Chapter

Integrated Biologics Manufacturing in Stirred-Suspension Bioreactor: A Stem Cell Perspective

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Abstract

Stem cell therapy is garnering attention as several clinical trials have taken place in the recent years by using human pluripotent stem cells (hPSCs). Hundreds of biotechnological companies are investing to find a permanent cure for difficult-to-treat diseases like age-related macular degeneration, Parkinson's disease, diabetes, etc. by using hPSCs. Therefore, clinical-grade cell manufacturing has become an important issue to make cell therapy products safe and effective. Current manufacturing practices are adopted from conventional antibody or protein production in the pharmaceutical industry where cells are used as a vector for producing the desired products. In cell therapy applications, cells are the products that are sensitive to physicochemical parameters and storage conditions anywhere between isolation to patient administration. Moreover, cell-based product manufacturing consists of multi-step processing, including isolation from patients, genetic modification, derivation, expansion, differentiation, purification, characterization, cryopreservation, etc. This can require long processing times and pose high risk of product contamination as well as high production cost. Herein, we discuss the current methods of biologics manufacturing and its limitations. We also review current practices for integrating and automating cell manufacturing facilities. Finally, we propose how to integrate multi-step cell processing in a single bioreactor to make the cell manufacturing practices more direct.

Keywords: biologics, stem cell therapy, genetic modification, integrated manufacturing, bioreactor

1. Introduction

Based on their self-renewal and differentiation capabilities, human pluripotent stem cells (hPSCs) including embryonic stem cells (ESCs) [1] and induced pluripotent stem cells (iPSCs) [2] are attractive tools in the field of regenerative medicine. After the discovery of hiPSCs in 2007, this field expanded vigorously and hundreds of biotechnological companies were established to use these cells for treating degenerative diseases. The most common degenerative diseases treated by the hESCs are age-related macular degeneration (AMD), type I diabetes mellitus, heart failure, Parkinson's disease, and spinal cord injury [3]. Although hiPSCs are a better source

for autologous cell therapy applications, they are less preferable for clinical trials because of less genetic stability compared to the hESCs. However, a few clinical trials have already been started using the patient-derived hiPSCs. The Takahashi group from the Riken Center for Developmental Biology has recently conducted a clinical trial for treating wet AMD [4]. Similarly, a Takahashi from Kyoto University is conducting a clinical trial for treating Parkinson's disease by using hiPSCs [5]. A few clinical trials are also ongoing in the USA for treating different diseases like β-thalassemia, liver diseases, diabetes, etc. using hiPSCs and their use is expanding worldwide day by day [6].

As stem cell therapy is garnering increasing attention, a lot of clinical trials are ongoing using both hESCs and hiPSCs cells. About 6849 clinical trials and 1415 stem cell-based therapies were found based upon searches we recently performed on clinicaltrials.gov (October, 2018) [7]. However, the percentage of success is not high enough as speculated from the previous clinical trials. Among the 315 clinical trials conducted (26.0% Phase 1, 40.6% Phase 1/2, 22.5% Phase 2, 3.8% Phase 2/3, and 6.7% Phase 3), only 0.3% went to Phase 4 [3]. The low percentage of completion of clinical trials depends on various factors. One of the major factors is manufacturing practices that can provide high safety and efficacy of cell therapy products. Moreover, production cost of multiple doses also hinders the success rate of clinical trials. As cell therapy revenue exceeded multi-million dollars and has been a profitable business in recent years, but much attention is needed to produce high quality cells for treating incurable diseases [8, 9].

The production of stem cell biologics is adapted from the conventional pharmaceutical protein and vaccine production. Conventional biologics production involves the following basic steps: isolation and identification of raw materials, formulation, filling, packaging, and storage, where the total processing stops at the storage of final products.

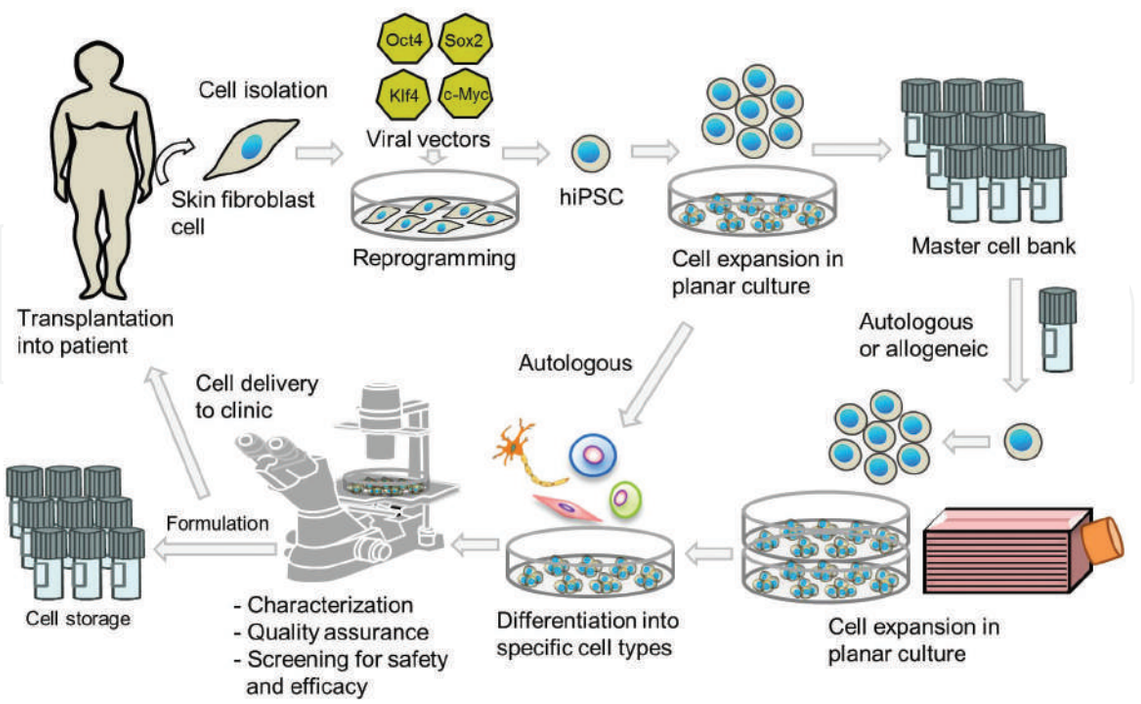

Figure 1.
Schematic illustration of current multi-step cell manufacturing strategies in planar culture for stem cell therapy applications. Skin cells are isolated from the patient and reprogrammed to hiPSCs using viral vectors. After reprogramming, hiPSCs are stored in a master cell bank or differentiated directly in autologous cell therapy applications. In some cases like allogeneic cell therapy applications, cells are expanded in a large amount and then differentiated. After performing characterization, quality assurance, and screening for safety and efficacy, cells are delivered to hospital or stored in a cell bank for future use.

There is a big difference between the production of conventional biologics and cell-based therapy products. For vaccines or pharmaceutical protein production, cells are used as a platform for obtaining desired proteins. After that, cells are discarded. However, in cell-based therapies, cells, which are sensitive to the physical or chemical attributes of the residing environment, are the final products. Therefore, much consideration is needed before translating cell-based products from bench to clinic. This extends to the acquisition of tissue samples and isolation of cells, initial cell purification, selection, activation and transduction, cell expansion in plate or bioreactor culture, differentiation, washing, harvesting and formulation, filling and cryopreservation, and finally, storage and delivery to the clinics (**Figure 1**) [10].

Cumbersome multi-step manufacturing systems can cause batch to batch variability, inefficacy, and low quality of cells for transplantation and need to be simplified and made more direct. In this context, we will discuss current limitations of cell manufacturing strategies and propose how to overcome these by integrating the total process in a single bioreactor to make cell manufacturing straight forward enough to deliver high quality cell therapy products to the clinic. In this review, we will also discuss how to integrate genetic modification—transfection or transduction, reprogramming, differentiation, purification, and formulation of final products in a single bioreactor.

2. Current manufacturing strategies for stem cell therapy

Current manufacturing strategies for cell therapy products are replicated from biologics manufacturing in the pharmaceutical industry. However, the processing of cells is far different from pharmaceutical proteins or vaccines. For pharmaceutical peptide production from microorganisms, the raw materials are extracted from bacteria or fungus [11, 12]. They are then separated, purified, and examined for quality assurance to meet the requirements of regulatory agencies, e.g., Food and Drug Administration (FDA), British Pharmacopeia, etc. The final products are stored or marketed in a dose-dependent manner.

Cell processing is more intensified when the pharmaceutical proteins are produced by using human, animal, or plant cells as a by-product. In this case, high quality products depend on the maintenance of high quality cells, and maintaining a sterile condition is very important. Therefore, good bioprocessing is required to optimize the production of desired proteins. After inoculating from a master cell bank, the cells are cultured for a specific period of time [13, 14]. The supernatant is then collected and the desired proteins are separated, purified, and concentrated. The isolated products then go through quality assurance to meet the criteria of the regulatory agency. Finally, the products are stored and marketed in a dose-dependent manner.

The manufacturing of stem-cell based products is not as straight forward as the production of pharmaceutical proteins or vaccines. This is because cells are the final product in stem cell therapy and are vulnerable to physical or chemical operations from isolation to delivery to patients. Cell manufacturing strategies also vary from source to source and depend on autologous or allogeneic transplantation (**Figure 1**). The major general steps are the acquisition of tissue samples and isolation of cells, initial cell purification, selection, activation and transduction, cell expansion, differentiation, washing, harvesting and formulation, filling and cryopreservation, and finally, storage and delivery to the clinics [10].

For stem-cell based products, cells are isolated from specific tissues of patients, e.g., blood, skin, etc. for autologous transplantation or can be used from cell banks for allogeneic transplantation. Heterogeneity of final products may arise from the cell isolation step because patients' tissues contain various undesired

subpopulations. For example, in chimeric antigen receptor T-cell (CAR-T) therapy, cells are isolated from patients' blood tissue, which contains abnormal levels of inhibitory factors and regulatory cells [15, 16] because patients are treated with chemo- and radiotherapies. As a result, heterogeneity occurs in the final products, which need much attention during the cell isolation step. Cells isolated from patients need to be purified by centrifugation, magnetic-activated cell sorting (MACS), or fluorescent-activated cell sorting (FACS). Then, initial cell culture is done for selection, activation, or transduction of specific interest.

After purification, cells are expanded in plate culture or bioreactor. Based on demand, large-scale expansion is required in a sterile condition, which also requires intensive consideration because it is the rate-limiting step for commercialization of cell therapy products. The most important considerations for large-scale expansions are: operational, economic, quality and safety.

Operational design for culture systems (2D or 3D) with manual or automatic (desirable) operation is important before large-scale expansion [17]. Bioreactors are superior to plate culture for obtaining a large number of cells. Online monitoring and control of process parameters (pH, DO, pCO_2, etc.) and considering the shortest possible culture time are also important parameters for operational consideration. A prediction model for medium consumption (glucose and glutamine) and toxic material production (lactic acid and ammonium) is very useful for determining medium feeding regimen. A dedicated single-use vessel is also a big operational consideration before large-scale expansion of cell-based products.

As cell-based products are costlier, economic considerations for medium, efficient cell lines and other indirect utilities are important. However, the most important consideration in large-scale expansion is product quality and safety. For this purpose, dedicated cell manufacturing facilities are required to maintain current manufacturing practices (cGMP) for high product purity and safety.

After large-scale expansion, cells are harvested by detaching them from the culture substrate using enzymatic treatment. Non-enzymatic detachment is also available by changing temperature or pH [18–20]. Aggregate culture in bioreactors may not necessarily need a detachment step for harvesting [21–25]. Next steps are washing and volume reduction, which can be done by centrifugation or tangential flow filtration on a large scale by using automated commercial devices (kSep systems and Terumo BCT).

Purified cells are formulated in a dose-dependent manner and checked for quality assurance. Quality assurance is done in three different stages: microbial contamination, chemical contamination, and quality or potency assurance. Microbial contamination is checked for bacterial, fungal, or viral contamination by sterility tests with various methods [26, 27]. The most commonly used sterility test is a 14-day incubation of cell products for bacterial and fungal contamination [28, 29]. Chemical testing includes checking for molecules accompanying the culture medium or other factors used during isolation, expansion, and storage. One commonly used chemical test is the LAL test for bacterial endotoxin. There is now an automated 15 min test for determining endotoxin in cell therapy products, which was developed following FDA regulations [30]. Other chemical testing concerns are checking for residual proteins of different origins, serum, and other harmful particles originated from cell processing.

In cell therapy products, quality is the major concern, especially because cell growth is a requirement. For that reason, a cell viability assay is done to determine live or dead cells in the product using a variety of staining methods. Colony forming unit (CFU) is also useful for determining biological activity of cell therapy products [31, 32]. Product potency is an important criterion to meet before releasing the product. For example, if a cell therapy product is applied for the chimeric antigen

receptor T (CART)-related cancer therapy, it needs to be examined for the secretion of cytotoxic cytokines (IFN-γ) and killing of target cells [33]. However, for hPSCs, the final products are differentiated cells, wherein potency should be checked via transplantation into disease models.

For hPSC-derived products, strict quality control is imperative before transplantation to the patients because there is high risk of oncogene transfer to patients. A clinical trial was halted in 2015 in Japan while treating AMD by autologous hiPSC-derived retinal pigmented epithelial cells because of genetic abnormality [34]. Since genetic abnormalities occur in hiPSC-derived products from reprogramming to finally differentiated cells [35], cells should be strictly screened for epigenetic signatures, karyotyping, telomerase activity, mitochondrial remodeling, etc. [36–38]. Rohani et al. summarized possible molecular cytogenetics for quality control that should be checked before releasing the final products [39]. Some of the proposed quality testings are whole-genome sequencing, single-cell genome sequencing, epigenomic analysis, and mitochondrial DNA integrity testing for maximizing the patient safety.

After passing the product quality assurance, cells need to be delivered to clinics immediately or stored for future use. Cells are shipped generally to the clinics on dry ice (−78°C) or in liquid nitrogen dry shippers (−160°C) if the cells are vitrified. The mostly used technique for cell storage is cryopreservation in liquid nitrogen at −196°C which is adapted from the conventional stem cell banking [40, 41]. For cryopreservation, dimethyl sulfoxide (DMSO), glycerol, sugars, or other polymers are used. Among them, clinical grade DMSO is widely used although it is detrimental and can cause harmful effects to cells [42, 43]. Therefore, removing it from cryopreservation protocols or lowering the concentration is important. However, developing appropriate protocols for freezing and thawing is also important for high recovery of cells. Generally, slow-freezing and quick thawing is highly applicable for better recovery of cryopreserved cells [44, 45]. Since intracellular ice crystal formation is a big obstacle in cryopreservation, using ice recrystallization inhibitors is also an effective process for cryopreservation of clinical cell therapy products [46, 47].

