD capsid II

We succeeded in loading GelStar into NLD capsid II. To achieve loading, 10 μg of NLD capsid II was incubated with GelStar at 45°C. Loading was then assayed by AGE at 10°C. Then, the gel was illuminated with ultraviolet light. The result was a fluorescent band of intensity that monotonically increased with decreasing GelStar dilution (left section of the right panel of **Figure 3**). The capsid amount was invariant, as judged by Coomassie staining of the same gel (left panel of **Figure 3**). At GelStar dilutions lower than those in **Figure 3**, down to 1/10, the band intensity reached a plateau (not shown). The dominant fluorescence, at all dilutions, was green, implying that the GelStar was bound to something capsid associated. GelStar did not detectably associate with NHD capsid II (not shown).

The following data indicated that the GelStar-binding capsid site was not on a DNA molecule associated with the capsid. As the dilution of GelStar decreased, the DNA-bound GelStar fluorescence underwent, first, an increase and then a decrease (**Figure 3**, right segment of right panel). However, the decrease was not observed for the binding to NLD capsid II. Second, although a minor NLD capsid II fraction has DNA [31], the DNA-containing NLD capsid II had been excluded during purification by selecting the low-density side of the NLD capsid II band after buoyant density centrifugation in a Nycodenz density gradient. Thus, the GelStar was apparently either self-bound or bound to capsid protein.

2.4 Loading of bleomycin in NLD capsid II

Association of bleomycin with T3 NLD capsid II was also achieved. However, the fluorescence signal was relatively weak (**Figure 4**). The bleomycin fluorescence signal of a NLD capsid II band did not change when the concentration of bleomycin was changed from 2 to 16 mg/ml. A bleomycin-associated NLD capsid II band is

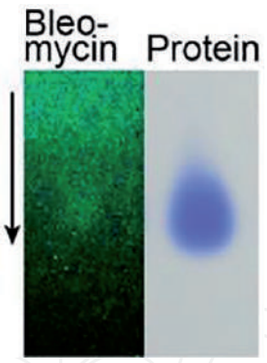

Figure 4.
*Association of bleomycin with T3 NLD capsid II. The experiment of **Figure 3** was repeated with bleomycin (8 mg/ml), instead of GelStar. The capsid region of the post-AGE gel is shown. The right (protein) panel has a single band of capsid stained with Coomassie blue. This band marks the position of the capsid-associated bleomycin fluorescence in the left panel. The arrow indicates direction of electrophoresis.*

shown in **Figure 4**. Most of the free bleomycin migrated toward the cathode (not shown), i.e., in a direction opposite to the direction of capsid migration.

The strength of the signal in **Figure 4** was weakened by the blue background and use of a green filter. In addition, a contaminant in the bleomycin preparation migrated close to the capsids, and is seen above the capsid band at the top of the left panel of **Figure 4**.

Calibration data for bleomycin, like the data for GelStar in **Figure 2**, were obtained. These data revealed that the amount of bleomycin loaded was 150–300 molecules per capsid.

3. Discussion

In the Introduction, we outlined a strategy that is expected to work, if we can achieve the following objectives: (1) high (~4 h) persistence of NLD capsid II in blood so that the EPR effect has time to work, (2) adequate loading and sealing of NLD capsid II and (3) tumor-specific, controlled release (de-sealing or unloading). Objective #1 is likely already achieved, given the high persistence of T3 phage. That is to say, if one considers this strategy to be engineering based, some of the engineering might already be been done by natural selection.

Concerning adequate loading, the volume of the internal cavity of NLD capsid II = 6.95×10^{-17} ml. For volume occupancy (F_V) of 0.5 (equal to the F_V of DNA packaged in mature phage [34]), the number of bleomycin molecules (1416 Da; density estimated at 1.6 g/ml as sulfate) per NLD capsid II particle is 2.4×10^4. The recommended dose of DDV-free bleomycin depends on the tumor, but is typically [39, 40] 10–20 units/m^2, corresponding roughly to 10–20 mg/m^2; 15 mg/m^2 is 1.76×10^{18} bleomycin molecules/m^2.

To calculate the number (N_D) of NLD capsid II particles needed for this dose at F_V = 0.5, we initially assume a 25 g mouse, which on average, has 78.6 cm^2 surface area [41]. Then, N_D is 3.6×10^{11}. A 6-liter culture yields 150–300 mouse doses of this size (cost ~ $1500), assuming (1) laboratory-scale production technique, (2) no development of procedures to increase the amount produced per bacterial cell, and (3) no drug-dose reduction caused by improved targeting. That is to say, if we can half-fill the volume of NLD capsid II, we have a viable beginning. However, thus far, we have filled no more than 2% of F_V = 0.5, NLD capsid II volume. So, increasing the loading is a major objective for the future.

An apparent obstacle to achieving this goal is the nonincrease in loading as bleomycin concentration increases above 2 mg/ml. At least two possible explanations

exist. (1) After passing through an open gate, the bleomycin eventually causes the gate to close. We were hoping to close the gate by lowering temperature. (2) After diffusing through an open gate, the bleomycin is prevented from diffusing in reverse by binding to internal proteins; the internal proteins become saturated as the concentration of bleomycin increases. In either case, increasing the loading is a problem of engineering.

An advantage of using a phage DDV is that the human-design engineering potential is relatively high. First of all, the capsids in a T7 NLD capsid II preparation are structurally uniform enough so that symmetric cryo-EM reconstruction is obtained at 3.5 Å [34] and asymmetric reconstruction, at ~8 Å [36]. Assuming T3 capsids to be comparably homogeneous, use of chemistry to improve gating should produce relatively uniform results.

Second, phages, in general, and phage T3 in particular, can be genetically manipulated, which is not possible with liposomes. Information for determining which nucleotides to change can be obtained from high-resolution cryo-EM structure. Structure of this type is not obtainable with liposomes.

Finally, we note that, as far as we know, the only phages tested for production of an NLD capsid II-like capsid are the related coliphages, T7, T3 and ϕII. All three of these phages produce a NLD capsid II-like capsid [42]. Other phages are potential sources of gated capsids, perhaps with properties even more DDV-favorable.

4. Materials and methods

4.1 T3 bacteriophage, capsids and DNA (nanoparticles)

We obtained bacteriophage T3 and T3 capsid II from 30°C-lysates of host, *Escherichia coli* BB/1, that had been infected by phage T3 in aerated liquid culture [43]. The growth medium was 2× LB medium: 2.0% Bacto tryptone, 1.0% Bacto yeast in 0.1 M NaCl. We initially purified both phage and capsids by centrifugation through a cesium chloride step gradient, followed by buoyant density centrifugation in a cesium chloride density gradient [43]. The latter fractionation separates capsid I from capsid II.

To separate NLD capsid II from NHD capsid II, we performed buoyant density centrifugation of capsid II in a Nycodenz density gradient, as previously described [32]. The purified NLD and NHD capsid II were dialyzed against 0.1 M NaCl, 0.01 M Tris-Cl, pH 7.4, 0.001 M $MgCl_2$. NLD capsid II, which formed a band near the top of the Nycodenz density gradient, had no detected contamination with NHD capsid II and vice versa, as previously seen by analytical ultracentrifugation [31]. Phage, NLD capsid II and NHD capsid II were dialyzed against the following buffer before use in the experiments described below: 0.2 M NaCl, 0.01 M Tris-Cl, pH 7.4, 0.001 $MgCl_2$.

T3 DNA was obtained from purified T3 phage by phenol extraction. The DNA was dialyzed against and stored in 0.1 M NaCl, 0.01 M Tris-Cl, pH 7.4, 0.001 M EDTA. DNA concentration was obtained from optical density at 260 nm.

4.2 Fluorescent compounds: test of fluorescence emission

GelStar was obtained from Lonza (Basel, Switzerland) in solution. The company recommends dilution by a factor of 1:10,000 for use as a nucleic acid stain after gel electrophoresis. Alexa Fluor 488 succinimidyl ester was obtained from Molecular Probes (Eugene, OR, USA) as a powder.

Bleomycin was obtained from Cayman Chemical Company (Ann Arbor, MI, USA) as a powder. The bleomycin was dissolved in the aqueous buffer indicated and diluted to the concentrations indicated before incubation with capsids and DNA.

Tests of fluorescence emission vs. fluorescent molecule concentration were made by pipetting 5 μl of diluted fluorescent molecule onto the surface of a 0.7% agarose gel (LE agarose, Lonza) that had been cast in a plastic Petri dish in the electrophoresis buffer of Section 4.3. The gel was then photographed by use of the procedures described in Section 4.3.

4.3 Loading experiments: AGE

To test for fluorescent compound/nanoparticle association, fluorescent compounds were mixed with one of the following T3 nanoparticles: NLD capsid II, NHD capsid II, phage, DNA. First, a 12.5 μl amount of fluorescent compound in 0.1 M NaCl, 0.01 M sodium citrate, pH 4.0, 0.001 M $MgCl_2$ (citrate buffer) was added to 4.5 μl of additional citrate buffer. Then, 8.0 μl of a nanoparticle sample was added and mixed (final pH, 4.1). This mixture was incubated at 45.0°C for 2.0 h.

To perform AGE, we added to this mixture 2.5 μl of the following solution: 60% sucrose (to increase the density for layering in sample wells) in the electrophoresis buffer below. This final mixture was layered in a well of a horizontal, submerged, 0.7% agarose gel (LE agarose, Lonza), cast in and submerged under the following electrophoresis buffer: 0.05 M Tris-acetate, pH 8.4, 0.001 M $MgCl_2$. The temperature of the gel and buffer had been pre-adjusted to 10°C in an effort to seal NLD capsid II and, therefore, prevent leakage of fluorescent compounds.

AGE was performed at 1.0 V/cm for 18.0 h with the gel and buffer maintained at 10°C by circulation through a controlled-temperature water bath. After AGE, the gel was soaked in 25% methanol in electrophoresis buffer for 1.5 h at room temperature, to cause leakage of fluorescent compounds from NLD capsid II and, therefore, to prevent auto-quenching.

Finally, the gel was photographed during illumination with a Model TM-36 ultraviolet transilluminator (Ultra Violet Products, Inc.). The camera used was a Canon Power Shot G2, 4.0 Megapixels. The following Tiffin emission filters were used as described in Section 2: Blue, 80A #290513; Green, 11Green 1—#287305; Yellow, Yellow 12—#282224; Orange, Orange 16—#289750. To detect capsid protein, the gel was subsequently stained with Coomassie blue and photographed during illumination with visible light [43].

