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CHEMICAL RECIPES, PATHWAYS AND MECHANISMS FOR THE SYNTHESIS OF NEW CHEMICAL ENTITIES AND/OR MATERIALS

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ACRONYMS

CT - Computed Tomography
DOTA - 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetate
DTPA - Diethylenetriamine Pentaacetic Acid
EDC - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
FT-IR - Fourier Transform Infrared
HER2 - human epidermal growth factor receptor 2
IO - iron oxide
hMSCs - human mesenchymal stem cells
kDa - kilo Dalton
MALDI-TOF - Matrix-assisted laser desorption/ionization- time of flight
MRI - Magnetic Resonance Imaging
MS - Mass spectroscopy
MW - Molecular weight
NHS - N-hydroxysulfosuccinimide
NMR – Neutron Magnetic Resonance
NP - Nanoparticle
PAMAM - Poly(amidoamine)
pDFEA - poly[*N*-(2,2-difluoroethyl)acrylamide]
PEG - polyethylene glycol
pHPMA - poly[*N*-(2-hydroxypropyl)methacrylamide]
pMOX - poly(2-methyl-2-oxazoline)
RAFT - Reversible Addition–Fragmentation Chain Transfer
SEC-MALS - Size Exclusion Chromatography Multi Angle Laser Light Scattering
SPPS - solid-phase polypeptide synthesis
UV/Vis - Ultraviolet/Visible

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1. Synthesis of Nanomedicines: a General Perspective

Nanomedicine is a result of application of nanotechnology to achieve breakthrough innovations in healthcare. Physical properties of materials change at the nanometer scale and nanomedicine exploits these specific properties to change healthcare treatment paradigms. Early detection of cancer cells is a major opportunity for an accurate diagnosis and efficient treatment. It drastically improves the chance of survival and recovery of patients. Nanoparticles can already be used as innovative contrast agents to improve the performances of imaging techniques as Magnetic Resonance Imaging (MRI), Computed Tomography (CT) scan, and fluorescence imaging. Nanoparticles can also be used to enhance the signal and better detect cancer biomarkers. These are molecules indicative of the presence of cancer in the body, whether produced by the tumor itself or by the body as a specific response to the presence of the tumor. Last, but certainly not least, nanomedicine products can improve the efficiency of chemical and biological based treatments, e.g., nano-carriers can encapsulate drugs to enable them to reach their target, the tumor, with higher accuracy, thus simultaneously improving treatment efficiency and reducing drug-related toxicity. In 2016, the nanomedicine market was estimated to be between $90 and $120 billion and was projected to steadily increase. 230 nanomedicine products were identified on the market or under clinical development for different therapeutic areas including cancer, diabetes, cardiovascular, neurodegenerative, osteo-articular, infectious diseases, etc. Focusing on cancer prevention, diagnosis and treatment, more than 80 products were identified worldwide under clinical development or on the market (including the first generation of nanomedicine products such as Abraxane, Doxil, DaunoXome, Evacet,Lipo-Dox, MyCare Assays, NanoTherm).

Translation of nanomedicines from laboratory to industrial scale is still a treacherous road, with one of the main obstacles being irreproducible procedures for nanoscale products. To address this issue, we have compiled a set of procedures that have been published and reproduced many times, and thus dependable procedures.