Product delivery is also an important step to consider before administration to the patients. Since the products are carried in an environment where temperature is extreme, the container should be made with such materials that can withstand extreme low temperature and do not cause any leakage compromising the product quality [48]. For autologous cell therapy applications especially for CAR-T cell therapy, a dedicated vessel, which can withstand extreme low temperature, is needed [49].

3. Integrated biologics manufacturing in bioreactors

The conventional production of pharmaceutical proteins or other biologics consists of multiple steps from raw materials to finished products. As biologics need to maintain stringent quality control, multiple steps in production facilities compromise the product quality significantly. They also reduce productivity and become prone to human errors, which decrease product efficacy and safety. Moreover, multiple steps in cell processing consume a lot of time, which indirectly increases production cost. To overcome these drawbacks, integrated pharmaceutical production has been attempted by various pharmaceutical companies. One of the significant attempts was made by the Novartis-MIT Center for Continuous Manufacturing of pharmaceutical products to fully integrate the cell processing system [50, 51]. Another attempt was taken by Genzyme™ for continuous production of pharmaceutical recombinant protein in bioreactors, where cell culture to product isolation and purification was integrated in a single flow [52]. By using this system,

they respectively reported successful production of monoclonal antibody as well as highly complex, less stable pharmaceutical protein with consistent product quality, high product output, and low cost. Process integrity is necessary for reducing cumbersome production steps and cutting cost significantly. One such integrated system developed by Johnson & Johnson has recently got FDA approval for large-scale HIV drug production [53] that reduces time and cost by one third compared to the conventional batch processing.

Since biologics production for cell therapies require multiple steps, integration of all of the steps will give high product quality and safety, as well as help overcome stringent regulatory requirements. In this context, we will discuss how to integrate some important basic steps of cell manufacturing especially genetic modification, cellular reprogramming, expansion, and differentiation in bioreactors to promote a single-step approach for cell-based therapies (**Figure 2**).

3.1 Genetic modifications in bioreactor

Genetic modification is one of the biggest steps in producing cell therapy products. In biologics manufacturing, it has been practiced for many years for producing antibodies, proteins, or other biotechnological drugs. It has also been used extensively in the cell therapy industry as various cell-based products have been applied for treating multiple incurable genetic diseases in recent years. Some genetic modifications affect patients directly and some indirectly. For example, in adrenoleukodystrophy (ALD), a neurological disorder occurs due to malfunction of oligodendrocytes and microglia where genetic modification can affect a patient directly. To recover from it, a corrected gene is inserted into the patient-derived hPSCs and transplanted into the

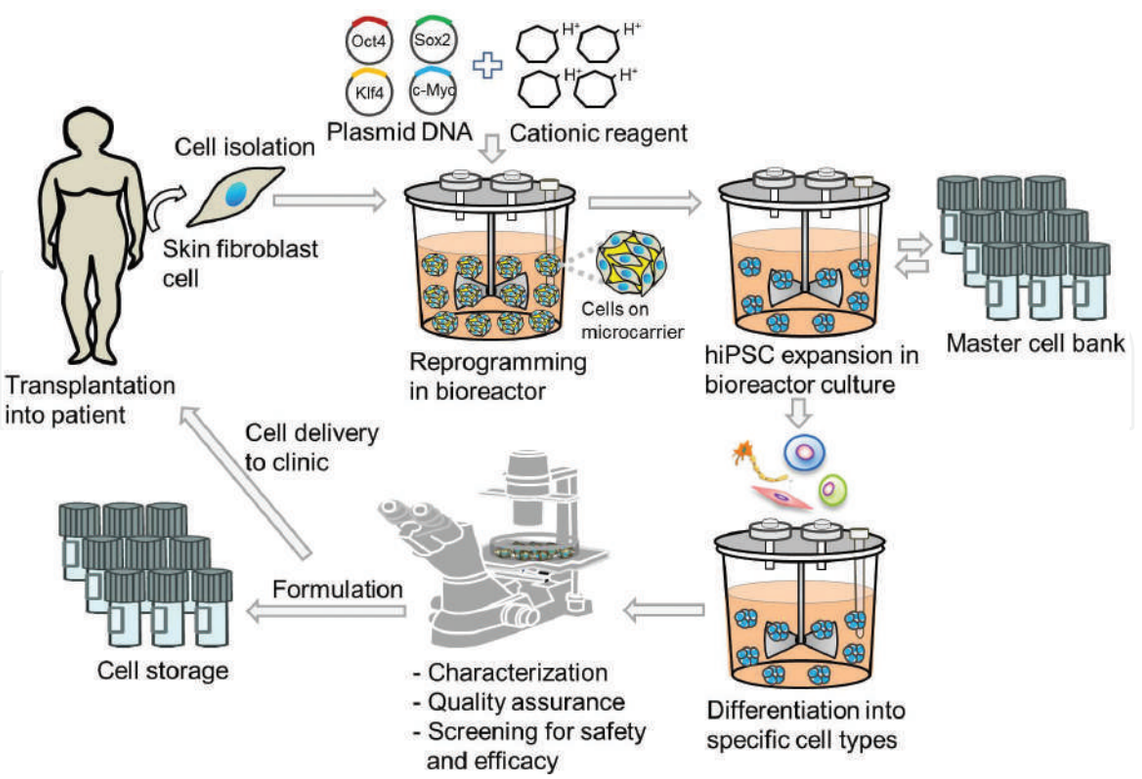

Figure 2.
Schematic illustrations of integrated single-step cell manufacturing strategies in bioreactor culture for stem cell therapy applications. Skin cells are isolated from the patient and reprogrammed to hiPSCs on microcarriers using a nonviral approach. After expansion as aggregates, hiPSCs are stored in a master cell bank or differentiated directly in bioreactor. After performing characterization, quality assurance, and screening for safety and efficacy, cells are delivered to hospital or stored in a cell bank for future use.

patient's brain, which is differentiated into microglia to promote production of myelin in the patient's brain that recovered the ALD [54].

In some gene therapy applications, patients are exposed indirectly to genetic modification. For example, in thalassemia, patient blood cells are extracted from the body and the cells are modified and enriched in *ex vivo* to target the specific antigens of patients' body [55]. Other indirect genetic modifications used for treating CAR or T-cell receptor (TCR) genes to T-cells [56], expression of CD40 ligand in dendritic cells [57], adenosine-deaminase severe-combined immunodeficiency [58], and beta-thalassemia [59], as well as deletion or insertion of desired genes in a specific genomic location. Among them, CAR-T cell therapy has got much attention for treating cancer-related diseases. These genetically modified T-cells can specifically target the antigens and kill the cancer cells efficiently [60]. CARs and TCRs are the mostly used receptors which are engineered to activate the T-cells [61]. Nowadays, a lot of CAR-T cell-based therapies are being established for treating advanced-stage lymphoma [62] and B-cell lymphoma [63] as well as other autoimmune diseases [64].

Viral vectors are commonly used to deliver genetic cargo to cells (**Figure 1**). This involves a two-step process: preparation for viral vectors and transduction for modifying the cells to express desired property. Lentiviral and gamma-retroviral are widely-used for their superior transduction efficiency but their transgenes are integrated with the host genome [65]. Another choice for viral transduction is adenovirus where viral transgenes are not integrated into the host genome but less efficient than lenti- and retro-virus. The major drawbacks in viral vector mediated transduction are concerns for safety of the products [66]. Viral vectors are widely used for reprogramming hiPSCs from skin fibroblasts cells [2].

Other methods for cellular transduction use nonviral approaches, including nucleofection or electroporation, or liposome-mediated delivery of DNA or RNA into cells. Although DNA vectors are easy to scale-up, carry large-size DNA with less immunotoxicity, this process is less efficient than the viral transduction. There are some other methods for skipping the use of viral vectors which are also efficient in doing the transgene expression [67–69]. Hsu et al. reported successful transfection by using commercially available nonviral cationic reagents, for example, TransIT-3D, TransIT-2020, XtremeGENE 9, XtremeGENE HP, JetPrime, Lipofectamine 3000, and Effectene and compared their transfection efficiency [70]. Warren et al. reported efficient reprogramming of hiPSCs from various cell sources by using mRNA and differentiated the cells into three germ layers [71]. hiPSCs were also reprogrammed by using recombinant protein that also maintained all the three germ layers [72].

Since transgene possesses high risk of cancer-causing agents; therefore, removal after transduction is highly desired. There are a few methods developed for the removal of these vectors. One of the methods is the piggyBac transposon system, which has been used to remove tandem Yamanaka reprograming genes Oct4, Sox2, Klf4, and c-Myc from iPSCs following reprogramming [73]. Removal of transgenes after incorporating CAR into T-cells used another transposon system called Sleeping Beauty, which successfully removed any genetic scar from the transduced cells [74, 75]. Likewise, transgene-free iPSCs have also been produced by Cre excision of reprogramming genes via loxP sites [76]. Integration-deficient viral vectors are also good candidates for producing transgene-free cell therapy products by mutating viral integrase [77]. Another approach is to use site-directed integration using targeting nucleases [78–80].

Various genome engineering technologies have been explored for gene addition, deletion, or correction in the cell therapy industry and are increasing day by day [81]. The most widely used targeting nucleases are zinc-finger nucleases (ZFNs), clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas

endonucleases, or transcription-activator like effector nucleases (TALENs) [82]. Although the CRISPR/Cas system has recently received much attention due to broad use in genome engineering of patient cells [83], ZFNs are also popular for treating graft-versus-host disease in T-cell therapy [84].

Recently, a nuclease dead variant of Cas9 bearing a transcriptional trans-activator has recently been used in cellular reprogramming by activating the transcription factors Oct4 and Sox2, which maintained pluripotency and expressed the markers for the three germ layers [85].

Although genetic modification is a rate-limiting step in the cell manufacturing industry, the conventional methods make it more complicated because it is a multi-step process. Conventional genetic modification in planar culture is also costly, labor-intensive, and time-consuming. The bioreactor is a better platform for producing large-scale genetically modified cells for commercial purposes because cell expansion is possible in the same vessel which makes the process straightforward (**Figure 2**). For genetic modification in bioreactor, Hsu et al. recently reported how to transfect reprogramming factors in bioreactor where they tried eliminating viral vectors for gene delivery by using cationic reagents [78]. Generally, transfection of reprogramming factors for generating induced pluripotent stem cells (iPSCs) is done in adherent culture and then cells are expanded in 2D or 3D which is a two-step process. By integrating the genetic modification step in bioreactor, it is possible to establish a single-step process which enables cell manufacturing in automated and closed bioreactor system.

Genetic modification is also a challenging step in CAR-T cell therapy-based products. In CAR-T cell therapy, generally cells are isolated from patients' blood sample and then the cells are expanded after selection and activation. Finally, the cells need to be transduced with the CAR or any other antigens depending on target diseases. Conventional methods for genetic transduction are based on planar culture where every step is performed in open culture system. Recently, a few steps are integrated in bag culture system where selection, activation, and expansion can be done in a single step using DynaMag™ CTS™ [86], whereas the Xuri cell expansion System developed by GE Healthcare can expand cells in large numbers [87–89].

Although washing and concentrating the final product are integrated by the COBE® 2991 system developed by Terumo BCT [90], the transduction step is still not integrated in any of the above systems. Integrating the transduction step with the expansion and formulation will make the CAR-T cell therapy straightforward and performing these steps in bioreactor is a good platform since the physiological parameters as well as automated operation is possible in bioreactor culture. Miltenyi Biotec developed a device named CliniMACS Prodigy™ which is based on bag culture for CAR-T cell therapy. This device integrated major steps especially cell preparation, selection, activation, expansion, transduction, washing, and formulation in an automated system [91–93]. Such integration in the bioreactor will pave a straightforward method for producing cell-based products in a closed and automatic method.

3.2 Integrated system for large-scale expansion and differentiation in bioreactor

Current manufacturing practices for stem cell-based products are multi-step: derivation, expansion, and differentiation. In this process, patient-derived skin fibroblast cells are transduced with reprogramming factors in the planar culture. After deriving hiPSCs, cells are expanded in planar or bioreactor culture to obtain a large number of cells. Then cells are differentiated to target cells of interest. The differentiated cells are characterized and transplanted to the patient in a dose-dependent manner. As this process is complicated with multiple steps, it poses high risk of contamination to the final products. Moreover, maintaining cGMP culture

platform is also mandatory for cell-therapy products [94–96], which makes the cell manufacturing process more complicated. Therefore, developing an integrated system that can combine all these steps from derivation to final products is required. Here, the bioreactor may be a good platform for doing this (**Figure 2**).

The bioreactor platform is widely used for the large-scale expansion of hPSC-based cell therapy production because bioreactor is easy to operate in an automated mode where various physicochemical parameters can be regulated in a closed-system. Two groups have demonstrated that the bioreactor is conducive to cellular reprogramming [97, 98]. Shafa et al. reported a significantly higher reprogramming efficiency in the bioreactor compared to the planar culture [97]. Since mesenchymal-epithelial transition (MET) is an important early step in cellular reprogramming [99], transformed fibroblasts that are moved into the bioreactor will form aggregates that are efficiently expanded in the bioreactor. Indeed because fibroblasts are substrate-dependent, bioreactor culture may be promoting aggregate formation and therefore cellular reprogramming.

Unfortunately, bioreactor reprogramming methods require genetic modification (retroviral, piggyBAC) prior to bioreactor expansion. It is theoretically possible to pursue cellular reprograming fully and completely in the bioreactor. Recently, for example, Hsu et al. has demonstrated that it is possible to transfect human fibroblasts directly on microcarriers [70]. Reprogrammed cells in theory will leave the microcarrier to form aggregates in the bioreactor via MET.

Following bioreactor derivation of hPSCs, the next big steps are expansion and differentiation. Generally, a large number of cells are required for an effective cell therapy application, which is ranging from 10^8 to 10^{10} cells per 70 kg patient [100]. In the conventional process, cell expansion is performed in planar culture. However, it has many drawbacks and limits the cell expansion in various ways. Planar culture is unable to provide enough growth surfaces for the unlimited expansion.

Another major drawback is surface coating. Extracellular matrix (ECM) is needed for surface coating which is initially derived from animal sources, which poses high risk in clinical-grade manufacturing. Currently, recombinant ECM has been discovered, which can be used efficiently for clinical applications [101]. The advancement in cell coating also stimulated the advancement of integration and automation of cell expansion in adherent culture.

Automated planar culture systems have been established for the expansion of hPSCs for clinical-grade cell manufacturing. One of the notable automated systems for cell manufacturing is CompacT SelecT™ developed by the TAP Biosystems. This system is based on T-flask where 90 T175 flasks can be accommodated for large-scale expansion of cells. All the cell culture steps, cell counting, seeding, medium change, passaging, and plating as well as transient transfection can be done automatically by using this robotic system. However, such systems are not used for differentiation since differentiation is a complicated process, which needs several components to add in the culture medium. As a result, the expansion and differentiation process in planar culture is mostly disintegrated.