5. Conclusions

Obtaining an increase in the current tumor-specificity of anticancer drugs should be possible via use of a DDV that implements multiple, independent stages of specificity increase. T3 NLD capsid II is an example of a bio-nanoparticle that has undergone some of the needed DDV-bioengineering via mutation/selection in the environment. Other examples, not yet found, are assumed to exist and potentially have even more favorable characteristics.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter

New Generation Peptide-Based Vaccine Prototype

Öznur Özge Özcan, Mesut Karahan, Palanirajan Vijayaraj Kumar, Shen Leng Tan and Yi Na Tee

Abstract

Synthetic peptide-based vaccine prototypes are the future potential vaccination. Antigens, which belong to minimal microbial component and produce antibodies such as peptides and polysaccharides, can promote long-term protection against pathogens that can cause infectious diseases. Production of peptides becomes simple with solid phase peptide synthesis and microwave-assisted solid phase peptide synthesis using automatic synthesizers. The use of synthetic peptides was approved by the health authorities for vaccine design. Peptides are themselves very weak immunogens and need adjuvants to provide an effective autoimmune response. For this reason, peptide antigens are conjugated with biopolymers and loaded with nanoparticles. The toxicity of vaccine prototypes is evaluated in cell culture, and non-toxic prototypes are selected for vaccinating experimental animals. The most effective peptide-based vaccine prototype is determined as the one with the highest antibody level. The goal of this book chapter is to illustrate the use of peptides vaccine systems and present their opportunities with their future development.

Keywords: biopolymers, nanoparticle systems, solid phase peptide synthesis (SPPS), synthetic peptide, peptide vaccine prototype

1. Introduction

The goal of this chapter is to review the importance of synthetic peptide-based vaccination, providing a brief knowledge about their new generation prototypes. In the first stage, relation to immunity and peptide vaccine with importance of using biopolymers was given under the title of solid-phase peptide synthesis including microwave system. After that, this review was focused on the established methods for peptide loaded nanoparticles or conjugated biopolymers preparation of peptide-based vaccine prototypes and nanotechnological particles as delivery system with touching on different methods. In addition, the impact of Contemporary Advancements in Peptide Based Vaccine like Liposome Based Subunit Vaccines was explained. In the last part, peptide-based vaccine prototypes studies *in vivo and in vitro* were given with their future perspective and development.

2. Peptide vaccines prototype and immunity

All vaccines generally are developed by using live or attenuated microorganisms. However, the use of whole microorganisms, their components or the biological

process for vaccine production has many weaknesses and a variety of approaches for synthetic peptide vaccination remain under investigation for the infectious diseases [1]. Peptides play an important role in a biological process, including the stimulate the immune response [2].

Peptide-based vaccination is an immunotherapy where a peptide is applied often with the use of an immunoadjuvant (nanoparticle or biopolymers) to stimulate T-cell and sometimes B-cell immunity. Peptide-based vaccinations are present in major histocompatibility complexes (MHC) the ultimate target for T cells in infection recognition and infection immune responses [3, 4]. Sometimes peptide-based vaccines play a role to stimulate innate and adaptive immunity both (**Figure 1**) and peptides are immunogen components of peptide-based vaccine and memory responses of peptide is weak in immune responses [1] without the biopolymer or nanoparticle system.

When producing a new generation of synthetic peptide vaccines, components of the pathogenic pathogen of interest are generally used. These components are linear peptides and produced by solid phase peptide synthesis (SPPS) method with high efficiency and purity [5–9]. When peptides are used in combination with a vaccine system, if they are used without a drug delivery system, there are risks of degradation by protease enzymes that break down proteins and phagocytosis by immune system cells such as antibodies [10]. In addition, drug delivery systems should be preferred as nanoparticles and biopolymers. A higher immune response

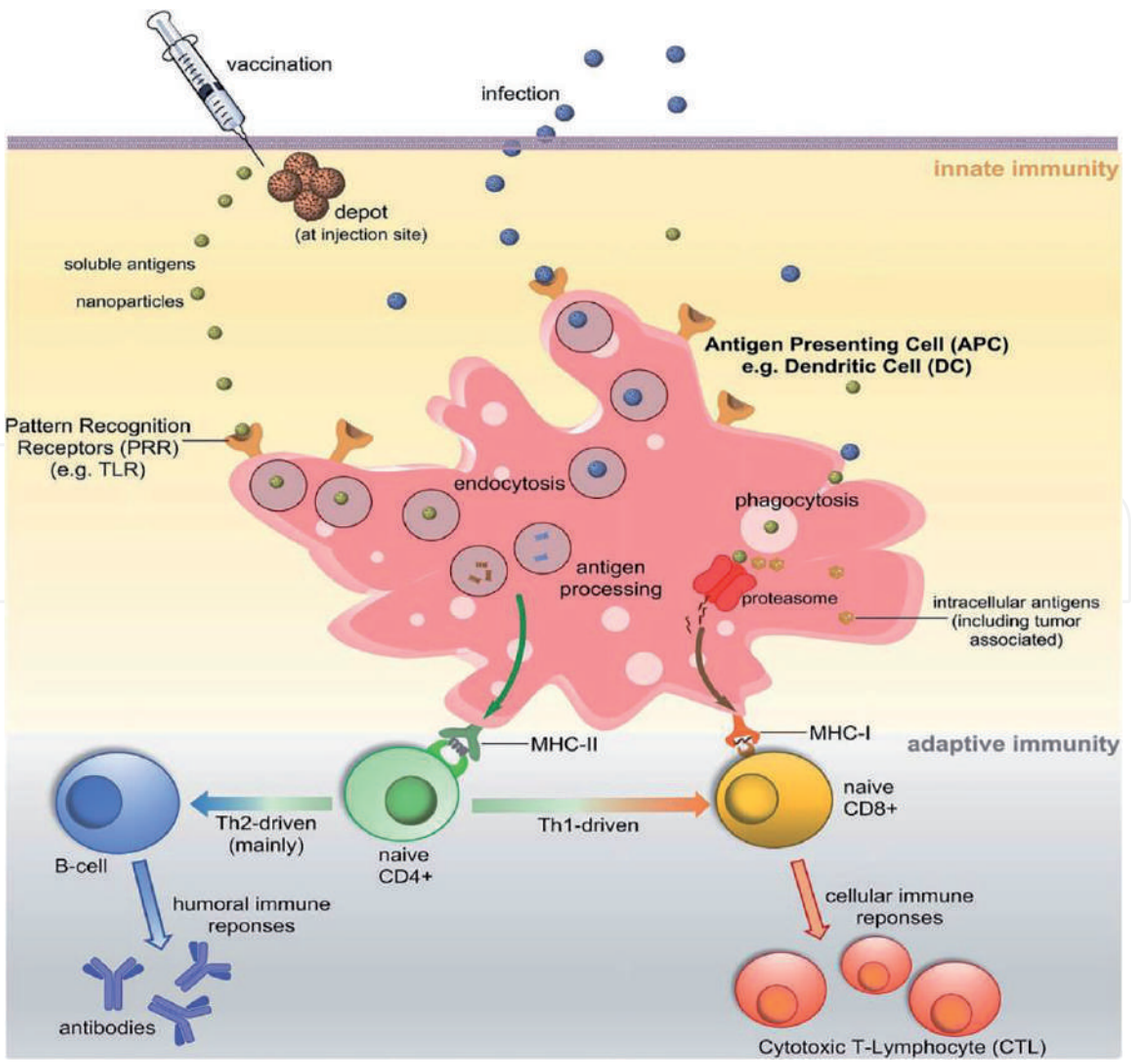

Figure 1.
Cellular representation of immune cells after vaccination [1].

and to protect peptides from harmful effects of degrading enzymes and aggressive antibodies, it is generally necessary to use nanosystems such as protein, biopolymer conjugations or nanoparticles (NPs). Peptide delivery systems based on nanoparticles are developing more and more for the development of peptide-based vaccines. Especially, biodegradable polymers offer very popular and patented vaccines [11]. For instance, poly(amino acid) and polylactic acid (PLA) are used for NPs and when antigenic peptides are encapsulated by them in order to vaccinate mice can provide significantly higher levels of total the antibodies like immunoglobulins (IgGs); IgG, IgG1, IgG2. This means they can able to stimulate humoral immune responses and also CD4+ and CD8+, T and B cell activation and for the cellular immune responses; interferon γ (IFNγ) which induce Ig class switching to IgG2a [12–14]. In another study, Murine model was used in an immunization study. An antigen of Hepatitis B disease loaded on Poly (lactic-co-glycolic acid) (PLGA) NPs (300 nm) provided better immune responses compared to the antigen alone. Immunization with PLA NPs (200–600 nm) can also provide higher levels of IFNγ production related to a Th1 response. In contrast, immunization of PLA microparticles (2–8 μm) promoted IL-4 secretion due to Th2 response [15]. Both PLGA NPs and liposomes are phagocytosed efficiently by cells to localize intracellular localizations and produce an immune response [16, 17]. Carbon NPs are promising in oral vaccine administration for the use of synthetic peptides [18].

Different approaches are available to develop synthetic peptide-based vaccines, using metal ions in combination with peptide sequences. In particular, the investigation of the complex formation biopolymer by peptide in the presence of metal ions contributes greatly to the technological development of peptide-based vaccine prototypes [19]. The contact of the peptides with the polyelectrolyte (PE) is found at the interface. Solubility of polyplexes and complexes with NPs and peptides; it depends on the structure of the peptides (such as hydrophilic and lipophilic) and correlates with the isoelectric points in this system. Metal ions such as copper (Cu^{+2}) generally promote two effects: (1) conjugation of polyelectrolyte to peptide molecules and (2) aggregation of polyplex particles in the intermolecular region. Some of these polyplexes exhibit strong immunogenicity and provide a high level of immunological protection for peptide vaccine prototypes, making them more efficient, but the solubility, composition and stability of these polycomplexes depend on pH, metal/PE and protein/PE ratios. These systems are based on conjugation of PE and antigen molecules with covalent bonds to NPs or biopolymers, which induce an immune response to the immunizing agent. The hydrophobic interactions in such a complex create an adjuvant effect for prototyping technology in vaccination. [19–22]. In the studies on the development of peptide vaccine prototypes previously made by our study group, it was observed that the purification of characterization of binding of synthetic peptides to various adjuvants and subsequent high immune response was obtained in BALB/c mice from experimental animals [23].