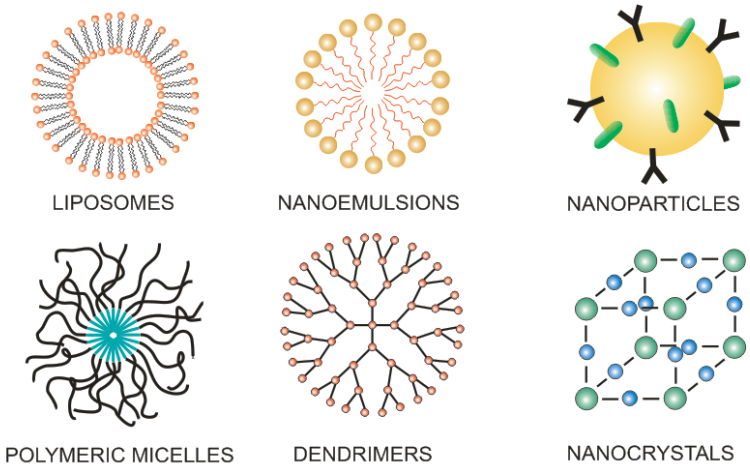

Figure 1. Examples of nanosystems with pharmaceutical potential (the systems are not drawn in the same scale).

2. Synthetic Procedures for Nanomedicines

2.1. SOFT NANOMEDICINES

Nanostructures can be formed in aqueous solutions by self-assembly of amphiphilic polymer architectures consisting of assembly-driving parts (hydrophobic permanently or temporarily in response to external stimulus) and hydrophilic parts providing sterical stabilization of such nanoassemblies in aqueous milieu or eventually biokompatibility due to stealth properties. If the self-assembly driving part is hydrophilic and becomes hydrophobic only at certain conditions or, oppositely, is hydrophobic and becomes hydrophilic or is degraded only at certain conditions, the self assembly/disassembly may become responsive to such external stimulus (such as pH, temperature or redox potential change, illumination causing photochemical change or the presence of certain ions and enzymes in solution).[1] Such stimuli-responsive systems are of extremely high importance in biomedicinal research as this stimulus triggering self assembly/disassembly can also trigger biological function of such system as, e.g., bioactive cargo release.[2] In such cases, the stimuli-responsive nanomedicines are designed to change properties in pathological tissues while being stable in physiological milieu. For pathologically influenced tissue such as solid cancer tissue is typical lower pH, higher temperature, hypoxia or oppositely presence of reactive oxygen species or the presence of enzymes such as matric metalloproteases.

2.1.1. Nanostructures Formed by External Stimuli-Responsive Self-Assembly of Amphiphilic Block, Gradient and Graft Polymer Architectures in Aqueous Solutions

The polymer architectures of this type can be in simplest case the AB or ABA block copolymers where A is hydrophilic block and B is the stimuli responsive/hydrophobic block. Such copolymers typically form micelles with stimuli-responsive block forming the core and hydrophilic block forming corona or, depending on blocks lengths and ratio, cylindric (worm-like) micelles or polymersomes.[3-5] The BAB triblock copolymers may form flower-like micelles at lower concentrations while at higher concentrations, especially if the block lengths are sufficiently high, they can form physically crosslinked gels as the terminal hydrophobic blocks can crosslink micelles into gel.[6] The graft copolymer architectures typically form nanoparticles and nanogels depending on whether the self-assembly driving block is the polymer backbone or grafts and also depending on the polymer backbone/grafts chain lengths.[7] Switching from block to gradient systems (where the blocks gradually penetrate into each other) changes properties of such micelles pronouncing “bitterball” structure with relatively hollow core and dense corona,[8] which is otherwise typical for dendrimers. There are also examples where e.g., ionic gelation or metal chelation is used instead of hydrophobicity[9,10] to drive self-assembly and examples where the systems are responsive to several stimuli simultaneously.

The block and graft copolymers forming such nanostructures are typically produced by stepwise polymerization with procedures allowing living [cationic – e.g., poly(2-

alkyl-2-oxazolines or anionic – e.g., poly(alkylene oxides)] or pseudoliving [RAFT polymerization of vinylic monomers] nature of the process, which allows us to fine tune block lengths and minimizes the presence of homopolymers. Especially, homopolymers of the self-assembly driving block may lead to undefined or anomalous micellization even at very low contents of such homopolymers (this was typical for older systems prepared by connecting of pre-synthesized blocks).