Cell expansion in bioreactors need not require surface coating except for microcarrier culture. Bioreactor also provides enough growth surface availability. Generally, a single bioreactor (100 mL working volume) is enough for providing clinically relevant number of cells for autologous cell therapy applications. Several types of bioreactors are employed for the expansion of hPSCs [102]. For anchorage-dependent expansion of hPSCs, microcarriers need to be coated with ECM for cell attachment in the bioreactor [100, 103–105].

After large-scale expansion, cells are harvested by detaching them from the microcarrier using enzymatic treatment. Nonenzymatic detachment is also available by changing temperature or pH [18–20]. Bioreactor expansion of hPSCs on

microcarrier is troublesome for clinical application because it needs an extra step for microcarrier separation from the final cell harvest. On the other hand, aggregate culture in bioreactors may not necessarily need a detachment step for harvesting [21–25] and clinically relevant numbers of cells can be produced in a single bioreactor as aggregate [21, 106–108].

A major drawback in aggregate culture is the size limitation. With the increase in aggregate size, the growth potential decreases in the large size aggregate due to diffusion limitation of oxygen and nutrients [109]. Therefore, maintaining aggregate size is an important issue to maintain high growth rate as well as high quality for cell therapy applications [21].

After expansion, cells can be differentiated in the same vessel which makes bioreactor culture a unique choice for integrated biologics manufacturing. Bioreactors were used for differentiation of hPSCs into various cell types, especially for cardiac [110–112], hepatic [113, 114], and neural [115] lineages. To provide straightforward methods for clinical applications, integration of expansion and differentiation is important and there are several reports published recently where expansion and differentiation were integrated [108, 116–118]. However, the integration of derivation with expansion and differentiation is still facing complications and there are a very few reports available.

Steiner et al. reported integration of derivation, propagation and differentiation of hESCs in suspension culture where hESCs were isolated from the inner cell mass in suspension culture that did not involve feeder cells or microcarriers [119]. However, the integration of derivation, expansion, and differentiation is not still realized for personalized medicine especially for autologous or allogenic cell therapy applications. Such integration is needed for overcoming the multi-step cell processing, which will reduce the risk of contamination and save cell processing time as well as reduce manufacturing costs for cell therapy manufacturing.

4. Concluding remarks and future directions

Cell therapy applications utilizing stem cells are increasing day by day and several clinical trials are ongoing to treat incurable diseases. With the growing need for cell-based products, the manufacturing facilities should be compatible for fulfilling the market demand by supplying safe and effective cell-based products. Since the current manufacturing systems are stuck with several drawbacks, especially multi-step processing which poses high risk of contamination as well as long processing time which contributes to increase culture cost, a more straightforward system is required. Bioreactor-based cell manufacturing system can provide a single-step and straightforward processing of cell-based products. Integration of different steps, especially genetic modifications, derivation, and expansion as well as differentiation in bioreactor will pave the future of manufacturing cell-based products. The integrated biologics manufacturing in stirred suspension culture will significantly reduce the risk of contamination of final products, increase product efficacy, and reduce cell processing time and provide a cost-effective platform for cell manufacturing for cell therapy applications.

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SCN conceptualized, designed, and wrote the manuscript. DER conceptualized and revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Chapter

A Simple Way to Produce Gold Nanoshells for Cancer Therapy

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Abstract

Gold nanoshells (GNSs), formed by a silica core surrounded by a gold shell, present a shift on their surface plasmon resonance (SPR) to the near-infrared (NIR) part of the electromagnetic spectrum when synthesized with specific dimensions. This chapter presents a simple method to prepare the nanoshells, a step-by-step characterization, as well as their absorbance spectrum. For the synthesis, silica spheres, with approximately 190 ± 5 nm in diameter, were prepared using the Stöber method and then functionalized with 3-aminopropyltriethoxysilane (APTES). The gold nanoparticles (GNPs), with a diameter of 7 ± 3 nm, were produced by the reduction of chloroauric acid. Then, the silica was seeded with the GNPs to later grow a gold shell with the help of $Au(OH)_4^-$ ions and formaldehyde. UV-Vis spectroscopy results showed an increase of absorbance starting at 520 nm. It reached its maximum around 600 nm and kept absorbing all through 1200 nm. Transmission electron microscope (TEM) and scanning electron microscope (SEM) images suggest that the absorption peak movement coincided with the completion of the shell. Furthermore, when the sample was irradiated with an 820 nm wavelength/3.1 mW laser, its temperatures increased by 6.3°C in 2 min, showing its absorbance in the NIR.

Keywords: gold, nanoshells, surface plasmon resonance, near-infrared absorption, silica core, core-shell particles

1. Introduction

There are hundreds of types of cancer, and each one has different characteristics [1]. Therefore, science utilizes the most innovative discoveries in an effort to find new treatments, and nanotechnology offers a wide variety of options. One example of this is the nanoparticle colloids. They can be designed to concentrate on specific organs (passive targeting), or their surfaces can be modified by an antibody or ligand to get attached to a specific target (active targeting) [2]. Furthermore, metallic nanoparticles, like GNS, present interesting optical properties. The shell, formed by GNPs, confines the plasmons to the surface of the particle, changing the plasmon frequency of the gold. Therefore, the GNSs absorb different wavelengths than gold

in bulk. Moreover, when the wavelength of the incident light is larger than the size of the nanoparticle exciting the plasmons at their natural resonance frequency, light is absorbed more strongly causing an increase in temperature. When the GNSs are synthesized with specific geometry and dimensions, their SPR changes causing their absorption to shift to the NIR region of the electromagnetic spectrum [3]. This shift offers a great potential for applications in the medical field because GNPs are bio-inert [4], and the cytotoxicity of the silica has been widely studied [5]. Additionally, the wavelengths of the NIR spectrum are considered the optical window of the human body. As a result, while most biological soft tissues have low absorption of these wavelengths [6], GNSs absorb them causing them to increase their temperature.

GNSs have been synthesized over different templates. Polystyrene cores claim to offer a narrower plasmon resonance absorption peak due to their higher reflective index [7]; iron oxide nanoparticles present a superparamagnetic template useful for magnetic resonance imaging [8]; silver nanoparticles have also been used as a mold for hollow gold nanoshells [9]. However, the functionalization of the polystyrene takes more time, reactants, and supervision which increases the chances of error as compared with the functionalization of the silica. Besides, the cytotoxicity of the silica nanoparticles makes them a good option for medical applications. Moreover, once the GNS is produced, the silica core can be diluted with hydrochloric acid to obtain hollow gold nanoparticles [10] that can be used for the controlled release of drugs [11] due to their capacity for encapsulating sensitive materials and their low thermal expansion coefficient. Therefore, providing a simpler and more efficient method of synthesis of GNS on silica templates provides a more promising variety of applications like for photothermal therapy [12], optical imaging [13], and drug release [2], as well as providing a near instantaneous in situ whole blood assay [14].

The synthesis of the GNSs has been extensively explored. Different methods, like reflux systems [15] or flow micro-reactors [16], can be used as well as procedures involving high temperatures [17]. But most of those methods last over 30 h [18]. In this chapter, we present a simple and effective method of preparation that shortens the time of the traditional procedures published before and uses only a magnetic stirrer with heating for the synthesis.

The reductions of the time were obtained by first modifying the Stöber method of synthesis of silica particles from 2 h to 30 min Samples were obtained at 30, 60, 90, and 120 min throughout the reaction to determine the minimum time of reaction needed. Also, the seeding process can be shortened from 2 h to 30 min. During the seeding process, where the silica is decorated with GNPs, a sample was obtained using only 30 min of resting time and compared with another sample obtained after the full 2 h of the resting time previously suggested. In both cases, SEM images were obtained showing that 30 min were sufficient to accomplish the synthesis of the silica as well as their seeding. In consequence, the total time of the process was reduced by 3 h.

2. Background

The "Birth of Nanotechnology" was the title used by David Thompson [19] on his article acknowledging Michael Faraday's synthesis of gold nanoparticles in 1857. What Faraday called "Colloidal Ruby Gold" [20] was, in fact, a solution of dispersed GNPs so small that no microscope of that time was able to observe them. It wasn't until 1985 that Turkevich et al. [21] used an electron microscope to corroborate that Faraday's ruby gold was formed by GNPs with an average size of 6 ± 2 nm. Separately, in 1967 Werner Stöber et al. developed a method of synthesizing silica spheres in the micron size range [22] to be used especially in the medical field due to its known cytotoxicity, and in 1998 they were used by Naomi Halas et al. as the templates of GNS [23].

3. Experimental

3.1 Materials

Ethanol (100%), tetraethyl orthosilicate (TEOS) (98%), 3-aminopropyl-triethoxysilane (APTES) (99%), trisodium citrate dihydrate, gold (III) chloride trihydrate ($HAuCl_4$, 49%), formaldehyde (37%), and sodium borohydride ($NaBH_4$, 98%) were purchased from Sigma-Aldrich. Potassium carbonate (K_2CO_3, 99%) and ammonium hydroxide (28%) were purchased from J.T. Baker. All the solutions were prepared with deionized water.

3.2 Characterization

Images were obtained using the field-emission scanning electron microscope (SEM, JEOL JSM-7401F) and the transmission electron microscope (TEM, HT7700 Hitachi). For the ultraviolet-visible (UV-Vis) spectra, the Evolution 220 spectrophotometer UV-Vis (Thermo Scientific) was used. The FTIR spectra were obtained with an IRAffinity-1S Fourier transform infrared spectrophotometer (Shimadzu). The sample was irradiated with an 820 nm wavelength/3.1 mW laser (Multi-Channel Fiber-Coupled Laser Source, Thorlabs), and the infrared images were taken with a Non-contact Digital IR Thermometer (TrueIR Agilent Keysight U5855A). Measurement of the particles and histograms were acquired with the Image J® software [24].

3.3 Preparation of silica spheres

Silica particles were prepared by modifying the Stöber method [22]. About 50 ml of ethanol, 2.5 ml of deionized water, and 4.25 ml of ammonium hydroxide were magnetically stirred in an 80 ml glass flask for 5 min. Then, 0.75 ml of TEOS was added dropwise. The solution was heated at 40°C. Temperature and agitation were kept for 2 h. The color of the solution changed from transparent to opaque white approximately 10 min after adding the TEOS as shown in **Figure 1**. This time corresponds to the induction period needed to form the SiO_2 nucleus from the concentration used of the TEOS monomer [25]. Samples were obtained at 30, 60, 90, and 120 min after adding the TEOS to observe the evolution of the process.

3.4 Functionalization of the silica spheres

In order to create open links over the silica to attach the GNPs, the silica was functionalized with APTES on a 1 ml:1 μl silica/APTES volume ratio. About 50 ml

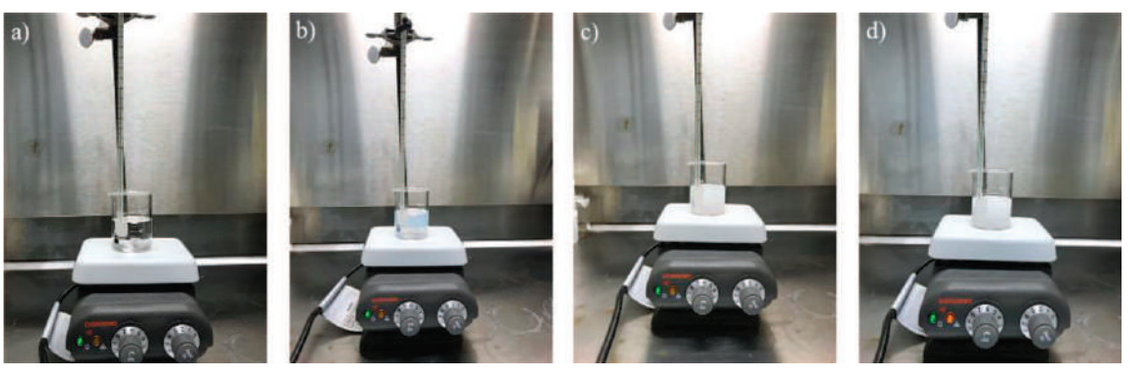

Figure 1.
Images of the synthesis of SiO_2 particles: (a) right after adding TEOS, (b) at 10 min of reaction, (c) at 30 min of reaction, and (d) at 2 h of reaction.

of the silica template solution was magnetically stirred for 5 min with 50 μl of APTES in an 80 ml glass flask. The solution was left still overnight at room temperature. The opaque white functionalized silica particles precipitated in the solution leaving a clear fluid at the top. To separate the functionalized silica, the mixture was centrifuged at 6000 rpm for 1.5 min and washed in deionized water three times. Finally, they were sonicated in 20 ml of deionized water final volume.

3.5 Synthesis of gold nanoparticles

The method presented by Abdollahi et al. [10] was followed to elaborate the GNPs. First, 100 ml of deionized water at room temperature was placed in a 140 ml flask under magnetic agitation. Then 1 ml of 1% $HAuCl_4$ solution, 2 ml of 1% trisodium citrate, and 1 ml of freshly made 0.075% $NaBH_4$ in 1% trisodium citrate were added in that order. The mixture was stirred for 10 min and used immediately to avoid the agglomeration. The GNP may also be stored at 4°C in an amber glass bottle for later use.

Throughout the synthesis, the gold solution changed its color from light yellow (**Figure 2a**) to wine red (**Figure 2b**). This is a characteristic of the GNP formation [26].

3.6 Seeding process

For the seeding process, 100 ml of GNPs and 10 ml of functionalized silica were magnetically stirred in a 140 ml glass flask for 5 min as shown in **Figure 3a**. Then, it was left still for 2 h. **Figure 3b** presents how the seeded silica spheres precipitated and changed their color from opaque white to lavender, while the mother solution changed from wine red to transparent. The mixture was centrifuged at 6000 rpm for 2 min and washed in deionized water three times. Finally, it was sonicated in 20 ml of deionized water final volume. The same procedure was followed, but the solution was left still for only 30 min to observe the development of the seeding process through time.