3. Solid-phase peptide synthesis (SPPS)

A historical overview of peptide chemistry from T. Curtius (who achieved the first synthesis of peptide in 1882) and Fischer (who synthesized the first dipeptide in 1901) to M. Bergmann and L. Zervas is first in presenting the Solid-Phase peptide synthesis. Next, the fundamentals of peptide synthesis with a focus on SPPS by R. B. Merrifeld are described. Although the peptides can be synthesized in three methods: in a solution medium, on a solid support, or as a combination of the solid and the solution synthesis, this chapter emphasizes an overview of peptide synthesis giving importance on SPPS. Currently, most of the peptides for research,

vaccination or therapeutic drugs for cancer and brain diseases are synthesized by SPPS methods. Successful peptide synthesis depends on the appropriate selection of suitable resins, linkers, amino acid derivatives and coupling reagents, as well as the side chain (de) protection and cleavage conditions, and the correct synthesis of the assay. In the SPPS method, the solid support is attached at the end of the first amino acid-COOH at the carboxyl end a polymeric support insoluble in the newly formed peptide chain is referred to as resin. A covalent binding step that binds the resin is important for the reaction [24]. The peptides may be gradually joined between the C and N terminus using N-protected amino acids. The Nα protecting group (Boc) is unstable in the presence of intermediate acid (trifluoroacetic acid; TFA), the side chain protecting benzyl (Bzl) based groups and the peptide/resin linkage are stable in the presence of intermediate acid and are variable in the presence of strong acid (HF). Fmoc group is important for solid-phase applications. Fmoc-based strategies are also available, and hydroxymethylphenoxy-based binders are used to add peptide to the resin with t-butyl (tBu) based side chain protection [25]. The solid phase peptide synthesis method consists of three basic steps. According to this, deprotection of the carboxyl group activation and peptide bond formation (Coupling). Following this procedure, the final deprotection of the last added amino acid is removed and the N- terminal is released. Cleavage and deprotection of the resin-bound peptide from the solid support [26].

The stepwise representation of solid phase peptide synthesis is illustrated in **Figure 2**. The starting amino acid masked by a non-persistent protecting group at the N-α terminus is loaded from the C-terminus to the resin. A semi-permanent protection group can also be used to mask the side chain if necessary (**Figure 3**, Step 1). The synthesis of the peptide, repeated deprotection of the N-α-transient protecting group, and binding of the next protected amino acid (**Figure 3**, Step 3).

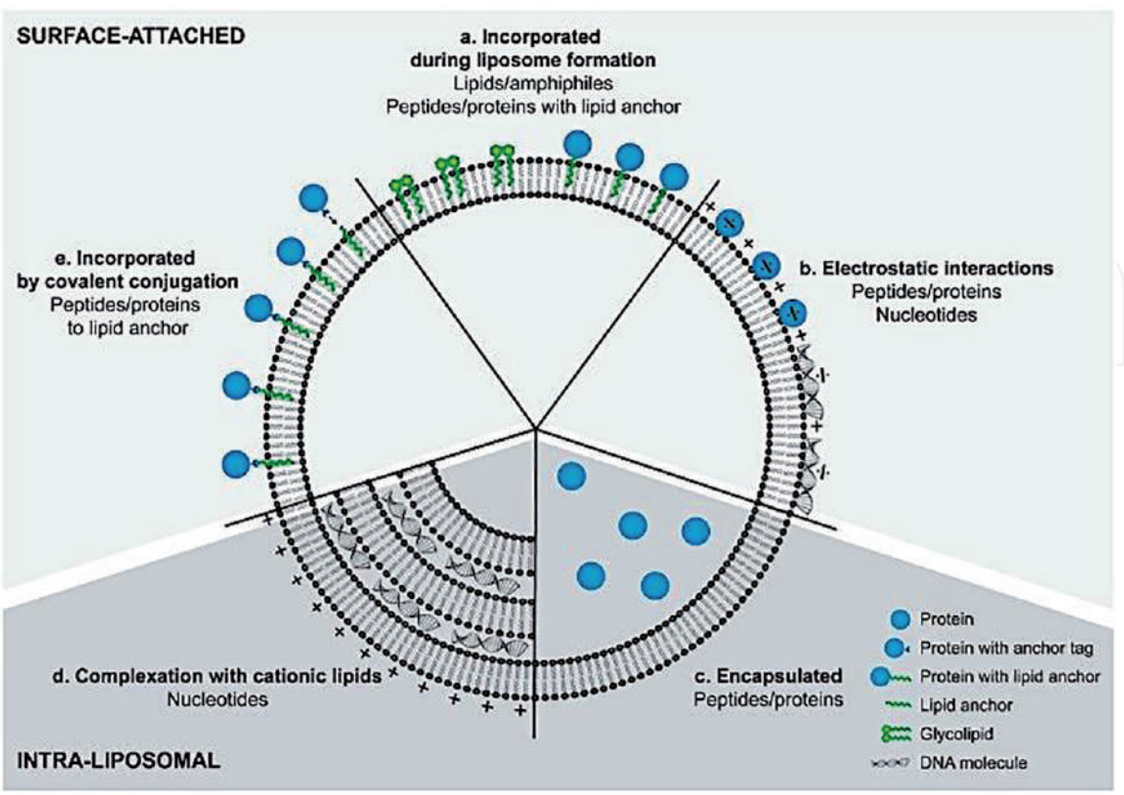

Figure 2.
The antigens and immunomodulators that can be used for inclusion in liposomes; it is shown in different strategies depending on the target and structure of the molecule [28].

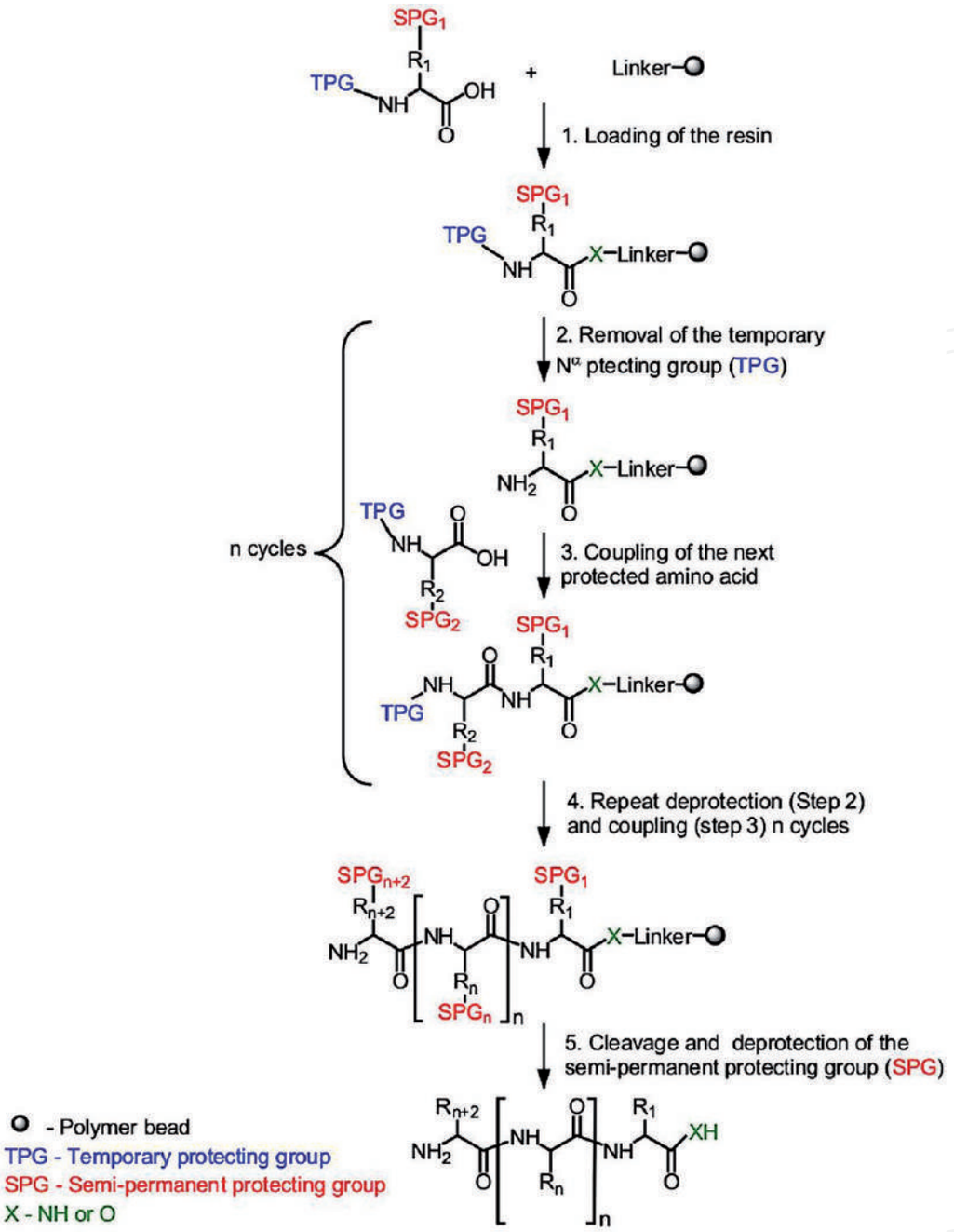

Figure 3.
Stepwise representation of solid phase peptide synthesis [25].

Synthetic cycle	Reagents	Time & conditions
Deprotections	Trifluoroacetic acid (Boc) and 20% piperidine in DMF (Fmoc)	1–5 min (70°C Fmoc and Boc)
Couplings	Amino acids, HBTU/HATU/HOBt/HOAt/DIC, DIPEA	5–15 min 50–70°C

Table 1.
Synthetic cycle and important reagents with time and conditions in microwave-assisted SPPS [27].

After the last amino acid is loaded (**Figure 3**, Step 4), the peptide is separated from the resin support and the Fmoc or Boc groups are removed [25].

The development of microwave-assisted solid-phase peptide synthesis has been developed by the synthesis of linear and complex peptide sequences and long

peptide sequences in a shorter time and high throughput. Time and temperature conditions, reagents and synthesis cycle for microwave assisted SPPS with using Boc or Fmoc are represented below in **Table 1**. The disadvantage of this technique could be the cost of resin (the binding procedure of the first binding amino acid to the resin in peptide synthesis can requires different and complex processes) and equipment [27, 29].

4. Peptide-based vaccine for nanotechnological prototypes

Synthetic peptides alone are not sufficient to develop vaccine prototypes because they cannot stimulate the cellular and humoral protection system sufficiently. So, different adjuvant systems are formed by conjugating the peptides biopolymers or loading them into NPs, resulting in a high immune response [30]. The use of peptide-polymer complexes and peptide loaded nanoparticles are the best way for the developing the peptide-based vaccine prototypes.

4.1 Type of nanoparticles

The binding of the antigenic peptides with the water-soluble polymer has multiple effects. Some of those are as follows:

- to provide modification of peptides,
- to increase the water solubility of those with hydrophobic properties,
- to raise regional impact,
- to increase immunogenic effects and immunoreactivity, and
- to be more effective in the living organism [31].