An example of such structures according to our approach[11] are nanoparticles or polymersomes (depending on polymer block lengths) based on using copolymers containing two blocks – hydrophilic poly[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA) or poly(2-methyl-2-oxazoline) (pMOX) and thermoresponsive poly[*N*-(2,2-difluoroethyl)acrylamide] (pDFEA). This composition corresponds with the amphiphilic character of these copolymers and contains sufficient concentration of fluorine atoms for ^{19}F magnetic resonance imaging. Block copolymers were prepared by RAFT (Reversible Addition–Fragmentation chain Transfer) polymerization of acryl and methacrylamide monomers and living cationic ring-opening polymerization of 2-methyl-2-oxazoline. Thermoresponsive function gives us controlled capability of forming nanoparticles upon heating in aqueous solution, which provides well soluble polymers in laboratory temperature and precipitate particles in temperature of human body.

2.1.2. Liposomes

Liposomes are a type of clinically well-established nanoparticle that have been commercially used to deliver cytotoxic drugs, antifungal drugs and vaccines. Some of the approved commercial liposome-based products. There are many more liposomal formulations undergoing clinical trials, which include new generations of liposomes as well as the delivery of genes and imaging agents.[12-18] Discovered by Alec Bangham in the 1960s, liposomes are simply formed from the self-assembly of lipids in aqueous media driven by their amphiphilic nature, resulting in spherical vesicles with an aqueous core enclosed by single or multiple concentric lipid bilayer.[19] The bilayer membrane structure of liposomes resembles the natural cell membranes, allowing them to be used as biological membrane model, for studying drug-membrane interactions.[20-21] Liposomes offer exceptional biological performances, namely biocompatibility, biodegradability, reduced toxicity, and capacity for size and surface manipulations.[22] These properties comprise the outstanding profile that liposomes offer compared to other delivery systems. They have been prepared using a wide range of techniques, where conventional lipid film method is still the most widely descried in the literature.[23]

2.1.3. Synthesis of Dendrimers and Dendrons Containing Peptides and Amino Acids

Dendrimers and dendrons containing or built entirely from amino acids offer several advantages over other drug carriers. They are assumed to be less degraded by specific proteases than linear derivatives and are characterized by biocompatibility and low immunogenicity. However, contrary to PAMAM dendrimers data on in vitro toxicity, plasma stability and organ deposition for polylysine, or polyornithine dendrons or dendrimers are scarce.[24-25]

At the laboratory level, stepwise solid-phase polypeptide synthesis (SPPS) is the most common due to well-developed methodology but usually yields milligram quantities of the sample.[26] In the SPPS method basic amino acids such as lysine or ornithine are used often as branching units, thus allowing formation of different types of dendrons and dendrimers due to the orthogonal approach.[27] To enhance the reaction efficency different coupling reagents and orthogonally protected amino acids are applied.[31] Moreover, the SPPS approach allows to obtain dendrons faster and remove the excess of reagents and by-products easily by washing and filtering the resin without purification and characterization of the intermediate products. However, regarding the SPPS method there are some limitations which should be considered – deletion impurities and by-products may occur because of incomplete coupling or deprotection step, final product may have some impurities since the intermediates cannot be purified.[26]

Optimization of solid support with predefined group occupancy and use of peptide synthesizers reduces time. Although fully automated, microwave peptide synthesizers enabling using of multiple reaction vessel sizes or tanks containing up to 15 L load equipped with mechanical stirring or/and N_2 mixing are available, to our best knowledge, no attempt of their application in attempt of up-scaling of dendrimer synthesis is published up to date.