3.7 Gold hydroxide solution

For the shell growth process, a gold hydroxide solution was prepared by mixing 100 ml of 2 mM K_2CO_3 solution and 1.5 ml of 1% $HAuCl_4$ in a 140 ml glass flask for

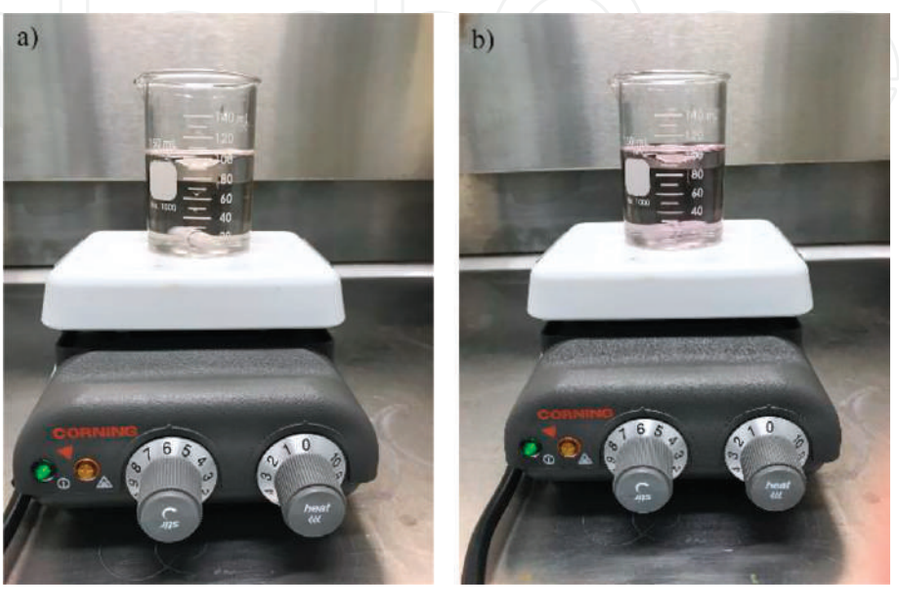

Figure 2.
Synthesis of GNPs at (a) the beginning of the reaction and (b) after 10 min of reaction.

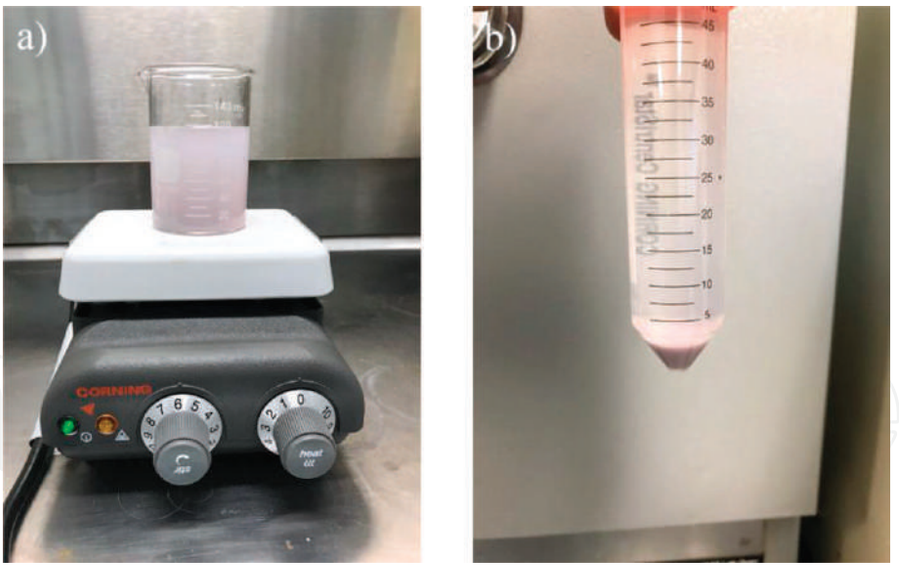

Figure 3.
Images at (a) the beginning of the seeding process and (b) after 2 h of resting time.

30 min. The color of the solution changed from light yellow (**Figure 4a**) to transparent (**Figure 4b**). It was left still overnight at room temperature in an amber glass bottle to facilitate the formation of $Au(OH)_4^-$ ions [18].

3.8 Shell growth

The shell was developed from the gold seeds deposited over the functionalized silica particles with the help of the $Au(OH)_4^-$ ions. About 100 ml of the gold hydroxide solution (**Figure 5a**) and 5 ml of seeded silica were magnetically stirred in a 140 ml glass flask for 5 min (**Figure 5b**). Next, 5 ml of formaldehyde was added to the solution (**Figure 5c**) and stirred for 10 min (**Figure 5d**). The solution was left still for 50 min. Finally, it was centrifuged at 6000 rpm for 2 min, washed, and dispersed in 10 ml of deionized water final volume.

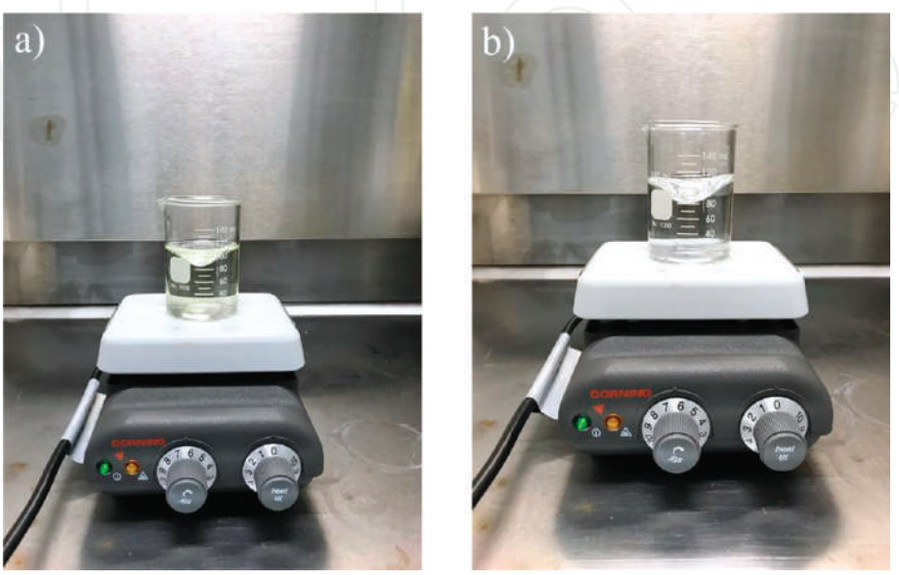

Figure 4.
Images illustrating the change of color of the gold hydroxide solution at (a) the beginning of the synthesis (light yellow) and (b) 30 min of reaction (transparent).

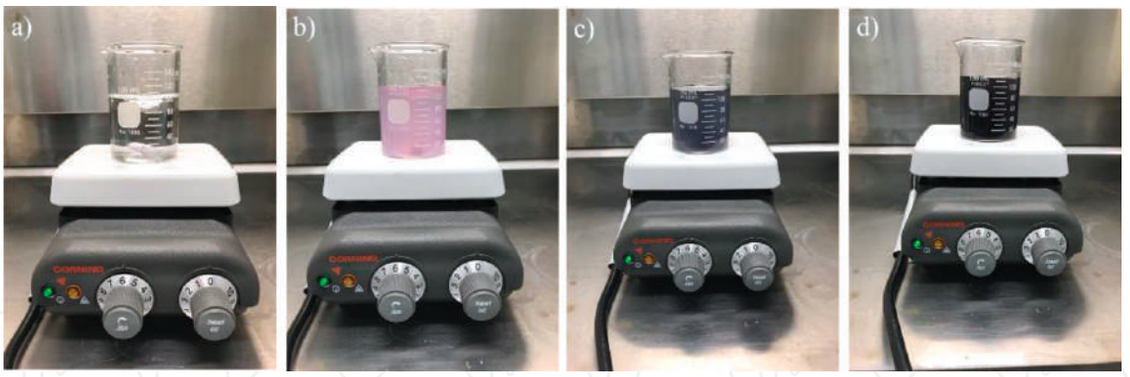

Figure 5.
Images of the shell growing process. (a) Gold hydroxide solution, (b) gold hydroxide + seeded silica, (c) gold hydroxide + seeded silica + formaldehyde, and (d) solution after 10 min of reaction.

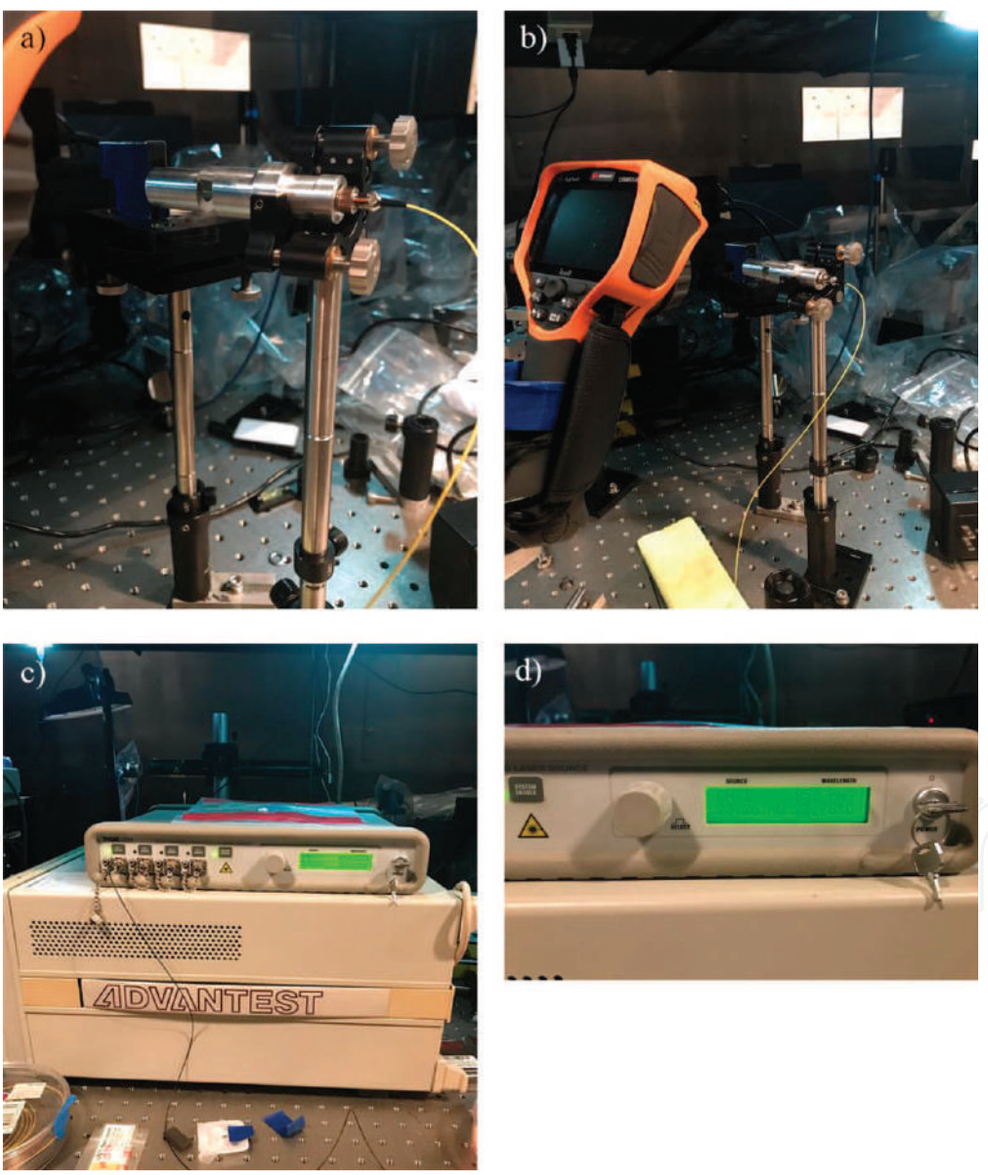

Figure 6.
Images of the installation of the (a) 820 nm wavelength laser, (b) non-contact digital IR thermometer, (c) connection to the multi-channel laser source, and (d) selection of the channel with the desired wavelength.

3.9 Thermography

To obtain the IR images, first, the 820 nm wavelength laser was fastened to the support for it to aim directly to the sample (**Figure 6a**). Then the Digital IR

thermometer was also secured and directed to the GNS (**Figure 6b**). Next, the laser was connected to the Multi-channel laser source (**Figure 6c**). Finally, the channel with the desired wavelength was selected (**Figure 6d**), and the irradiation was started.

4. Results and discussion

4.1 Characterization of the silica templates

TEM images obtained from the silica samples taken at 30, 60, 90, and 120 min after adding the TEOS are presented in **Figure 7**. When comparing the images, no significant variation in the size of the silica particles is noticeable.

To corroborate that the silica particles do not change substantially when the reaction time is over 30 min, the images were studied with the software Image J®, and the diameter distribution of the particles was analyzed. Over 1000 particles from the different samples were measured to obtain the histograms presented in **Figure 8** where samples A, B, C, and D correspond to 30, 60, 90, and 120 min of reaction time, respectively. They illustrate that the diameter distribution of the silica spheres throughout the synthesis oscillates around the 190 ± 5 nm on all the samples.

To have a better understanding of the information, **Table 1** contains useful statistic information from the samples.

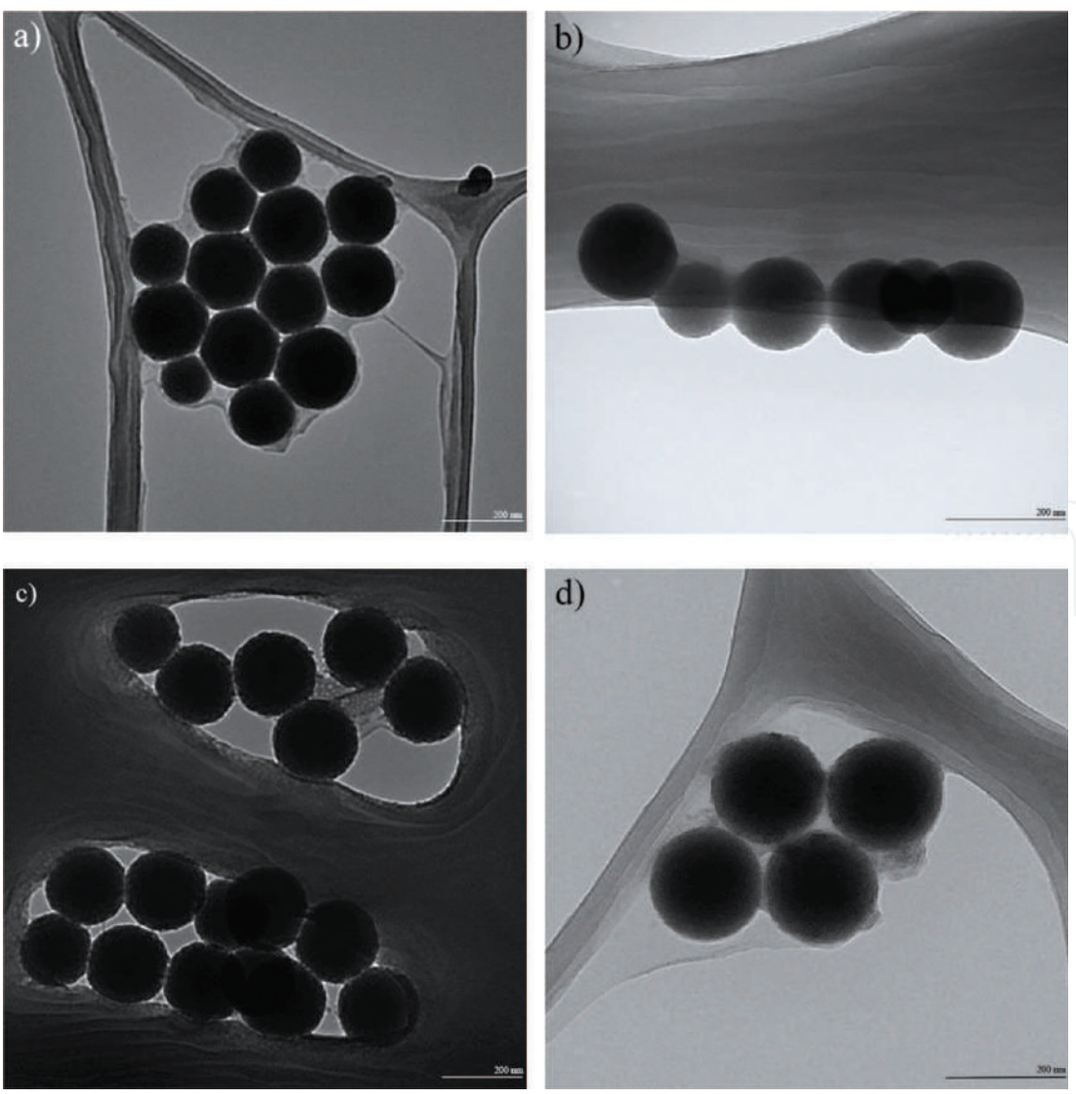

Figure 7.
TEM images of silica particles at (a) 30, (b) 60, (c) 90, and (d) 120 min after adding TEOS.