NPs are spherical polymeric carriers. The particles that are below 1000 nanometers (nm) are called nanoparticles. These particles with superior properties are used in many fields such as electricity, electronics, biotechnology, automotive, medical. NPs are morphologically and physicochemically influenced by the physical and chemical properties of the starting material used. The nanoparticles used as polymeric carriers are solid colloidal structure. The active substance can be encapsulated, absorbed or dissolved in the particle. Polysaccharides, polyanhydride, polycaprolactone, polyacrylic acid and polylactic-co-glycolic acid is also used for producing an effective nanoparticles and produce a co-polymer system such as poly(ethylene glycol) (PEG)-Nps and poly(ethylene glycol)-poly(ε-caprolactone) copolymers (PEG-PCL) copolymers. The copolymers of N-vinyl-2-pyrrolidone with acrylic acid (P(VP-co-AA)), PLGA, NPs loaded with the antigenic peptide can be used for future vaccine prototypes [32]. PLGA NPs is approved by U.S. Food and Drug Administration (FDA) and using for peptide carrier in vivo because of strong immune response [33].

Polymeric NPs are used for therapeutic applications and some of popular NPs are as follows:

1. Pluronics®
2. PEG–PLA

3. PEG-PCL
4. PEG-Lipid
5. PEG-PLGA
6. PEG-poly (amino acids)
7. Stimuli-sensitive polymeric micelles
8. Endogenous stimuli-sensitive polymeric micelles
9. pH-sensitive polymeric micelles
10. Reduction sensitive polymeric micelles
11. Thermo-sensitive polymeric micelles
12. Exogenous stimuli-sensitive polymeric micelles
13. Light-sensitive polymeric micelles
14. Magnetic field-sensitive polymeric micelles
15. Ultra-sound sensitive polymeric micelles
16. Margination of micro/NPs: Requirement for optimum drug delivery

5. Established methods for peptide loaded NPs or conjugated biopolymers preparation

We have mentioned that the peptides alone cannot produce an adequate immune response and also have poor stability with the internalization problem while crossing cell membranes. To solve all these limitations, peptides are loaded nanoparticle systems or conjugated biopolymers. Biopolymers are generally nontoxic products are generally preferred for producing continuously release systems with long term effect [34]. Here, the applicable and most common strategies for the synthesis of peptide-based NPs and encapsulation or conjugated methods of biopolymers are shown.

5.1 Emulsification-solvent evaporation method

The emulsion solvent evaporation technique is known as the most successful and useful method in the preparation of peptide loaded NPs and this technique is studied under two groups as single and double emulsion solvent evaporation methods [35].

5.2 Conjugation methods

Conjugation is a technique for achieving peptide and biopolymer complexes. The covalently linked peptide biopolymer conjugates can be linked using the water-soluble carbodiimide method as a cross-linker and synthesis with microwave

energy methods [36]. Peptides conjugates biopolymers can be synthesized in organic media using microwave energy. Also, there are another methods, including complex formation of biopolymers and peptides and electrostatic complex formation and metal coordination via ion coordination [35]. Specific antibody titers were observed in mouse experiments against peptides containing polymeric conjugates and complexes. The molecular weights of these conjugates are also very important. Biopolymer conjugation is crucial to obtain a high immune response to antigens at low molecular weights [37–39].

5.3 Nanoprecipitation

Nanoprecipitation is the most strategic method for the preparing of vaccine prototypes. Reducing the pH is very important to stabilization of system. Also, salt concentration under the solubility conditions is another important thing for the encapsulation method [70]. If the experiments cannot move on then adding a non-solvent phase in the quality of the solvent technique in which the parent compound of the NPs is dissolved can help [40]. Nanoprecipitation is frequently used in encapsulation of peptides. A pH-controlled precipitation rather than a non-solvent precipitation is a more preferred approach for passing the polymer to a non-dissolved phase with a simple pH change in the medium. For NPs or biopolymers prepared by nanoprecipitation, these solvents are known as the organic phase of acetone and ethanol [41].

5.4 Encapsulation of peptide

Encapsulation is carried out simultaneously by synthesizing NPs and biopolymers in all of the methods mentioned the encapsulation method for peptides should be selected based on the hydrophobic or hydrophilic facilities of peptide. Using of peptide encapsulation is important because of

- peptide release controlling,
- modeling of targeted delivery systems,
- mask unfavorable organoleptic properties (taste, odor, color),
- protection of peptide from immune attacks and enzyme degradation,
- insurance of bioconjugate molecules stability,
- decrease toxicity, and
- design of new dosage forms [42].

5.5 Peptide characterization

After purification of the peptides, they are commonly characterized by liquid chromatography-electrospray ionization-mass spectrometry (LS-ESI-MS), fluorescence spectroscopy and possible three-dimensional structures of the synthetic peptide (PEP-FOLD) server. It has validation since the chromatographic method has positive properties in terms of linearity, accuracy, precision and repeatability. Synthetic peptide vaccines are immunogens that can be used when creating vaccine

prototypes, especially because of their lipophilic structure (which also allows cell permeability to pass easily) [43]. Methods such as Fourier transform infrared (FT-IR) and nuclear magnetic resonance (1H- and 31P-NMR) are frequently used to visualize the physical structure of the copolymer and peptide biopolymer conjugates and to perform characterization studies. The conjugation of molecular weights is measured via size-exclusion chromatography (SEC) [44].

5.6 Characterization of peptide vaccine prototype

Ultraviolet (UV) and FT-IR Spectrophotometers and ZetaSizer are used for studying the nanoparticles and Scanning Electron Microscope (SEM) is used for morphological examination of Polymers or bioconjugate [45].

5.7 Toxicity studies

Peptide-based vaccine prototypes need to be tested in a cell culture medium to be feasible because they may have physiological, biological and chemical effects, causing cytotoxicity. The method used to investigate the cytotoxic profiles of peptide-based vaccines is also called *in vitro* cytotoxicity assays or cell culture-based measurement methods [46, 47]. Tetrazolium salts are compounds used in cell lines to measure the metabolic pathways of cells of microbial origin. Tetrazolium salts are the heterocyclic organic structure of these compounds and their reduction to colorless or weak colored aqueous solutions known as formazans has been the basis of their use as vital dyes in redox chemistry, biological and chemical applications [46, 47]. The tetrazolium ring can only be broken by active mitochondria, so viable cells and dead cells can be distinguished by discoloration. The fact that this change can be made only by living cells in vitro has made tetrazolium compounds a highly biologically important to measure toxicity of peptide-based vaccine formulas. The mechanism of toxicity assays, such as 3-(4,5-dimethylthiazolyl)-2,5 diphenyltetrazolium bromide (MTT) [19], 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2Htetrazolium inner salt (WST), 5-methyl-phenazinium methyl sulfate(PMS), 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4- sulfophenyl)-2H-tetrazolium inner salt; (MTS) are used [49, 50] and in our studies, we generally use MTT analysis. For example, our technological vaccine prototype example is Zika peptide loaded PLGA nanoparticles which were determined on ECV304 human epithelial cells via MTT assay, which is the cytotoxicity test, was performed to determine the cytotoxic effects of the peptide, peptide loaded NPs [45]. The importance of toxicity studies is to determine the non-toxic vaccine prototype and to switch to in vivo animal studies.

5.8 Contemporary advancements in peptide based vaccine

5.8.1 Liposome based subunit vaccine

Live attenuated vaccine is highly immunogenic and considered as well-tolerant for healthy individuals. However, live attenuated vaccine should not be administered to immunocompromised individual as it would cause systemic infection. An alternative vaccine technology, subunit vaccine, is safer and more suitable for immunocompromised individual. It uses fragment of a pathogen (antigen) to trigger an immune response and stimulate immunity against the pathogen. However,

Figure 4.
General features of virus-like particle [48].

subunit vaccine has low immunogenicity and often combined with adjuvants to induce protective immunity. The adjuvants are capable to enhance vaccine effectiveness and stimulate immune responses [28].

Human alphaherpesvirus 3 (HHV-3) is known as Varicella-zoster virus (VZV), which is the causative agent of varicella (chicken pox) and herpes zoster (shingles). In 1978, the first commercial Varicella-zoster immune globulin, Vzig™ (Massachusetts Public Health Biologic Laboratories, Boston, Massachusetts) became available. However, the supply was removed from the U.S. market in 2006. The alternative preparation, Varizig® (Cangene Corporation, Winnipeg, Canada) was licensed by FDA in 2012 and has shown to be comparable to Vzig™ [Saol Therapeutics Inc. 2012]. Varizig® is supplied as a sterile solution containing human Varicella-zoster immune globulin (IgG) which showed for post-exposure prophylaxis in high-risk patients [51].

Zostavax® is the vaccine licensed for herpes zoster prevention in individuals above the age of 50. It is a lyophilized preparation which is given as subcutaneous injection [52]. A non-replicating liposome-based subunit vaccine (HZ/su) is the new development for zoster prevention. The HZ/su is a non-live recombinant VZV glycoproteins E with the adjuvant ASO1B [53]. A randomized placebo-controlled study has shown that HU/su poses age-independent defense against HZ and has better efficacy compared to Zostavax® in reducing the risk of HZ for immunocompromised adults with the age above 50. Unlike HZ/su, Zostavax® lose efficacy as age increase [54]. HZ/su vaccine is not yet approved by FDA.

Liposomes are nano-carriers and they are useful in delivering vaccine antigen by forming liposome-based vaccine delivery systems. It is advantageous over other carriers due to its biocompatibility, non-toxic and biodegradable features [55]. Besides, liposomes can be customized to achieve desired immune profiles by optimizing their composition, antigen-loading strategies and the use of adjuvants system [28, 56].

5.8.2 Virus-like particle

Eculizumab (Soliris®) is a humanized monoclonal antibody (mAb) which function as a terminal complement inhibitor [57]. It was the first therapeutic agent approved by the FDA for atypical hemolytic uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH)-associated with thrombotic microangiopathy (TMA) in 2007 and 2011 respectively [58]. Soliris® (Alexion Pharmaceuticals, New Haven, Connecticut, USA) is in the form of sterile solution for i.v. injection. Eculizumab increases the patient's susceptibility to meningococcal infection (Neisseria meningitidis), all patients must be vaccinated against meningococcal infections prior to or at the time of initiating Eculizumab.