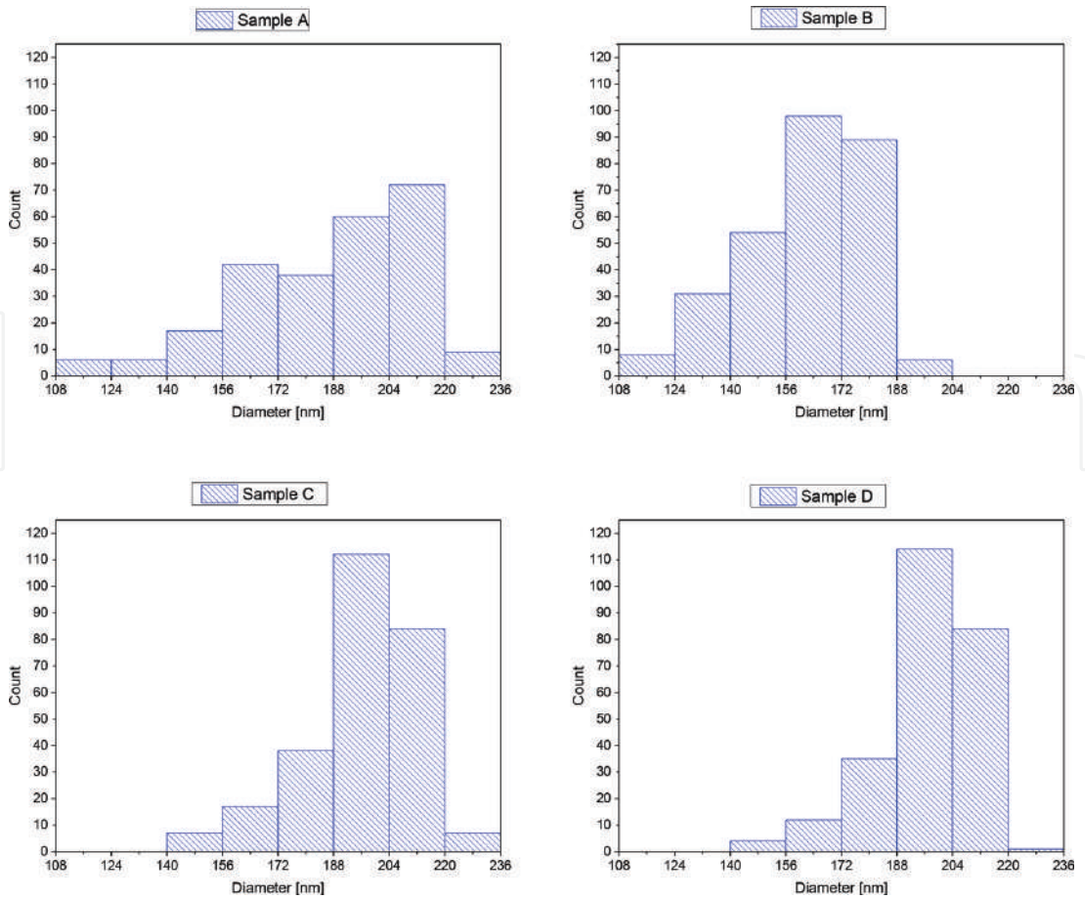

Figure 8.
Histograms illustrating the diameter distribution of the silica particles throughout their synthesis.

	Count	Mean	Std dev.	Mode
Sample A	250	187	25	197 (79)
Sample B	287	162	18	162 (80)
Sample C	265	196	16	201 (92)
Sample D	250	197	13	198 (105)

Table 1.
Statistic information obtained by measuring the diameters of silica particles from the different samples.

The mean, standard deviation, and mode obtained after analyzing the samples show that, in general, the silica templates keep their size and shape after 30 min of synthesis. Therefore, the objective of synthesizing silica particles with diameters of 190 ± 5 nm was achieved within 30 min of reaction time. More than 30 min of synthesis does not result in any relevant change in the sample. For this reason, the total process time can be reduced from 2 h to 30 min, shortening the reaction time by 1 h and 30 min when compared with similar published works where the synthesis time is at least 2 h [10, 17, 27, 28].

4.2 Characterization of the functionalized silica templates

The functionalization of the silica with a primary amine group ($–NH_2$) was accomplished by the use of APTES which changed the superficial charge of the

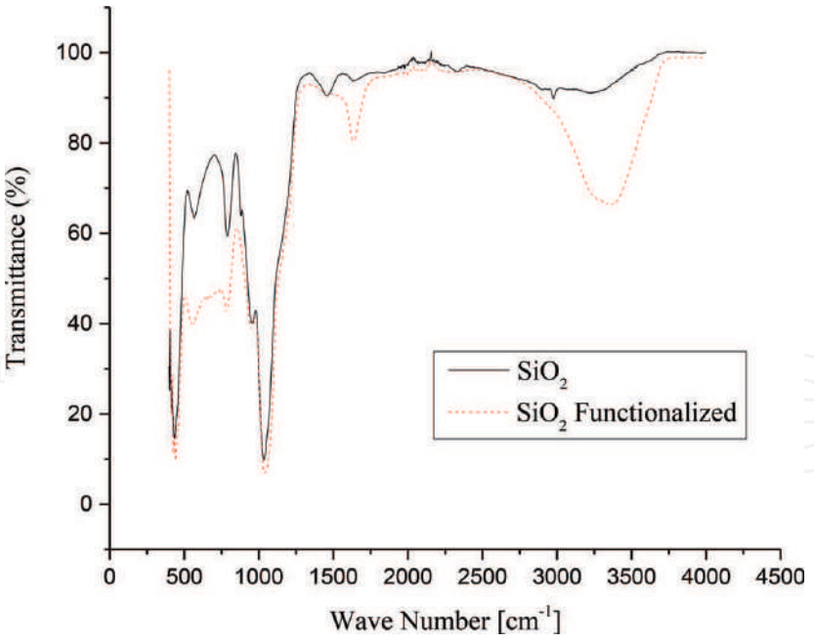

Figure 9.
FTIR spectrum of silica particles and silica particles functionalized with APTES.

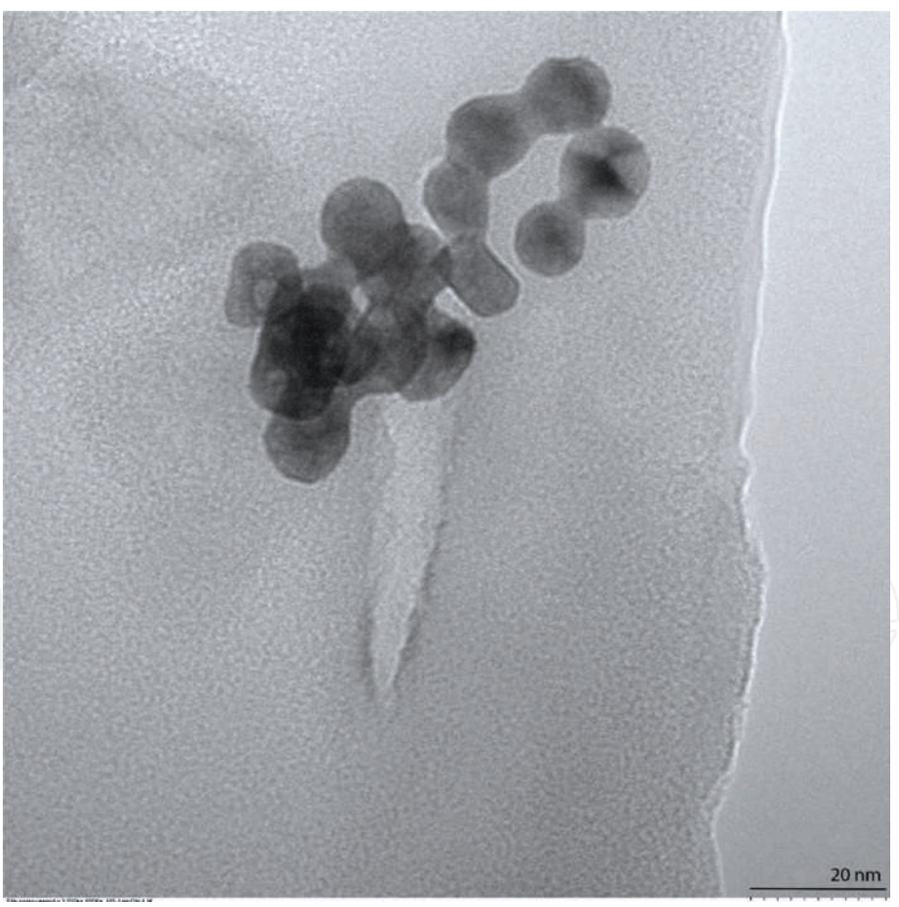

Figure 10.
TEM images of GNPs.

silica providing an electrostatic link for the GNPs to attach [29]. This superficial modification was verified by the FTIR spectrum shown in **Figure 9** where the vibrations of primary amines are found between 3550 and 3330 cm^{-1} which correspond to the vibrations of a primary amine group [30].

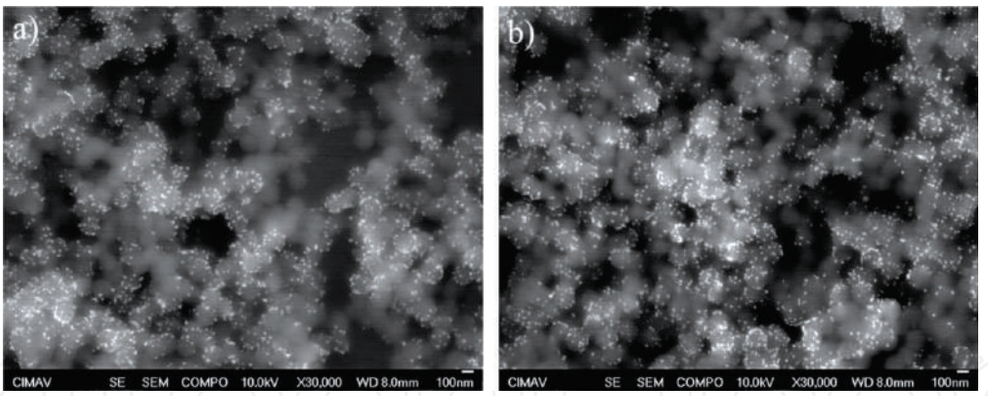

Figure 11.
SEM images illustrating the seeding process with (a) 30 min of resting time and (b) 2 h of resting time.

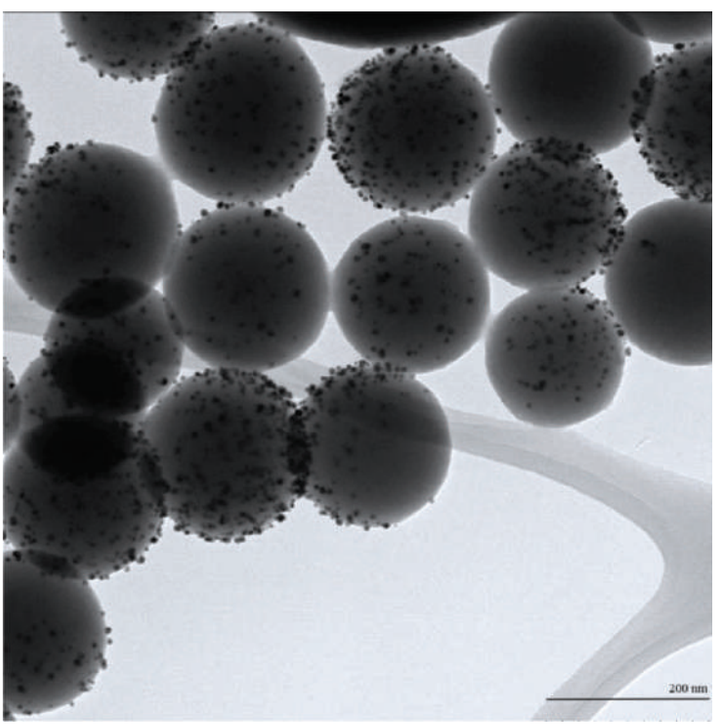

Figure 12.
TEM image of a gold decorated silica particle.

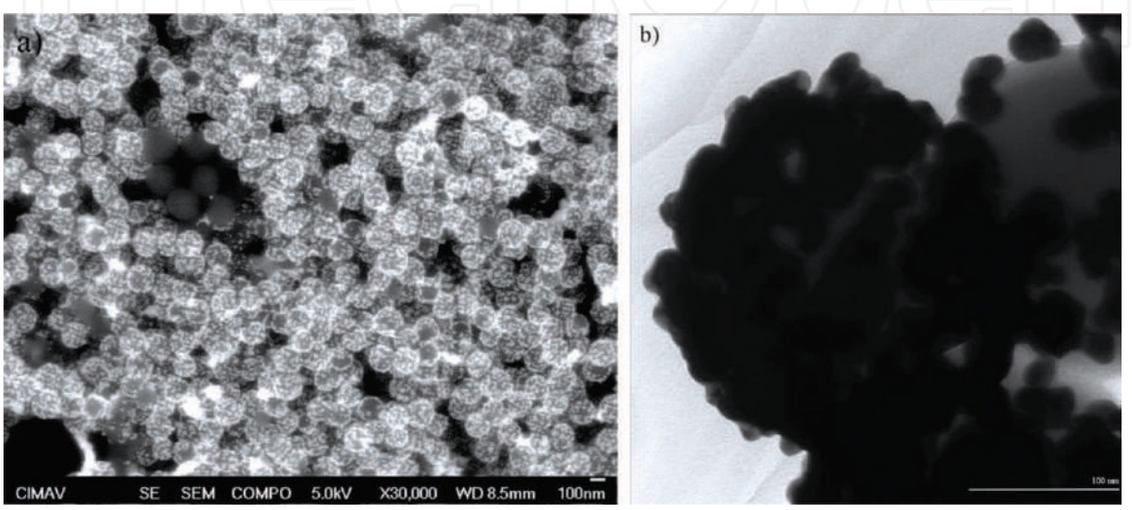

Figure 13.
(a) SEM and (b) TEM images of GNS.

4.3 Characterization of the gold nanoparticles and seeded silica

The GNPs were analyzed under a TEM. **Figure 10** illustrates the GNPs with a diameter of 7 ± 3 nm and spherical shape overall.

The seeding process was followed with 2 h of still time as well as with 30 min of still time. The first and second samples were observed under the microscope. The samples were taken with the purpose of observing the development of the seeds. **Figure 11a** presents an SEM image of seeded silica with 30 min of resting time, while **Figure 11b** presents an SEM image of seeded silica with 2 h of resting time. The images show that 30 min is enough time to create the seeds because both images display approximately the same number of nucleus per silica particle.

Even though a complete shell was not formed, the seeds are ready to grow the gold shell on the next step. A TEM image of a seeded silica particle is presented in **Figure 12**. This image corroborates the seeding process as well as the silica functionalization.

4.4 Characterization of the gold nanoshells

Figure 13a and **b** presents SEM and TEM images of the synthesized GNS, respectably. They illustrate that the silica particles are almost surrounded by gold. The higher density of gold, the separation of the GNPs [31], and the dielectric properties of the silica [3] contribute to the absorption of the NIR wavelength, which causes the increase in temperature.

4.5 The UV-Vis spectrum

Figure 14 presents the UV-Vis spectrum of the particles through the process. Silica particles, as well as functionalized silica particles, do not show significant

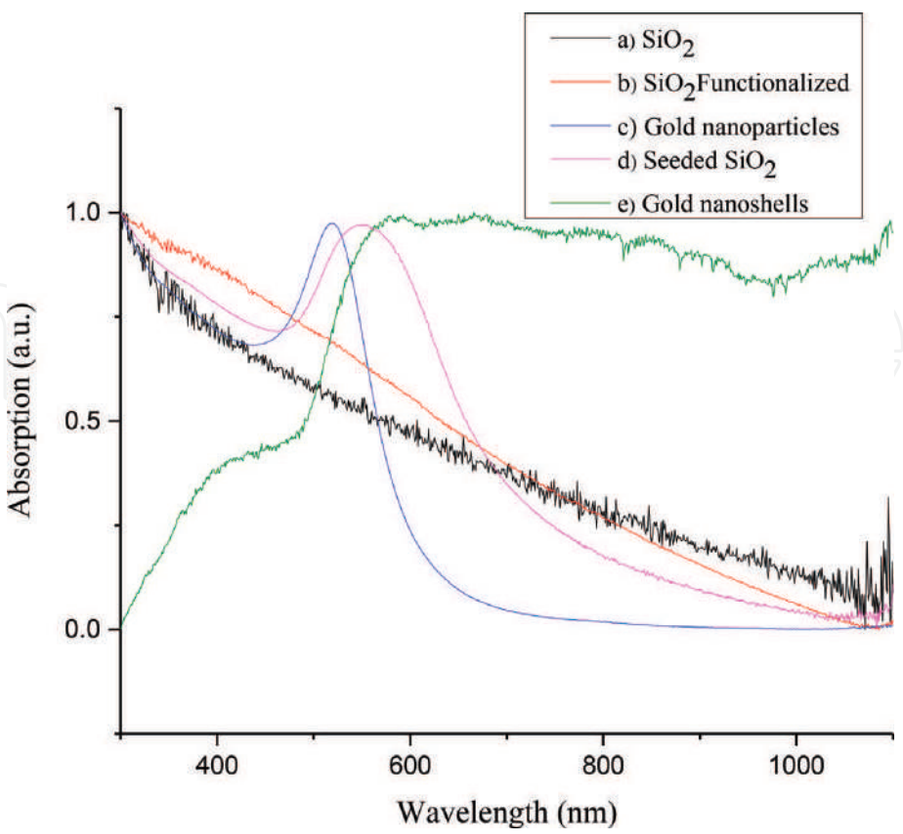

Figure 14.
UV-Vis spectrum of (a) silica, (b) functionalized silica, (c) gold nanoparticles, (d) gold seeded silica, and (e) gold nanoshells.

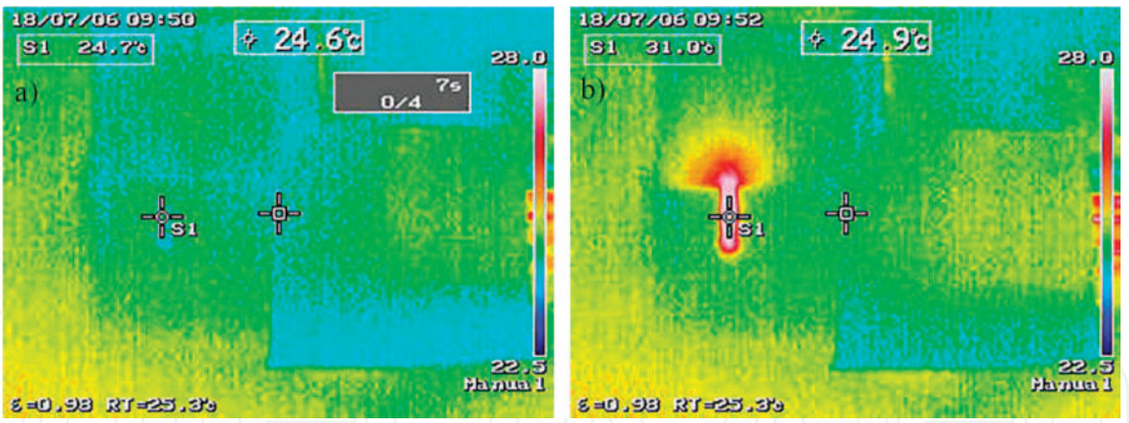

Figure 15.
Thermography images of the GNS taken (a) before and (b) after being irradiated with 840 nm wavelength laser for 2 min.

absorption on the NIR. As for the GNPs, they exhibit their characteristic absorption between 520 and 530 nm [29]. However, on the seeded silica particles, the slight shift to the NIR is noticeable. While on the GNS, the peak not only shifted to the NIR, but it kept a high absorbance all the way to 1100 nm. This range is part of the optical window of the human body [5]. The absorbance of the GNSs is due to the SPR that creates an electric field on the surface increasing the absorption of these wavelengths. SPR happens when metal nanoparticles are irradiated with a wavelength bigger than their size, exciting the electrons of the conducting band [2].

4.6 Heat generation of GNS from light energy

To verify the absorbance of the GNS, they were irradiated with an 840 nm laser with a power of 3.1 mW. **Figure 15a** and **b** displays the thermography images of the sample while being irradiated at time zero and 2 min later. The temperature of the sample increased from 24.7 to 31.0°C. This confirms that GNSs are able to absorb light from the NIR and convert it in heat.

5. Conclusions

Synthesizing GNS by seeding and growing a gold shell over silica spheres with GNPs showed to be an effective method to tune their absorption to the NIR. The SEM and TEM images show the evolution of the process, while the absorbance spectrum displays the GNS shifting over the NIR. Therefore, we obtained a simple technique of producing GNS that can be used for medical applications thanks to the bio-inert GNPs [3] and the widely studied cytotoxicity of the silica [5]. This method does not require long periods of time, when compared with previously published mechanisms, and does not need sophisticated equipment.

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Nomenclature

GNSs	gold nanoshells
SPR	surface plasmon resonance
NIR	near-infrared
APTES	3-aminopropyltriethoxysilane
GNP	gold nanoparticles
TEM	transmission electron microscope
SEM	scanning electron microscope
TEOS	tetraethyl orthosilicate

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Chapter

Engineering of Surface Proteins in Extracellular Vesicles for Tissue-Specific Targeting

Stefan Vogt, Gerhard Stadlmayr, Johannes Grillari, Florian Rüker and Gordana Wozniak-Knopp

Abstract

Extracellular vesicles (EVs) have in the recent decades gained an important stand as vehicles enabling cell-to-cell transport and communication. With the advanced development towards their clinical use and increasing versatility of potential applications, improving their tissue-specific targeting in order to enhance their functionality in drug delivery opened as a challenging engineering field. In the past, the question of specific intercellular contact has been addressed by decoration of the EV surface with agents able of specific target recognition. An attractive possibility here is the modification of strongly overexpressed EV surface marker proteins towards recognition of target cells. As these proteins are involved in a plethora of biological functions in EV biogenesis, cargo targeting and intercellular transfer, a minimal impact on protein architecture upon modifications is desirable, which would also increase the stability of the exosomal preparation intended for therapeutic use. This chapter focuses on the possibilities of engineering of the EV marker proteins towards antigen-recognition units broadly applicable to endow EVs with tissue-targeting functionality.

Keywords: extracellular vesicles, exosomes, tetraspanin, tissue-specific targeting, exosomal drug delivery

1. Introduction

The transfer of extracellular vesicles (EVs) has emerged in the last two decades as a novel mechanism for intercellular communication. Nomenclature of EVs has by now been agreed to be based on biogenesis pathway. Therefore, EVs budding from plasma membranes are termed ectosomes or microvesicles, while exosomes are formed via the endosomal compartment within multivesicular bodies (MVB) which then are released by their fusion with the plasma membrane. Finally, when blebbing from apoptotic cells, the term apoptotic bodies has been retained [1]. Biomarkers to differentiate these EVs are still questionable, and in general, size is used to differentiate exosomes from ectosomes, while apoptotic bodies are considered to present phosphatidylserine on the outside and thus can be stained by annexin V. Exosomes (40–150 nm in diameter) are produced by formation of endosomal intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and are secreted by fusion of these vesicles with the plasma membrane [2, 3] (**Figure 1**).

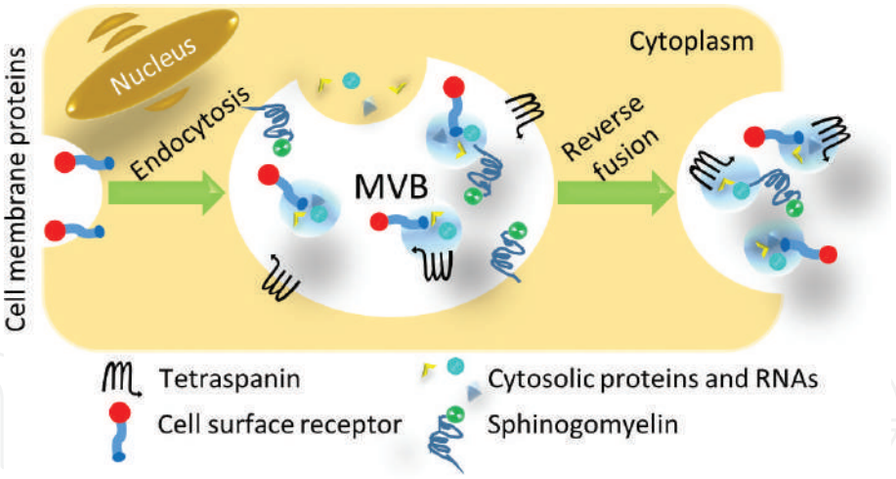

Figure 1.
Biogenesis of EVs.

Since they have been known to mediate directional intercellular transfer of their content, typically proteins, mRNAs, microRNAs (miRNAs) and a variety of non-coding RNAs [4], several studies were oriented towards their application as drugs *per se* or as therapeutic tool for drug delivery [5]. Their membrane is strongly enriched in sphingomyelin and cholesterol, which contributes to their unique buoyancy coefficient and enables practical isolation from other particles of cellular origin using differential centrifugation methods. Another discernible feature of exosome membranes is that it reflects the composition of the MVB membrane and has a high density of endosomal membrane proteins, such as proteins involved in MVB biogenesis (Alix and TSG101), membrane transport, in particular the components of the endosomal sorting complex required for transport (ESCRT) and most prominently tetraspanin proteins (CD9, CD37, CD53, CD63, CD81, CD82 and CD151) [6, 7]. Some tetraspanins such as CD9, CD81 and CD151 are more broadly expressed, while others are restricted to specific EV subsets [8, 9]. The overexpression of tetraspanins, as shown for CD9, can in several production cell lines enhance the exosome production and, in addition, cause a reduced overall size of the vesicles [10].

Tetraspanins play an important role in several physiological processes [11] and have been discovered to cooperate in states of health and disease in signal transduction, cellular activation, polarization, motility, adhesion, tissue differentiation, angiogenesis, tumorigenesis and metastasis [12–14], both by regulating cellular interactions as cell-membrane bound molecules and indirectly through exosomes. They are involved in each step of the metastatic cascade due to their ability to interact with cell surface receptors, adhesion molecules, matrix-remodeling proteases and signaling molecules. In this pathological state, they hence regulate cell proliferation, participate in epithelial-mesenchymal transition, modulate integrin-mediated cell adhesion and mediate the invasion through modulation of angiogenesis, tumor-endothelial cell interactions and regulation of cancer cell migration through the regulation of tumor microenvironment, as well as direct influence on extracellular matrix [15]. In this chapter, we introduce the characteristics of EVs and the engineering approaches aimed at their surface proteins to achieve tissue-specific targeting.