Virus-like particles (VLPs) is one of the alternative types of nanoparticles delivery system [59]. A recent research has shown that the development of autologous C5 vaccine in nanoparticle form is able to elicit strong humoral responses [60]. A peptide epitope (PADRE peptide) in the C5 vaccine is used to create a recombinant virus-like particles (VLPs). It showed a reduction in hemolytic activity and protect the mice from complement-mediated intravascular hemolysis [60]. Based on the study's result, it is showed that the recombinant VLPs could be used as an alternative or supplement for Eculizumab.

VLPs is known as an emerging class of targeted delivery vehicles with potential of overcoming the limitations of other nanoparticles [48]. VLPs is a potential delivery system due to their immunogenic nature, well-defined structure, ability to present a wide variety of potential epitopes, and ease of production [60]. They lack natural genome thus it is non-infectious. Besides, it can turn as self-adjuvant which is proficient in breaking the immune tolerance.

One of the limitations of VLPs are phagocyte-mediated clearance [59]. Besides, a recent study showed that ellipsoid nanoparticles can extravasate from the blood vessel more effectively than spherical nanoparticles. Meanwhile, the ellipsoid shape is possible for conventional polymeric NPs, but is not feasible for icosahedral VLPs. However, this limitation can be overcome through the modification of VLP surface by adding a variety of useful ligands [61]. VLPs may be able to efficiently extravasate from the vasculature of the blood vessels by showing multiple ligands with high affinity for the tight connections between endothelial cells [59].

6. Importance *in vitro* and *in vivo* experiments using peptide-based vaccine prototypes

After forming a synthetic vaccine prototype, the cytotoxicity of the bioconjugate of the peptides and biopolymers is first determined (generally we use MTT analysis). After the apoptotic effect of the prototype on living cells is measured by flow cytometric detection, the vaccine prototype with the most viable cell number should be selected for further study [62]. After all these methods, immunization is the next step. We immunize BALB/c mice with each one of the peptides biopolymer conjugates or peptides loaded nanoparticles following conventional immunization protocol. The goal is to identify the most antigenic vaccine prototype. The

antibodies are measured in blood (for humeral response such as; T and B lymphocytes, IgGs) or splenic (cellular response like ILs and IFNs) samples from the immunized BALB/c mice via the indirect enzyme-linked immunosorbent assay (ELISA) to determinate the highest antibody level. Thus, the most suitable peptide-based vaccine prototypes will be identified for future clinical phase studies. In brief, cell culture and toxicity studies are important before the analyses the effect of vaccine prototype in vivo [62].

7. Current situation and future perspective

Peptides can affect important brain regions that are essential for life-sustaining functions. There are studies about Peptide drug and Peptide-Polymer Vaccines and drugs using for brain disorders. In this future perspective we will explain the using of peptides in common brain disorders such as Alzheimer Disease, Parkinson Disease (PD), Multiple Sclerosis. In a study using an interference-inducing peptide (TAT-DATNT) to elute a protein complex consisting of interaction between DAT and the dopamine D2 receptor (D2R), it was determined that locomotor behaviors were induced in Sprague–Dawley (SD) rats. This peptide can provide potential therapy for regulating the activity of DAT and dopaminergic neurotransmission of Attention Hyperactivity Deficit Disorder (ADHD) therapies [63]. Alpha Synuclein (A-syn) aggregate is very important for the PD. Against of this aggregate, an immunogenic peptide the sequence of CGGVDPDN [64] is developed with solid phase peptide synthesis method as a vaccine in PD. Peptides can also be neuroprotection for the PD. TFP5 peptide, FITCGGGKEAFWDRCLSVINLMSSKMLQINAYARAARRAARR; TP5 peptide, KEAFWDRCLSVINLMSSKMLQINAYARAARRAARR; SCP peptide, FITCGGGGGGFWDRCLSGKGKMSSKGGGINAYARAARRAARR are reduction in neuroinflammation and apoptosis. **MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)** is a neurotoxin drug of MPP+, which causes permanent symptoms of PD by destroying dopaminergic neurons in the substantia nigra of the brain for mouse modeling [65]. PD01A is a Phase 1 epitope vaccine in experimental — a synthetic a-syn mimicking peptide-polymer based on a-syn aggregate by inducing an immune response that generates antibodies specifically against it [66]. In general, vaccines are developed according to the T cell response, but a peptide epitope with a three-celled peptide epitope with a three-cell universal peptide such as Syn85-99 (AGSIAAATGFVKKDD), α-Syn109-126 (QEGILEDMPVDPDNEAYE), α-Syn126-140 (EMPSEEGYQDYEPEA) and P30 (*FNNFTVSFWLRVPKVSASHLE*) epitope vaccines comprising three peptide-based epitope vaccines comprising different α-Syn peptides, but consisting of different B cell epitopes as follows, are noted for their high immunogenicity [67]. So peptides can have different immune cell response in brain disorders. Peptides can also protect cell biological agents such as microtubules activity. This is very important for cell stability while the diseases are seen in the cells. The dysregulation of ADNP / ADNP2 expression in the relevant brain tissue and animal model may improve the prognosis of schizophrenia because these genes are responsible for the regulation of interacting microtubules. The microtubule-interacting drug candidate, NAP (davunetide) is a small peptide and belong to activity-dependent neuroprotective protein (ADNP) which contains a small peptide motif, *NAPVSIPQ* sequence that provides potent neuroprotection for tau pathology, neuronal cell death as well as social and cognitive dysfunctions [68]. Especially in Amyloid beta pathology, *DAEFRHDSGY* peptide, Wang et al. synthesized peptide immunogens, A1-14 peptide immunogens for *UBITh*® AD immunotherapeutic vaccine by using automated SPPS for the Alzheimer Disease.

Wang's group has developed a synthetic peptide vaccine prototype for the prevention and treatment of AD and is conducting *phase II* clinical trials. The occurrence of Alzheimer's disease constitutes a strong immune response to Amyloid Beta (Ab) Plaques; *UB-311* was constructed with two synthetic Ab1-14 targeting peptides (B cell epitopes), each bound to different helper T cell peptide epitopes and formulated in a Th2 delivery system [69].

8. Conclusion

Consequently, this chapter provides a brief manual for anyone in the fields of solid-phase peptide synthesis, peptide vaccines, Nanotechnological importance for effective vaccine prototypes, and their future perspective for other diseases such as brain disorders.

Conflict of interest

The authors declare no conflict of interest.

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Chapter

Self-Emulsifying Drug Delivery Systems: Easy to Prepare Multifunctional Vectors for Efficient Oral Delivery

Khaled AboulFotouh, Ayat A. Allam and Mahmoud El-Badry

Abstract

Self-emulsifying drug delivery systems (SEDDS) have been mainly investigated to enhance the oral bioavailability of drugs belonging to class II of the Biopharmaceutics Classification System. However, in the past few years, they have shown promising outcomes in the oral delivery of various types of therapeutic agents. In this chapter, we discuss the recent progress in the application of SEDDS for oral delivery of protein therapeutics and genetic materials. The role of SEDDS in enhancing the oral bioavailability of P-glycoprotein and cytochrome P450 3A4 substrate drugs is also highlighted. Also, we discuss the most critical evaluation criteria of SEDDS. Additionally, we summarize various solidification techniques employed to transform liquid SEDDS to the more stable solid self-emulsifying drug delivery systems (s-SEDDS) that are associated with high patient compliance. This chapter provides a comprehensive approach to develop high utility SEDDS and their further transformation into s-SEDDS.

Keywords: solid self-emulsifying drug delivery systems, solidification techniques, oral delivery, P-glycoprotein (P-gp), cytochrome P450 3A4 (CYP3A4), multidrug resistance (MDR), protein therapeutics, plasmid DNA (pDNA)

1. Introduction

Lipid-based drug delivery systems (LBDDs) have been intensively investigated to overcome various obstacles encountered in oral drug delivery including poor aqueous solubility, limited permeability, low therapeutic window, first pass metabolism as well as inter- and intraindividual variability in drug response [1]. Lipid-based nanoparticles can achieve high loading capacity of hydrophilic and hydrophobic drugs [2]. The delivery features of these drug delivery systems could be tailored to achieve either immediate or sustained release properties depending on the appropriate selection of lipid composition. Most of lipids employed in the formulation are generally recognized as safe (GRAS), biocompatible and biodegradable [3]. LBDDs can enhance both transcellular and paracellular transport of drugs by transient disruption of lipid bilayer cells and alteration of tight junction by products of lipid digestion, respectively. Interestingly, they could permeate challenging physiological barriers such as blood brain barrier without surface

modification due to their lipophilic nature [4]. Further, they are promising carriers for protection of therapeutic peptides against harsh GI environment [3]. Ease of preparation, cost effectiveness and possibility of large-scale production make LBDDs more attractive compared to polymeric nanoparticulate delivery systems [5].

2. Classification of lipid carriers

Lipid carriers can be classified into various categories depending mainly on their method of preparation as well as their physicochemical properties. They include liposomes, niosomes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), micro and nanoemulsions, self-emulsifying drug delivery systems (SEDDS), and lipid-drug conjugates [2, 4].

Liposomes are uni- or multilamellar spherical vesicles which are composed of cholesterol and other natural or synthetic phospholipids enfolding an aqueous compartment [6]. They were first introduced by Bangham et al. in 1965 [7]. Thus, liposomes have been considered as biocompatible and biodegradable carriers that possess efficient delivering capability of hydrophilic and hydrophobic drugs. Advantages of liposome-based drug delivery systems include reduction of systemic and of target toxicities as well as targeting potential to achieve the desired outcome [8]. Thus, many liposome formulations have been approved for commercial use such Ambisome® (amphotericin B), Depocyt® (cytarabine), DepoDur® (morphine sulfate) and many others. However, their poor stability and rapid elimination by reticuloendothelial system limit the widespread applicability of liposomal formulations [4].

Niosomes are first described by Handjani-Vila et al. in 1979 [9]. They are nonionic surfactant-based vesicles in which the hydrophilic surfactant heads are oriented toward the exterior and the interior of the bilayer while, the hydrophobic tails are enclosed inside the bilayer. Therefore, like liposomes, niosomes have the ability to encapsulate hydrophilic or lipophilic molecules [10]. Niosomes also have cholesterol in their structure which enhances the rigidity of bilayer and reduces premature drug release [11]. Niosomes are superior carriers to liposomes in terms of production cost, chemical and physical stability, and loading capacity [12].

SLNs and NLCs are the most widely described solid-core lipid-based nanocarriers in the scientific literature [3]. SLNs were first described in 1991 to replace the liquid oil of O/W emulsions by a single solid lipid or mixture of solid lipids [2]. SLNs are composed of either solid lipid or mixture of lipids, that do not melt at room or physiological temperature, in an aqueous dispersion stabilized with the help of nonionic surfactants [3].SLNs offer the advantage of avoiding the use of organic solvents during preparation, effective delivery of both hydrophilic and lipophilic drugs, feasibility of surface functionalization with specific moieties to enhance their targeting potential, possibility of extended or controlled drug release, long shelf-life, biocompatibility, lower acute or chronic toxicity and effective large-scale production [2, 13, 14]. On the other hand, efficient drug delivery by SLNs is challenged by low drug loading capacity due to lipid crystalline nature, expulsion of loaded drug due to perfect crystalline lattice formation of the lipid and erratic gelation tendency that results in particle aggregation during storage [4, 14].

NLCs were developed to overcome the problem of drug expulsion during phase transition or crystallization of lipids comprising SLNs [15]. They also exist as a solid lipid matrix at temperature up to about 40°C. However, they are composed of solid lipid mixed with an oil which in turn reduces the lipid crystallization capacity and enhances the drug loading efficiency [16].

Nanoemulsions are kinetically stable heterogeneous systems composed of ultrafine oil droplets dispersed in aqueous media and stabilized by the aid of surfactants

and cosurfactants. Nanoemulsions have gained increasing attention as promising drug delivery systems due to their multiple advantages including high surface area for drug absorption, biocompatibility, increasing drug solubility and improving mucosal permeability. Further, many FDA approved nanoemulsion-based products of water insoluble drugs are now available for clinical use including Restasis®, Estrasorb® and Flexogan®. Microemulsions also offer favorable characteristics such as thermodynamic stability, ease of production being formed spontaneously without the need for high energy input and high penetration due to the large surface area of internal phase [17].

Lipid-drug conjugates (LDCs) are lipid nanoparticle formulations which are characterized by the conjugation ability of the lipid matrix with the hydrophilic drug moieties, and thus provide novel pro-drugs to achieve many therapeutic outcomes in oral drug delivery [18]. Like other lipid-based nanocarrier systems, LDCs possess several advantages including biocompatibility, being solid at body and room temperature, high capacity for loading hydrophilic drugs, high permeation through GI tract, enhanced drug absorption through lymphatic uptake, improving stability and bioavailability loaded drugs, and feasibility of large scale production [19].

3. Self-emulsifying drug delivery systems

Self-emulsifying drug delivery systems (SEDDS) are lipid-based formulations that encompass isotropic mixtures of natural or synthetic oils, solid or liquid surfactants and co-surfactants [20]. When they are exposed to aqueous media (e.g., gastrointestinal fluids), they undergo self-emulsification to form O/W nanoemulsions or microemulsions with a mean droplet size between 20 and 200 nm [21]. Consequently, SEDDS are usually referred to as self-nanoemulsifying drug delivery systems (SNEDDS) or self-microemulsifying drug delivery systems (SMEDDS) depending on the nature of the resulting dispersions formed following their dilution [20].

SEDDS have been reported to enhance the oral bioavailability of poorly water-soluble drugs particularly those belonging to class II of the Biopharmaceutics Classification System by multiple underlying mechanisms [22]. Among these mechanisms, the enhanced drug solubilization was the most widely investigated. Lipidic components of SEDDS stimulate lipoprotein/chylomicron production thus promoting drug absorption [23]. The ultrafine droplet size range of the resulting emulsion provides a large surface area of interaction with gastrointestinal (GI) membranes [24]. Importantly, the bioactive effects of various ingredients employed in SEDDS formulation have significantly contributed to the enhanced oral bioavailability of the loaded drugs. These bioactive effects include tight junction opening and increasing membrane fluidity by the high surfactant content employed in SEDDS formulation [25]. Furthermore, stimulation of the intestinal lymphatic pathway as well as inhibition of intestinal drug efflux pumps such as P-glycoprotein (P-gp) and intestinal cytochrome P450 3A4 (CYP3A4) are considered promising strategies for enhancing the oral delivery of P-gp substrates and bypassing intestinal and hepatic first pass metabolism [26].

P-gp is an energy-dependent membrane bound protein and the most abundantly distributed ATP-binding cassette transmembrane transporter throughout the body [27]. P-gp prevents the accumulation of endogenous substances and xenobiotics in cells by transporting them back to the extracellular space [28]. Unfortunately, intestinal P-gp transporters hamper the intestinal uptake of substrate drugs thus, reducing their oral bioavailability. Additionally, overexpression of P-gp transporters is involved in the development of multidrug resistance (MDR) in numerous human tumor types [29]. Hence, many strategies have been developed to inhibit P-gp

activity for enhancing the oral bioavailability of P-gp substrate drugs and reversing MDR in tumor cells. Among these strategies, nanocarriers have been widely investigated [26]. Nanocarriers have the advantage of protecting P-gp substrates against premature release and interaction with the biological environment [30]. They control drug tissue distribution and favorably accumulate in tumor tissue [31, 32]. Among various nanocarriers, SEDDS have been widely explored to enhance the oral bioavailability of P-gp substrate drugs and reverse MDR in tumor cells.

Interestingly, the unique combination of SEDDS properties allows the enhancement of oral bioavailability of both hydrophobic and hydrophilic drugs [33]. The oral delivery of protein therapeutics and genetic materials represents a real challenge due to their hydrophilic nature and their large molecular weight. In this chapter, we discuss the recent progress in the application of SEDDS for enhancing the oral bioavailability of P-gp substrates, reversing MDR in tumor cells and oral delivery of protein therapeutics and genetic materials. The aim of the current discussion is to call attention to the unique combination of SEDDS properties that makes them multifunctional delivery systems acting *via* various mechanisms to enhance the oral delivery of target therapeutic agents.

3.1 SEDDS overcome P-gp-mediated efflux and reverse MDR in tumor cells

Over the past 2 decades, SEDDS have been widely investigated to overcome P-gp-mediated efflux of substrate drugs to enhance their oral bioavailability. The potential of SEDDS to inhibit P-gp activity relies mainly on the excipients with established P-gp inhibition activity that are employed in the formulation [26]. Nonionic surfactants are the most widely employed excipients and are considered the mainstays of P-gp inhibition by SEDDS [29]. Cremophor EL, Cremophor RH40, vitamin E TPGS 1000, Labrasol, Transcutol P and Tween 80 are the most frequently employed. P-gp inhibition activity of a given surfactant depends on its HLB value and the structure of its hydrophobic domain [34]. There is no obvious correlation between surfactants' HLB values and P-gp inhibition activity. Structurally, the hydrophobic moieties of the surfactant should be linked to polyoxyethylene hydrophilic side chains to inhibit P-gp activity [34].

The binding affinity of nonionic surfactants to the hydrophobic portion of P-gp molecule is different from that of ionic surfactants [35]. Nonionic surfactants can change the secondary or tertiary structure of P-gp molecule resulting in the loss of its function [36]. Additionally, non-ionic surfactants were reported to modulate P-gp activity by inhibiting P-gp ATPase activity and either membrane fluidization or rigidization [37, 38]. At concentrations below the critical micelle concentration, nonionic surfactants are most effective in reducing P-gp activity; however, surfactant micelles showed some P-gp modulation activity [26].

SEDDS have superior formulation efficiency and *in vivo* performance compared to their individual components [39]. Various formulation aspects of SEDDS can potentiate the P-gp inhibition activity of their ingredients. The entrapment of P-gp substrate within the ultrafine emulsion droplets provides a protection against recognition by P-gp efflux pumps at GI epithelium [33]. In addition, SEDDS allow the co-administration of several excipients which are co-localized in close proximity to GI epithelium [22]. Further, pharmaceutical excipients with established P-gp inhibition activity (e.g., curcumin) or traditional P-gp inhibitors (e.g., elacridar) could be loaded into the SEDDS formulation to further augment their P-gp inhibition activity [40, 41].

On the other hand, the efflux of chemotherapeutic agents by P-gp transporters, which are overexpressed in tumor cells, represents a major obstacle in cancer chemotherapy [42]. SEDDS are extensively investigated to overcome MDR in tumor

cells which is partly attributed to the overexpression of P-gp efflux transporters. SEDDS allow the combinational delivery of multiple chemotherapeutic agents acting *via* independent pathways in the same vector to produce a synergistic anticancer activity [42]. Interestingly, SEDDS could be employed for the co-delivery of various antioxidants for overcoming the oxidative stress in cancer cells [43].

3.2 SEDDS enhance the oral delivery of protein and peptide therapeutics

Protein therapeutics have a significant role in almost every field of medicine. However, the extensive application of protein therapeutics is challenged by their route of administration, being administered by parenteral route which is associated with reduced patient compliance [44]. Consequently, there is a great interest in the development of noninvasive strategies for delivery of protein therapeutics [45]. Oral delivery systems have been extensively investigated for the administration of protein drugs [46]. Unfortunately, the oral delivery of protein and/or peptide therapeutics is challenged by several barriers including the acidic environment

Protein	SEDDS composition	Bioavailability increase	Control	Animal species	Ref.
β-lactamase	Lauroglycol FCC (41.7%) Cremophor EL (33.3%) Transcutol HP (25%)	1.29-fold	β-lactamase solution	Sprague–Dawley rats	[54]
Insulin	Miglyol 840 (65%) Cremophor EL (25%) Co-solvent (DMSO and glycerol, 1:3) (10%)	3.33-fold	Insulin solution	Sprague–Dawley rats	[55]
Insulin	Ethyl oleate (35%) Cremophor El (32.5%) Alcohol (32.5%)	6.5-fold	Insulin solution	Male Wistar rats	[56]
Leuprorelin	Capmul MCM (30%) Cremophor EL (30%) Propylene glycol (10%) Captex 355 (30%)	17.2-fold	Leuprolide acetate solution	Sprague-Dawley rats	[57]
Pidotimod[†]	**Oil phase:** SoyPC (9.6%) Span 80 (21.1%) Oleic acid (36.1%) MCT (12%) 0.5% gelatin solution (3%) H_2O (12%) **Surfactant phase:** Tween 80 (6%)	2.56-fold	Pidotimod solution	Sprague–Dawley rats	[48]
Enoxaparin	Captex 8000 (30%) Capmul MCM (30%) Cremophor El (30%) Propylene glycol (10%)	2.25%▪	Enoxaparin IV solution	Sprague-Dawley rats	[58]
	Labrafil 1944 (35%) Capmul PG 8 (25%) Cremophor EL (30%) Propylene glycol (10%)	2.02%▪			

Abbreviations: DMSO, dimethyl sulfoxide; MCT, medium chain triglycerides.
▪*Absolute bioavailability.*
[†]*Self -double emulsifying drug delivery system.*

Table 1.
SEDDS-mediated enhancement in the oral bioavailability of various proteins. Reprinted with permission from Ref. [33] © Elsevier (2018).

in the stomach, degradation by GI enzymes, mucus barrier as well as low cellular penetration [47]. Several strategies have been developed to overcome these barriers [48–50]. As shown in **Table 1**, SEDDS have been extensively investigated as promising carriers for oral delivery of protein and peptide therapeutics. Various surfactants and oils that are employed in SEDDS formulation have a permeation enhancing effects; thus, they increase the cellular uptake of hydrophilic macromolecules such as protein therapeutics. The ultrafine droplet size provides a large surface area for rapid intestinal permeability. The anhydrous nature of SEDDS protects proteins against aqueous hydrolysis. Other bioactive effects of SEDDS such as tight junction opening, and enhanced lymphatic uptake also contribute to the enhanced oral bioavailability of loaded protein therapeutics [49]. However, loading of protein therapeutics into SEDDS is challenged by their hydrophilic nature. Thus, the lipid solubility of protein therapeutic should be increased before their incorporation into the SEDDS preconcentrate. This could be achieved by various techniques including, hydrophobic ion pairing [51], double emulsification [48], using hydrophilic solvents or co-solvents [52] and chemical modification of the peptide molecule [53].