2. Structure of the tetraspanin proteins

The rod-shaped structure of a tetraspanin consists of four transmembrane helices that connect the two extracellular loops [16] (**Figure 2**). The short extracellular loop (SEL), which has not yet been reported to contain any element of a

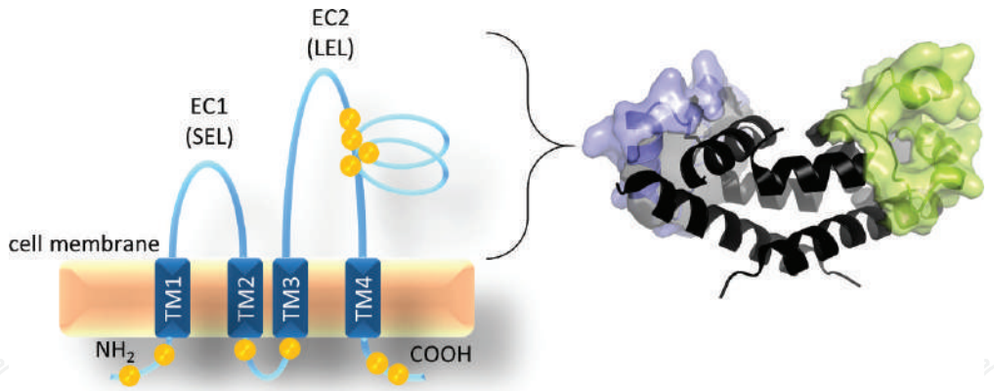

Figure 2.
Organization of tetraspanin CD81: 4 transmembrane helices (TM) span 2 extracellular loops. Positions of cysteine residues are indicated with yellow dots. Crystal structure of EC2 domain is presented as a cartoon diagram of the dimeric form (PDB: 1G8Q); the conserved three-helix bundle is black and the variable domains C and D are surfaced in blue or green for each protomer. The figure was prepared using PyMOL (PyMOL molecular graphics system, version 1.3 Schrödinger, LLC).

particular secondary structure, is of 12–31 amino acids in length. In the studies oriented towards tetraspanin engineering, the EC1 domain has until now been less addressed due to its lower degree of structural organization. Its replacement for a strand of glycine-serine residues has led to an unusual cell surface expression and a distribution dissimilar to the wild-type parental molecule, which may suggest the importance of this structural subunit for the stability of the membrane-bound tetraspanin [17].

The large extracellular loop (LEL) or extracellular domain 2 (EC2) has on the other hand been well characterized. The crystal structure of CD81 [18] demonstrated the four invariant cysteine resides within EC2 to form the 2 disulfide bridges, as a hallmark stability feature of the conserved tetraspanin fold [8, 19] (**Figure 2**). Moreover, the topology of several other tetraspanins was predicted by molecular modeling studies in the absence of an available crystal structure, using the CD81 EC2 structure as a template: these were the tetraspanins CD37, CD53, CD82 and CD151 [20, 21]. It has been early established that binding of CD81-specific antibodies depends on the formation of the disulfide bonds [22]. One class of tetraspanins harbors the EC2 composed of five α-helical elements, forming stalk and head elements of a mushroom-like structure, and the second class of tetraspanins is postulated to contain an additional helical element, stabilized with another pair of cysteine residues. The EC2 fold structure is evolutionarily conserved across species for any particular tetraspanin in spite of a high degree of variability at the amino acid level [23], with a subdomain consisting of a three-helix-bundle fold and a second subdomain variable in size among members of the tetraspanin family [18, 20].

The presence of an energetically unfavorable hydrophobic patch and the dimeric form of CD81 LEL in a crystal hinted at the high likelihood of tetraspanin assembly into dimers or multimers in the cell membrane. As shown later, clustering of tetraspanins leads to the organization of tetraspanin-associated proteins in a tetraspanin web or tetraspanin-enriched microdomain (TEM) [24, 25]. Biologically, such domains induce partitioning of the complexes into lipid rafts and clustering of the lipid rafts [26].

Apart from the hydrophobic interactions of the extracellular domains, the membrane-proximal cysteines residing in transmembrane domains contribute to the dimer formation and stability of tetraspanins. For the CD9 tetraspanin, it has been demonstrated that these residues are positioned close to the dimerization interface and influence homotypic and heterotypic tetraspanin association depending on their reversible palmitoylation status [27]. Similarly, the mutation of N-terminal

and C-terminal transmembrane cysteines to serine eliminated palmitoylation of CD151, which turned out to be deleterious for the assembly with other cell surface proteins, including tetraspanins CD9 and CD63, their organization to TEMs and subsequently their subcellular distribution and cell morphology [28]. At the same time, it had minimal influence on the density of tetraspanin protein complexes and was dispensable for CD151-$\alpha_3\beta_1$ integrin association. Depalmitoylation of CD81 did not impact its surface expression and stability, but rendered it less available for contact with its natural interaction partner CD9 and the relevant epitopes less accessible for binding of structurally dependent antibodies [29].

The four transmembrane helices of tetraspanin proteins form two largely separated pairs of antiparallel helices: one pair comprises TM1/TM2 and the other TM3/TM4. The two pairs of helices only converge close to the cytoplasmic side of the membrane through contacts between TM2 and TM3. In the recently solved crystal structure of full-length CD81, this cone-like structure has been shown to harbor a binding pocket for cholesterol [30], and mutations within transmembrane domains in certain tetraspanins have been connected with pathological states [31]. The short cytoplasmic tails show no obvious functional significance in signaling processes, suggesting that their signaling competence relies on association with other molecules [32]; nevertheless, its mutation can lead to different assembly with association partners as shown for CD9 [33].

3. Natural ligands of tetraspanin proteins

3.1 The tetraspanin web and intertetraspanin contacts

The ability of members of the tetraspanin family to assemble into a unique biological feature known as tetraspanin-enriched microdomain (TEM) is due to their mutual interactions; however, these structures include also receptors, integrins and signaling molecules such as phosphatidyl-kinase C (PKC) and phosphatidylinositol-4-kinase (PI4K) [9]. These interactions are fundamental for cellular functions such as cell adhesion, proliferation and motility. Interactions between tetraspanin members are important in maintaining the integrity and stability of the tetraspanin web and providing binding sites for different ligands. The multimers of newly synthesized proteins are formed in the Golgi apparatus. The predominantly cross-linked tetraspanin species are homodimers, but also higher order complexes and low amounts of heterodimeric tetraspanins (CD81/CD9, CD9/CD151, CD81/CD151) were identified [27]. It has been suggested that tetraspanin homodimers, formed in the Golgi and present at the cell surface, serve as building blocks in the assembly of higher organized tetraspanin protein complexes. Interestingly, the exosomes originating from cell lines overexpressing CD9 are believed to be enriched in more stable TEMs [10]. Overall, although most tetraspanins can interact with most other tetraspanins, and similarly engage with several other proteins, the nature of these interactions has been until recently classified only according to their stability in the presence of detergents of different stringency, which does not necessarily reflect their significance in the cellular milieu [34]. A thorough characterization of strength and abundance of the interactions between the members participating in a tetraspanin web in a particular cell and physiological situation is therefore needed and will support the understanding of its mediated biological effects. Similarly, most data on tetraspanin functionality come from studies on their localization on cell membranes, while functional data in vesicles are still scarce. Therefore, we here summarize the known cellular functions, while speculating how this might translate to EVs.

An important step towards the understanding of the specificity of the tetraspanin interactions in TEM has been achieved by delineation of the involved tetraspanin regions by dissecting the model tetraspanin into domains, differently amenable for modification. Early experiments that addressed the relatively unstructured and at the same time antigen-binding competent regions that appeared attractive for mutagenesis resulted in a protein that showed aberrant clustering involving both homo- and heterodimerization of resulting full-length tetraspanins [35], albeit the mutagenesis method employed in this study was a complete deletion of targeted domains. The CD81 D-region was studied in more detail: the CD9 and CD151 tetraspanins were more competent of clustering with CD81 when homologously engrafted with CD81 D-region [36]. When the mutagenized CD81 EC2 molecular subunits were transplanted to other tetraspanins, the extremely flexible conformation of the solvent-exposed D-segment of CD81 EC2 was sufficient to overcome the orientational restrictions to initiate the homotypic contact for dimerization, and this finding has been corroborated with both wet-lab data and the insights from molecular dynamic simulation of the cell membrane-embedded protein [37].

3.2 Interaction of tetraspanins with integrins and matrix-degrading enzymes: role of tetraspanins in cancer and metastasis

Important association partners of tetraspanins are the integrins. The role of such complexes in invasive growth *in vivo* as well as the effect of integrin-mediated binding events on cell proliferation and invasion is well established. Especially, the laminin-binding integrins ($\alpha_6\beta_4$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_7\beta_1$) exhibit extensive interactions with tetraspanin proteins [12, 38]. The functionality of integrins may depend critically on their interaction of tetraspanins: it has early been described that the remarkably stable association of the tetraspanin CD151 and the integrin $\alpha_3\beta_1$ leads to a high level of activation of cellular PI4K [39]. Further, CD151 interacts directly with the α_3 subunit and links it to other tetraspanins, CD9 and CD81. Loss of CD151 abrogates the $\alpha_3\beta_1$ mediated mobility on its ligands, laminin-332 and laminin-551. CD9/CD81 complex may even regulate the integrin-mediated functions independently of CD151 by forming a complex with the integrin and directing the PKCα-$\alpha_3\beta_1$ association [40]. Another example of tetraspanin-integrin association reveals its proangiogenic role through VEGF induction, mediated by cooperation between TM4SF5 and integrin α_5 of epithelial cells [41]. Interestingly, removal of CD151 palmitoylation sites did not disrupt the CD151–$\alpha_6\beta_4$ complex in epithelial cells but strongly influenced $\alpha_6\beta_4$-integrin–dependent cell morphology [42]. The rat tetraspanin D6.1A (human homolog is CO-029) was able to induce systemic angiogenesis by initiation of an angiogenic loop that reached organs distant from the tumor, probably due to the abundance of D6.1A in tumor-derived exosomes. This is in line with reports claiming that EVs prepare niches for metastatic tumor cells at tissues distant from the primary tumor [43]. This tetraspanin associates with integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$, as well as with tetraspanins CD9 and CD81, and is similarly to CD151 linked to tumor-promoting activities [44].

Active complexes of tetraspanins and integrins influence biological processes other than cellular signaling by interacting with cellular metalloproteinases, important players in the remodeling of extracellular matrix. A study of MDA-MB-231 cells, a breast cancer cell line, has indicated that the $\alpha_3\beta_1$-tetraspanin protein complex may be linked to an invasive phenotype of tumor cells via modulation of various signaling pathways, including activation of membrane metalloproteinase-2 (MMP-2), an enzyme associated with invasive migration of the cells, and affecting phosphatidylinositol-3-kinase (PI3K) signaling pathways, which control actin cytoskeleton dynamics [45]. By the incorporation of the members of a disintegrin and metalloproteinase (ADAM) family members the tetraspanins are able to influence the

cellular ectodomain cleavage and release activity of these enzymes [46]. The tetraspanins of the TspanC8 group (tetraspanins with 8 cysteines) have a significant impact on the cellular exit and catalytic activity of ADAM10 [47], in particular the activity of Tspan15/ADAM10 promoted N-cadherin cleavage [48–50]. Different TspanC8/ADAM10 complexes seem to have different substrate specificities [51]. The silencing of CD9 enhanced shedding of ADAM17-substrates TNF-α and ICAM-1 [52].

Important discovery of the possible consequences of fine differences in composition of TEMs has been delivered by the study of exosomes enriched in Tspan8-α_4 complex that were preferentially taken up by the endothelial and pancreatic cells [53]. The fact that such modifications can allow selective targeting *in vitro* and *in vivo* holds promise to achieve improved exosomal delivery by engineering of their membrane components.

3.3 The role of tetraspanins in immune complexes

In antigen-presenting cells (APCs), tetraspanins integrate into TEMs protein-recognition receptors binding to conserved repeated motifs of microbes, such as Toll-like receptors, and MHCII molecules into tetraspanin web platforms, as well as Fc_γ receptor I in phagocytic cells, Fc_γ receptor IIb and III upon the activation of macrophages and Fc_ε receptor I in monocytes and skin-derived dendritic cells [54].

The particular role of CD81 protein in the formation of specialized microdomains in the plasma membrane of the cells of the immune system was discovered by elucidating its function of recruiting various adhesion molecules, receptors and signaling proteins to the central zone of the immune synapse in T-lymphocytes and APCs [55]. Therefore, it has early been proposed for CD81 to play a key role during antigenic presentation, since it colocalizes with the T-cell receptor/CD3 [56], and CD81 indeed turned out to be a regulator of CD3 clustering and sustained CD3 signaling [57].

Further, the T-cell side of the immune synapse is densely populated by tetraspanins CD9 and CD151. The abolishment of their expression reduces markers of activation of T-lymphocytes conjugated to the APCs, such as IL-2 secretion and expression of CD69 [58].

Another role in the immune response of tetraspanin CD81 is amplifying and sustaining B-cell receptor signaling from lipid rafts by ligation to the co-receptor CD19/CD21 complex. The signaling through a variety of cell surface protein complexes implies a role of lipid rafts, again highlighting the ability of tetraspanin to facilitate raft association [9].

3.4 Tetraspanins in pathogen infection

Several studies have been oriented towards the research of tetraspanins as ligand molecules for pathogen entry. CD81 has been identified as a ligand for hepatitis C virus (HCV) recognition and viral entry [59]. The ligand for viral glycoprotein E2 is the D-domain of the LEL, a dynamic region positioned within the triple-bundle helix, whose conformation in solution differs substantially from the one suggested by crystal structure. Challenging for structure-based design, this region nevertheless presents an attractive target for design of therapeutically relevant ligands with methods such as NMR [60]. Apart of the EC2 domain, other regions of CD81 have proven important for virus infection. Experimental evidence here was based on the exchange of the structural domains of the molecule with the ones of tetraspanins of different degrees of homology, and it was found that closely related substitutions were more efficient at functional complementation of CD81. Viral entry has been shown to correlate with surface expression of the chimeric protein and to depend on the presence of the cholesterol-coordinating glutamate residue [61]. EWI-2wint,

a cleavage form of EWI-2, a member of the immunoglobulin superfamily, has an inhibitory effect on HCV infection by obstructing the interaction between CD81 and HCV E2 [62]. The related factor EWI-F inhibits *Plasmodium* infection, whereas its silencing increases infection efficiency [63].

Tetraspanin microdomains have been described to regulate HIV-1 entry, assembly and transfer between the cells [64, 65]. CD81 influences importantly the early stages of virus replication by controlling the stability of HIV-1 restriction factor and consequently the activity of viral reverse transcriptase [66], while CD63 facilitates endocytosis of the HIV receptor CXCR4 [67] as well as supports the replication steps in macrophages [68, 69]. Also Coronaviruses and low-pathogenicity Influenza A viruses utilize TEM domains as entry portals to co-engage with cellular receptors and proteases, which enable viral proteolytic priming [70]. As shown in an *in vivo* model with CD151 null-mice, this tetraspanin is a critical novel host factor of nuclear export signaling of Influenza A virus, used complementary to the viral nuclear export proteins [71].

Tspan9 modulates the early endosome compartment to make it more permissive for membrane fusion of early-penetrating viruses, and its depletion strongly inhibits infection by alphaviruses that fuse in early endosomes but does not alter the delivery of virus to early endosomes or change their pH or protease activity [72]. It is unclear, what function then EV-based tetraspanins might have in the context of viral infection, and it might be speculated that cells use EVs to titrate away virus into membrane structures that are unable to provide replication and protein synthesis machineries for the virus. This is supported by the findings that EVs might have anti-influenza infection activity *in vitro* [73].