Figure 1 summarizes various hypotheses for the enhanced oral delivery of protein and peptide therapeutics by SEDDS. Protein therapeutics incorporated within the ultrafine oil droplets are effectively protected against degradation by GI enzymes. Further, these cargoes are absorbed when the nanosized oil droplets are absorbed. Thus, the protection against enzymatic degradation is achieved *via* controlling the release rate of loaded protein therapeutic [59]. Burst release could result in rapid degradation of protein molecules within the GI lumen before reaching the absorption site [60]. Another suggested mechanism for the enhanced oral bioavailability by protein therapeutics incorporated in SEDDS is based on the bioactive effects of SEDDS ingredients. They include mucus penetration, enhanced paracellular transport *via* opening of tight junction, and enhanced cellular uptake by transcytosis-mediated transcellular transport [61, 62]. Finally, enhancing the lipid solubility of protein molecules *via* hydrophobic ion pairing could increase their intestinal uptake and bioavailability. However, this hypothesis is challenged by the rapid dissociation of hydrophobic ion paired complexes within the GI fluids.

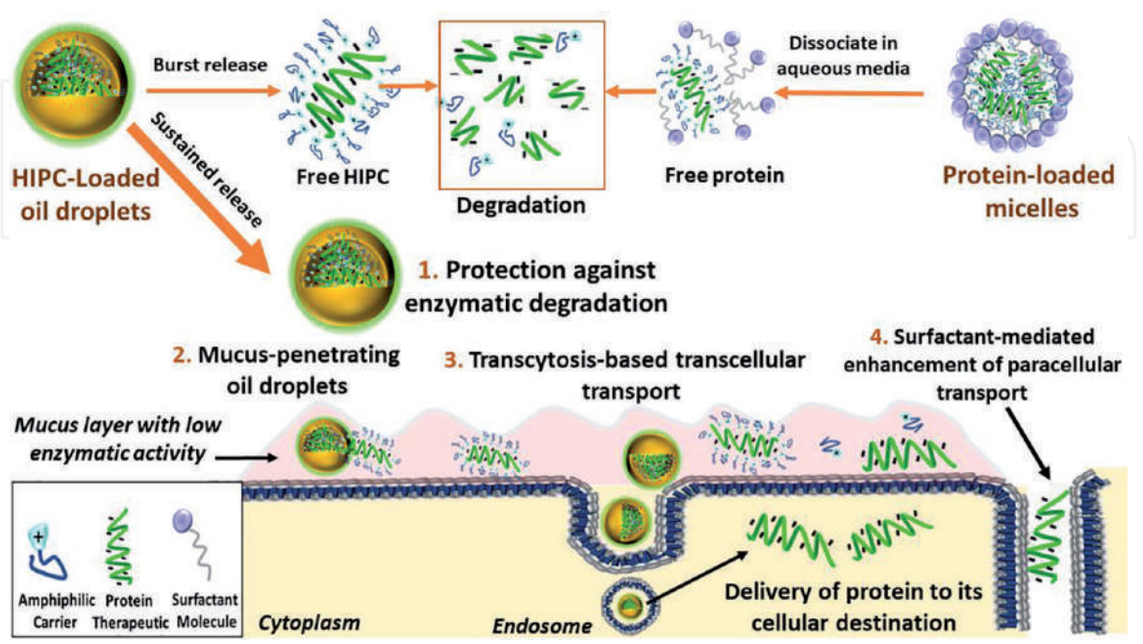

Figure 1.
A schematic representation of some underlying mechanisms for the enhanced oral bioavailability of protein therapeutics by SEDDS (HIPC, hydrophobic ion paired complex). Reprinted with permission from Ref. [33] © Elsevier (2018).

3.3 SEDDS as promising vectors for oral delivery of genetic materials

Oral gene therapy allows the sustained production of therapeutic proteins locally at the disease site as well as for systemic absorption [63]. Unfortunately, the oral delivery of plasmid DNA (pDNA) as well as other nucleic acid products is challenged by their safe and efficient delivery as well as cellular internalization and processing [64]. SEDDS have been investigated as promising non-viral vectors for oral delivery of genetic materials. The superior cellular permeation and stability of pDNA loaded into SEDDS could be mainly attributed to its entrapment within the ultrafine nanoemulsion oil droplets.

3.4 Characterization of SEDDS

3.4.1 Stability of SEDDS preconcentrates

SEDDS preconcentrates should have sufficient stability to avoid drug precipitation as well as creaming or phase separation of the resulting nano- or microemulsions. If some components of SEDDS preconcentrate undergo physical or chemical instability, the resulting emulsion may become unstable [20]. Thus, the stability of SEDDS preconcentrate should be evaluated by subjecting the nano- or microemulsion, resulting from aqueous dilution of the preconcentrate, to a centrifugation study at 5000 rpm for 30 min [65]. Then, SEDDS preconcentrates are subjected to heating–cooling cycle which includes six cycles of storage at 4 and 40°C for 48 h at each temperature followed by freeze–thaw cycle which involves three cycles of storage at −21 and 25°C for 48 h at each temperature [66].

3.4.2 Robustness to dilution

Robustness of the resulting emulsion to dilution guarantees the absence of drug precipitation when SEDDS preconcentrates are subjected to high dilution folds *in vivo* [21]. Thus, SEDDS preconcentrates should be exposed to different dilution folds (e.g., 50-, 100-, and 1000-folds) with different media (e.g., 0.1 N HCl and phosphate buffer, pH 6.8) to mimic *in vivo* conditions [20].

3.4.3 Assessment of self-emulsification efficiency

Self-emulsification efficiency is assessed by determining self-emulsification time and the efficiency of preconcentrate dispersibility when it is exposed to aqueous dilution. The SEDDS preconcentrate is added drop wise to aqueous media with different pH values and composition in a standard USP dissolution apparatus. Self-emulsification time is determined visually as the time required for the preconcentrate to form a homogenous dispersion [21]. The efficiency of preconcentrate dispersibility is also determined visually and is given in grades according to previously reported grading systems [20, 67, 68]. The selection of the appropriate grading system depends on the dilution fold to which the preconcentrate is exposed. This test ensures the ability of SEDDS preconcentrates to disperse quickly in order to form fine emulsions when they are exposed to aqueous media under mild agitation provided by the GI peristaltic movement.

3.4.4 Cloud point measurement

Cloud point could be measured after 100-fold dilution of the preconcentrate with distilled water which is then placed in a water bath with gradual increase

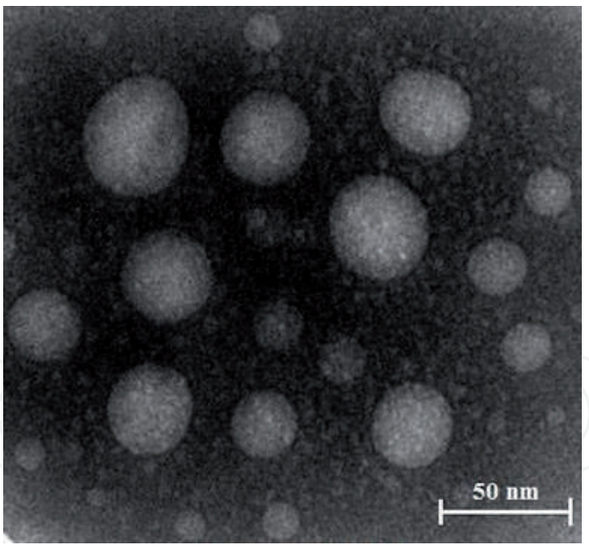

Figure 2.
Transmission electron microscope photograph shows spherical nanoemulsion droplets without any signs of aggregation. Reprinted with permission from Ref. [20]. © Elsevier (2017).

in temperature. Cloud point is determined as the temperature above which the emulsion clarity turns into cloudiness which is attributed to the dehydration of polyethylene oxide moiety of non-ionic surfactants [69]. Cloud point values should be sufficiently higher than 37°C (i.e., normal body temperature) to avoid phase separation in the GI tract [70].

3.4.5 Determination of zeta potential, mean droplet size and polydispersity index

Mean droplet size affects the *in vivo* performance of SEDDS. Small mean droplet size provides large interfacial area for drug absorption and ensures the kinetic stability of the resulting emulsion. Small value of polydispersity index suggests good uniformity of droplet size distribution. High zeta potential values confirm the electrical stability of emulsion droplets and absence of aggregation [66].

3.4.6 Droplet morphology

The morphology of emulsion droplets could be determined by transmission electron microscopy after appropriate dilution of SEDDS preconcentrate (about 1000-fold) using 2% solution of either phosphotungstic acid or uranyl acetate for negative staining. Droplets should possess a spherical shape without any signs of aggregation or drug precipitation as shown in **Figure 2**.

3.4.7 In vitro lipolysis

Drugs incorporated into lipid-based formulations are already present in a dissolved form. Thus, the assessment of the applicability of these formulations should be more properly based on the rate of drug precipitation over time. On the other hand, the drug solubilization capacity of lipid-based formulations is not a function of formulation characteristics alone. Rather, formulation dispersion and digestion result in the formation of colloidal species that account for the intestinal solubilization capacity [71]. Consequently, possible changes to solubilization capacity that could be attributed to digestion of formulation ingredients or interaction with biliary solubilizing agents should be assessed. *In vitro* lipolysis models simulate the GI environment and better predict the *in vivo* behavior of lipid-based formulations

such as SEDDS. They also assess the extent of drug precipitation as a result of digestion of formulation ingredients and changes to solubilization capacity [72].