4. Extracellular vesicles as mediators of cell-cell interaction

4.1 Biological basis for therapeutic applications: EVs as mediators of intercellular interactions

EVs are secreted by most cell types and are taken up by recipient cells, where their cargo consisting of a cocktail of proteins, mRNAs and non-coding RNAs alters the behavior of the recipient cells in a way that might be even considered similar to hormones or cytokines [74], e.g. in the context of skin or bone cell paracrine signaling [75–77]. siRNAs (small interfering RNAs) and miRNA-based inhibitors have been recognized as potent novel drug candidates for many years. As EVs can be loaded with different drugs *in vitro*, they qualify as an attractive drug delivery system. The specificity of the recipient cell targeting *in vivo* is understood in a limited way only, although there is evidence of accumulation of specific EVs [43]. For example, EVs from human mesenchymal stem cells accumulated in the liver, spleen and sites of acute kidney injury [78]. Such tropism for a specific cell type, a requirement for targeted drug delivery, appears to be determined by surface proteins of the source cells. The composition of EV membrane reflecting the one of their source cell makes these particles non-immunogenic, and their small size allows them to pass the immune surveillance of the host organism [79, 80]. The reported engagement of exosomes in physiological processes in normal and diseased central nervous system makes them attractive vehicles for delivering neurotherapeutic agents across the blood-brain barrier [81–83]. Nevertheless, their delivery in humans seems so far limited to liver and kidney as they are reported not to reach therapeutic amounts in brain, heart and other tissues due to lack of specific targeting and thus low enrichment of the intended therapeutic ingredient in the target tissue. Modifications of the EV surface membrane to achieve enhanced targeting of a specific cell type are hence a common strategy embodied in several different engineering approaches.

4.2 Mechanisms of EV entry into the target cell

When an EV attaches to the target cell surface, it can in some cases activate the cognate receptors without internalization or transfer of the content to the recipient cell via its fusion with the target cell membrane or via endocytosis [84]. Endocytosis is an active process that requires cytoskeletal remodeling dependent on actin dynamics and includes clathrin-dependent endocytosis, phagocytosis and macropinocytosis. The clathrin-dependent endocytosis has been established as cellular entry for EVs based on the experiments with specific inhibitors of this pathway. Additionally, endocytic uptake of EVs can involve lipid rafts, sometimes dependent on caveolin proteins. The size of EVs may be a limiting factor for cellular entry via endocytosis [85]. The EV uptake by phagocytosis was monitored by their high level of accumulation in phagocytic cells and localization into the phagolysosome [86], as well as the identification of the crucial role of the phosphatidylserine binding T-cell immunoglobulin and mucin domain containing (TIM4) receptor for the uptake of exosomes into macrophages [87, 88]. The contribution of macropinocytosis pathway was revealed with studies where exosomal uptake was decreased by the inhibition of cytoskeletal rearrangements that normally lead to membrane ruffles [89], as well as with its promotion caused by the activation of the agonistically acting epidermal growth factor [90].

5. Molecular engineering to facilitate EV labeling and delivery to target cells

5.1 Modifications of EV membrane with non-covalent and chemical modifications

The role of EVs as encapsulated intercellular messengers makes them attractive for development into nanoscale therapeutic agents [91], and therefore, the need of augmenting the interaction with the recipient cell has been widely recognized. The higher rigidity of the membrane of EVs in comparison with their source cells does not appear to obstruct the efficient application of common hydrophobic insertion strategies to EVs. The introduction of small lipophilic ligands, such as membrane dyes, works effectively for their labeling and aids in monitoring in *in vitro* and *in vivo* experiments. Furthermore, hydrophobic loading is used for encapsulation of certain drugs and leads to their increased stability and therapeutic effect [92, 93].

Due to the negative charge on the recipient cells, binding and uptake of EVs were enhanced by increasing the charge interactions with an artificially introduced positive surface potential, as exemplified in their derivatization with cationic lipids [94]. A downside of this method can be that the extremely charged cationic reagents can cause cytotoxicity and the cellular uptake of the modified particles can proceed differently from the usual pathways, which results in an unpredictable cellular fate of the particle and its cargo and possibly its undesired degradation. The innate slightly negative electrostatic potential exhibited by unmodified EVs should contribute to the longer half-life of such particles *in vivo* as inferred from liposomal studies [95]. Further, the extremes of unbalanced positive charge may negatively impact the storage stability and the application of such reagents in high concentrations.

As an alternative to other methods, labeling of EV surfaces with fluorescent probes has been achieved using click chemistry without an apparent effect on the size and function of the particles [96]. Nevertheless, there is an immense complexity behind the engineering and downstream methods developed for other

biologicals conjugated with small molecules, especially when intended for therapeutic purposes [97, 98].

The idea of decoration of EV membranes with functional ligands further led to the consideration of receptor binding strategies. Such EV functionalization can readily be achieved by modification of the source cell. A resulting opportunity harvesting the specificity of this approach could also be used as a strategy to eliminate vesicles implicated in pathological processes, such as cancer metastasis, or to neutralize the undesired activity of therapeutically applied vesicles. Transferrin-conjugated superparamagnetic nanoparticles, reactive with surface-expressed transferrin receptor of exosomes, enabled their isolation from blood and endowed the vesicles with superior targeting properties [99]. A robust labeling approach of microvesicles was the expression of biotin acceptor peptide-transmembrane domain (BAP-TM) receptor on the source cells in combination with biotinylation *in vivo* [100].

Another example of employing an EV-localizing protein for efficient presentation on the EV surface involved a peptide or a protein fused with the C1C2 domain of lactadherin, which binds to EV outer membrane due to its affinity to phosphatidylserine, strongly enriched in exosome membranes. This method was used to generate antibodies against tumor biomarkers [101] and to increase the host immune response to tumor-associated antigens [102, 103].

Strong binding of lactadherin to the exosome membrane was also the basis of an efficient labeling protocol utilizing overexpression of a fusion protein composed of *Gaussia* luciferase and a truncated lactadherin in source melanoma cells. After harvest by ultracentrifugation, the labeled exosomes were successfully used for *in vivo* biodistribution studies [104].

5.2 Engineering of tetraspanin proteins for detection and monitoring of EVs

Regarding the tetraspanins not only as very abundant, but as prominent structural and stability elements of the exosomal membrane, it is sensible to engineer genetic fusions of these proteins enriched in EVs to ensure a high density of the expressed product and optimize the derivatization for the different purposes of engineering: cognate ligand binding, labeling for visualization or isolation, modifying cargo uptake and transfer and stability (**Figure 3**). Most practically, transgene

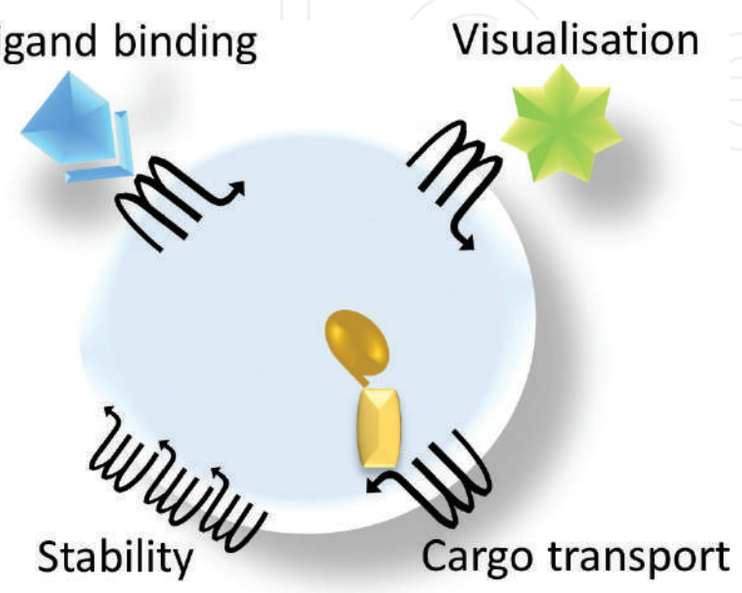

Figure 3.
Main engineering goals utilizing the modification of tetraspanins.

expression in the parent cell would be induced to deliver EVs enriched in the modified protein. The success of such set-up depends on the understanding of both molecular biology and protein architecture of the targeted species.

In one of the pioneering studies, certain sites on the tetraspanin CD63 have been chosen to allow the integration of fluorescent fusion proteins on the extra- and intravesicular side of the exosomal membrane [105]. CD63-GFP fusions have proven valuable reporters in the elucidation of the role of immune synapse in secretion of exosomes from T-cells to APCs and their fusion with recipient cells [106], in determining differential uptake properties of different immune cell subsets for EVs originating from a cancer cell line [107] and in an *in vivo* study imaging the fate of EVs produced by breast cancer cell line in *nude* mice [108].

5.3 Engineering of EV surface proteins for enhanced cell-type specific targeting: introduction of target specificity, stability and improved cargo delivery

In the pioneering example of derivatization of a protein enriched in exosomal membrane to achieve specific targeting [109], the rabies virus glycoprotein (RVG)-peptide fused with lysosome-associated membrane glycoprotein 2b (Lamp-2b) was used to target exosomes to the central nervous system in an *in vivo* mouse model. Immature dendritic cells-derived exosomes, enriched in an N-terminal fusion of an α_v integrin-specific internalizing RGD-sequence containing peptide with this scaffold, could internalize efficiently into target-positive breast cancer cells [110]. The display of such constructs could be efficiently enhanced by introducing a protective glycosylation motive, which improved the surface expression of exosome-bound N-terminally fused peptides by preventing their acid-mediated proteolysis during endosomal passage and indeed led to a more efficient specific cellular uptake of exosomes engineered in this way [111]. Specific targeting of IL3-receptor overexpressing chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells has been shown for exosomes armed with a fusion protein of IL3 and Lamp2b and loaded with Imatinib, a tyrosine kinase inhibitor, and led to a the reduction of tumor size *in vivo* [112]. Exosomes engineered in this way achieved a higher abundance at the tumor site and were hence able to inhibit xenograft growth more efficiently than the active ingredient alone or than loaded control exosomes.

Glycosylphosphatidylinositol (GPI)-mediated anchoring of the specific targeting unit was demonstrated to be a stable alternative to Lamp2b fusions. EVs expressing GPI-anchored nanobodies, specific to epidermal growth factor receptor (EGFR), displayed enhanced binding to the EGFR-overexpressing cancer cells. This, however, did not lead to an increased uptake, and it was suggested that in this particular biological system not only the affinity but also the density of the targeting ligand must be high enough to induce receptor clustering and subsequent internalization [113]. Exosomes derived from a HEK cell line transfected with a construct of platelet-derived growth factor (PDGF)-anchoring sequence and an EGFR-binding peptide were on the other hand efficient in targeting EGFR-expressing tumors *in vivo* and reducing their size with delivery of microRNA Let7 [114]. Enhanced uptake has been achieved for EVs enriched in a fusion protein of tetraspanin CD63 and stearylated octaarginine, a representative cell-penetrating peptide, by their ability to induce active macropinocytosis [115].

The concept of enhancing the stability of the targeted exosomal surface protein intended for fusion with a targeting agent to allow a higher degree of versatility for their modification for an improved target recognition has raised interest in their engineering at the protein level. A significant increase in thermal stability has been achieved by introduction of additional disulfide bonds in the EC2 of tetraspanin CD81, with the best variant exhibiting a positive shift in the melting temperature

for 45°C comparing with the wild-type protein [116]. When engrafted with a human transferrin-receptor specific peptide, the stabilized scaffold exhibited significantly better biophysical properties than the analogously engrafted wild-type protein. In the same study, a mutant has been discovered that exhibits reversible unfolding behavior up to a temperature of 110°C in contrast to the wild-type CD81 EC2, which presents another option for extensive engineering required for directed evolution of tetraspanin proteins towards novel antigen binding.

Not only can the overexpression of tetraspanins in source cell lines increase the production and stability of exosomes, but also the exosomes engineered to contain adeno-associated viruses (AAVs), designed for improved delivery of genetic material to target cell [100], were superior in their yield and functionality when CD9 was overexpressed in AAV-producer cells [10]. CD9 overexpression has also increased the speed and transduction efficiency of lentiviral gene delivery into numerous cell lines, confirming the important role of this tetraspanin in gene transfer [117].

The current scope of EV engineering reaches beyond receptor targeting systems and aspires towards modifications with complex modules, assigning them simultaneously with multiple novel functionalities, such as specific recognition as well as enzymatic activity, to enhance their potential therapeutic effect. Recently, regression of orthotopic Her2-positive tumors has been achieved by applying exosomes, able of specific targeting via their surface decoration with a fusion protein of high-affinity anti-Her2 single-chain Fv, and containing mRNA encoding a bacterial enzyme Hchr6, which in the strongly Her2-positive cells catalyzed the conversion of the prodrug CNOB to cytotoxic MCHB [118]. Tetraspanin engineering could support a sophisticated concept to aid intracellular delivery of exosomal cargo proteins, directionally incorporated during vesicle biogenesis [119]. Genes encoding two recombinant proteins, a fusion of photoreceptor cryptochrome 2 (CRY2) and the protein of interest and a fusion of tetraspanin protein CD9 and CRY-interacting basic-helix–loop–helix 1 (CIB1), were co-transfected into a single cell line. The blue light-induced binding of CRY2 and CIB1 enabled docking of CRY2-fused target proteins into nascent exosomes, and in the absence of the blue light, the cargo protein was released into the exosomal lumen. Transfer of Cre recombinase confirmed the efficiency of this system *in vitro* and *in vivo*.

6. Conclusions

The tetraspanins, well established as the biomarker proteins of extracellular vesicles, have been addressed for increasing EV stability and improving their function as delivery vehicles, both by assigning them with target recognition properties and modulating their cargo transfer. From the current point of view, the complexity of the tetraspanin-mediated interactions and signaling networks formed in a cell is yet to be discerned. Tetraspanins are known to interact naturally with a plethora of cell surface-bound ligands, which results in potent biological effects conveyed through different pathways; however, systematic evaluation of the affinity of the association with the interaction partners would assist in prediction of the consequential cellular processes as well as in determining optimal choice of the tetraspanin targeted for modification. There are recent reports describing modified tetraspanins mediating both surface protein interactions and an intracellular fusion-mediated enzymatic activity, which underline the feasibility of engineering versatile functions into tetraspanin proteins as fusion partners. The structural details on tetraspanins modified in this way, as well as the read-out revealing their actual behavior in the foreseen role and the influence of such modifications on

the fate of an EV preparation, will pave the way into the design and production of EV-based reagents as therapeutics.

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Conflict of interest

The authors declare no conflict of interest.

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