4. Solid self-emulsifying drug delivery systems

It was reported that ~70% of newly discovered drug molecules and ~ 40% of marketed drugs for oral administration are classified as practically insoluble in water. Therefore, various strategies have been explored to enhance the aqueous solubility and thus oral bioavailability of these drugs. SEDDS have been investigated as an efficient strategy that allows drug administration in pre-solubilized form ready for absorption. Consequently, drugs loaded into SEDDS preconcentrates avoid the dissolution step that frequently limits their absorption. However, the widespread application of liquid SEDDS is challenged by low stability during handling or storage [73] and irreversible drug and/or excipient precipitation [74]. Thus, the majority of marketed liquid SEDDS are filled into soft gelatin (e.g., SandimunneNeoral®, Norvir®, Fortovase®, and Convulex®) or hard gelatin capsules (e.g., Gengraf® and Lipirex®) to be administered as a unit dosage form [75]. However, this approach still possesses the possibility of drug precipitation upon exposure to aqueous media. Additionally, capsule technologies have some limitations such as high production cost and the risk of interaction between the active pharmaceutical ingredient and excipients with the capsule shell. Also, the possibility of drug leakage out of the capsule shell and capsule aging represent further obstacles [76]. Further, the storage temperature is an important consideration since the drug and/or excipients could undergo precipitation at lower temperatures [75]. The tendency of volatile excipients to evaporate into the capsule shell results in drug precipitation and a consequent alteration of drug release [77].

Thus, to address these limitations solid self-emulsifying drug delivery systems (s-SEDDS) were developed by converting the conventional liquid SEDDS into powders which are subsequently filled into capsules or formulated as solid dosage forms such as self-emulsifying tablets, granules, pellets, beads, microspheres, nanoparticles, suppositories and implants [74, 78]. Various solidification techniques for converting liquid SEDDS into s-SEDDS are discussed below.

4.1 Solidification techniques for converting liquid or semisolid SEDDS to s-SEDDS

4.1.1 Adsorption to solid carriers

Adsorption to highly porous and/or high specific area solid carriers is the most intensively explored approach to obtain s-SEDDS [75]. This technique could be effectively used to produce s-SEDDS by simple mixing of solid carriers with the liquid formulation in a blender [74]. The most frequently employed carriers for adsorption of liquid SEDDS formulations are: (i) silicon dioxide such as Aerosil® (fumed silica) and Sylysia® (micronized amorphous silica); (ii) Neusilin® (magnesium aluminometasilicate) which is available in different surface properties and particle size; (iii) Fujicalin® (porous dibasic calcium phosphate anhydrous) and (iv) calcium silicate [75].

Advantages of this solidification technique include:(i) good content uniformity of the produced powders [79]; (ii) high drug loading efficiency (up to 80% *w/w* without affecting flow properties) [80]; (iii) absence of organic solvents [81]; (iv) cost effectiveness because small number of excipients and basic equipment are required for the final formulation and (v) production of free-flowing powders that can be filled into capsule or compressed into other solid dosage form [82].

During the formulation of s-SEDDS by adsorption technique, careful consideration should be given to the possible interactions between the solid carrier and the drug or other excipients in liquid SEDDS which could result in delayed or incomplete release of loaded drug [83]. Additionally, the particle size, specific surface area, tortuosity of pores as well as type and liquid SEDDS: carrier ratio should be considered [75].

4.1.2 Spray drying

Spray drying is also a promising technique for transforming liquid SEDDS to s-SEDDS using different carriers (i.e., hydrophobic or hydrophilic carriers) which preserve the self-emulsifying properties of the formulation. It is a simple and economical technique which involves mixing of lipids, surfactants, drug and solid carriers followed by solubilization and spray drying. The solubilized mixture is atomized into a spray of fine droplets that are introduced into a drying chamber where the volatile phase evaporates forming dry particles under controlled conditions of temperature and airflow [74]. The type of carrier can affect the rate of release and thus the oral bioavailability of loaded drug by affecting the droplet size of the nano or microemulsion formed after reconstitution [84]. Also, careful consideration should be given to the atomizer, the airflow pattern, the temperature and the design of the drying chamber which should be selected according to the powder specifications. Low yield is a disadvantage of solidification by spray drying technique which could be attributed to the removal of non-encapsulated drug with the exhausted air [85].

4.1.3 Extrusion/spheronization

Extrusion/spheronization is the most explored technique for the production of uniformly sized self-emulsifying pellets [75]. Extrusion is a procedure of converting a raw material with plastic properties into a spaghetti-shaped agglomerate having uniform density. Extrusion is followed by spheronization where the extrudate is broken into spherical pellets (spheroids) of uniform size [86]. The produced pellets have good flowability and low friability. Before pellet production, the wet mass is composed of liquid SEDDS, lactose, microcrystalline cellulose (MCC) and water. A disintegrating agent could be added to enhance drug release [87]. MCC acts as adsorbent for the liquid SEDDS to ease pellet formation and avoid problems such as poor flow properties, pellet agglomeration and low hardness. Larger amount of liquid SEDDS can be loaded into the pellets when a greater quantity of MCC on the account of lower amount of lactose is employed in the formulation. The ratio of lactose: MCC and liquid SEDDS: water affects the pellets' disintegration time and surface roughness as well as the extrusion force [88].

4.1.4 Microencapsulation

Co-extrusion technique is a promising strategy for microencapsulation of liquid SEDDS into polymeric matrices. This technique employs a vibrating nozzle device equipped with a concentric nozzle. The formed microcapsules are then hardened by ionotropic gelation. Ionotropic gelation is based on the gel formation ability of polysaccharides (e.g., pectin, alginate, carrageenan, and gellan) in the presence of multivalent ions (e.g., Ca^{+2}) [89]. Alginate and pectin are the most intensively investigated natural ionic polysaccharides for formation of microcapsule shell. However, Ca-alginate microcapsules clog the nozzle during the microencapsulation process. On the other hand, pectin microcapsules lack sufficient hardness. Thus, microcapsules composed of an alginate-pectin matrix could be more acceptable than those composed solely of one polymer. Various hydrophilic filling agents

(e.g., lactose) could be added to the shell formation phase in order to prevent core leakage and microcapsule collapse during the drying process. Advantages of microcapsules include predictable GI transit time and large surface area that allow faster drug dissolution. Additionally, they are composed of biocompatible, non-toxic and biodegradable natural polymers [90].

4.1.5 Wet granulation

Different carriers (e.g., Aerosil® 200) were employed to prepare the self-emulsifying granules where the liquid SEDDS acts as a binder. However, granulation with SEDDS produces a broader size distribution and difficult to control aggregation compared with granulation procedure where water is employed as granulating agent [91].

4.1.6 Melt granulation

In this process, powder agglomeration is attained by the addition of binding agent which melts at relatively low temperature such as Gelucire®, lecithin, partial glycerides or polysorbates [92]. While the liquid SEDDS is adsorbed to neutral carriers such as silica and magnesium aluminometasilicate [93]. Melt granulation is advantageous compared to wet granulation since it is a 'one-step' process in which the addition of granulating liquid and the following drying phase are absent [74].

4.2 Characterization of s-SEDDS

SEDDS are combinations of SEDDS and solid dosage forms. Therefore, the characterization of s-SEDDS is the sum of the corresponding evaluation criteria of both SEDDS and solid dosage forms.

4.2.1 Solid state characterization

4.2.1.1 Differential scanning calorimetry (DSC)

DSC is mainly employed to ensure drug incorporation into the s-SEDDS as well as the absence of drug-solid carrier interaction. It is also used to investigate the physical state (i.e., crystalline or amorphous) of the incorporated drug in the final formulation [94]. Transition from the crystalline to amorphous state is common in SEDDS formulations which lowers the drug melting point and improves its solubility and dissolution rate [95].

4.2.1.2 X-ray diffractometry (XRD)

XRD is employed to investigate the physical state of the incorporated drug because it affects both *in vitro* and *in vivo* performance.

4.2.1.3 Scanning electron microscopy (SEM)

SEM is employed to elucidate the structural and morphological features of s-SEDDS and the raw materials as well as to confirm the physical state of loaded drug [96].

4.2.1.4 Fourier-transform infrared spectroscopy (FT-IR)

FT-IR is usually employed to investigate any potential interaction between the incorporated drug and the solid carrier or other formulation excipients [76].

4.2.2 Determination of micromeritic properties

The flow properties of powders are crucial aspect of large-scale production of solid dosage forms because it affects feeding consistency, reproducibility of die filling and dose uniformity. Powder flowability is affected by various physical, mechanical and environmental factors. Thus, various parameters such as angle of repose, bulk density, Carr's index and Hausner's ratio should be assessed to determine s-SEDDS flowability to overcome the subjective nature of individual tests. The angle of repose is a measure of internal cohesiveness of particles. Powders having angles of repose <30° are considered as free flowing powders; while, powders with angles of repose >40° are regarded to have extremely poor flowability. On the other hand, powders with angles of repose up to 35° are regarded passable; while, those between 35 and 40° indicate poor powder flow which requires the addition of a glidant [97]. Powders having Carr's index up to 21% are considered to have acceptable flow. Hausner's ratios <1.25 are usually corresponded to free-flowing powders with minimum interparticle frictions. On the other hand, Hausner's ratios between 1.25 and 1.5 indicate moderate flow which could be acceptable [98].

4.2.3 Droplet size of reconstituted s-SEDDS

The droplet size of reconstituted s-SEDDS should be similar to that of liquid SEDDS to ensure that the self-emulsification performance of liquid SEDDS is preserved.

5. Conclusion

SEDDS are promising nanocarriers for overcoming various obstacles encountered in the oral delivery of drugs and bioactive agents. The inhibition of P-gp activity by SEDDS relies mainly on the employment of ingredients (i.e., oils and surfactants) with established P-gp inhibition activity in their formulation. Thus, selection of excipients with established P-gp inhibition activity is the first step in the formulation of SEDDS for overcoming P-gp-mediated efflux of substrate drugs and reversing MDR in tumor cells. The effective concentration range for inhibiting P-gp activity should be considered while selecting the formulation ratios. P-gp inhibition activity of SEDDS can be further enhanced by loading other pharmaceutical excipient with established P-gp inhibition activity or traditional P-gp inhibitor. SEDDS are also considered promising systems for the oral delivery of protein therapeutics and genetic materials; however, this role is still in its infancy. Entrapment of these macromolecules within the nanosized emulsion droplets guarantees effective delivery. The bioactive effects of SEDDS ingredients could further enhance the oral bioavailability of protein therapeutics. Liquid SEDDS could be transformed into s-SEDDS to further enhance the formulation stability, allow cost effective large-scale production as well as to enhance the patient compliance.

Conflict of interest

None.

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