2.1.4. Carbosilane Dendrimers

First carbosilane dendrimers were independently reported by two research groups. Van der Made and van Leeuwen applied the catalytic hydrosilylation and allylation steps to grow the carbosilane dendrimers up to the fifth generation using tetraallylsilane as a core.[32,33] Roovers et al. performed a similar synthetic route using tetravinylsilane as the core molecule and methyldichlorosilane in the hydrosilylation step.[34]

Carbosilane dendrimers represent a subgroup of the dendrimer family with a silicon atom as a branching point in an exclusively carbon-silicon framework. Therefore, carbosilane structures are inert, non-polar, neutral and chemically, hydrolytically and thermally stable. The absence of polar bonds allows to modify the dendrimer surface using a wide range of

organic reactions. A strong physical-chemical contrast between the inner and outer layers of carbosilane dendrimers enables a formation of, for example, monomolecular micelles. In addition, the carbosilane domain can be only a part of the dendritic structure incorporated in the distinct parts of macromolecules.[35] The non-toxic carbosilane framework offers interesting opportunities for the further investigation in the field of biomedical research.[36,37]

The synthesis of carbosilane dendrimers is almost exlusively divergent process from the core to the interior generations and to the periphery.[38] The divergent synthesis of carbosilane dendrimers comprises a repetitive sequence of two clean, high-yield reactions: (a) a catalytic hydrosilylation and (b) a nucleophilic displacement using Grignard or organolithium reagents. The hydrosilylation reaction introduces branching point, therefore, creates the next generation, while the subsequent nucleophilic substitution forms the branches of the dendrimer. By varying the multiplicity of the branching points the length of the branches, new architectures of the carbosilane dendrimers can be designed. The synthesis of carbosilane dendrimers is very flexible - diverse architectures can be constructed by varying the length of spacers and the branching multiplicity.

2.1.5. Synthesis of PAMAM-Dendrimers

General information: Solvent & reagent purity is highly important! We used HPLC-grade methanol and toluene. The ethylenediamine was distilled at atmospheric pressure and is stable for long periods if kept cold (refrigerator or freezer). Temperatures during evaporation & drying should not exceed +40 °C.

G0: DAB-PAMAM-$(NH_2)_4$[39]

DAB-PAMAM-Core (71.55 g, 0.167 mol) was dissolved in methanol (850 mL). Ethylene diamine (524 g, 8.7 mol, 13 eq. per surface group) was dissolved in methanol (110 mL) and cooled to 0 °C, using an ice bath. The dendrimer solution was added to this solution dropwise over 1 h under nitrogen atmosphere. The ice bath was kept for another 3 h and the reaction was then stirred for 4 days at room temperature. Workup was done by removing as much methanol and EDA as possibly by applying vacuum. After that, azeotropic distillation with a mixture of methanol/toluene 1/9 was performed until all EDA had been removed in vacuum. The excess toluene was removed by azeotropic distillation with methanol. The final compound was gained as a colorless oil in a quantitative yield (91.5 g, 0.167 mol). **MW:** 544.7 **- ^{1}H-NMR:** (500 MHz, MeOD-d_4) [ppm]: δ = 1.30-1.36 (m, 4 H); 2.26 (t, 8 H,^3J=6.8 Hz); 2.34-2.43 (m, 4 H); 2.62 (t, 8 H,^3J=6.4 Hz); 2.65 (t, 8 H,^3J=6.8 Hz); 3.14 (t, 8 H,^3J=6.4 Hz) - **^{13}C-NMR:** (125 MHz, MeOD-d_4) [ppm]: δ = 25.84; 34.59; 42.07; 43.05; 50.86; 54.45; 175.32 - **MS:** MALDI-TOF: m/ z calc. = 567.425 $[M+Na]^+$; m/ z found = 567.425 $[M+Na]^+$; SEC-MALS: Elution time = 19.55 min - **UV/Vis:** ε_{215nm} = 3560 $M^{-1}cm^{-1}$, ε_{280nm} = 30 $M^{-1}cm^{-1}$- **Fluorescence:** λ_{exc} = 360 nm, $\lambda_{em,}$max = 470 nm - **FT-IR:** $\tilde{\nu}$[cm^{-1}]= 3263 (m, ν_{strech}(N-H

Amide); 3076 (w, ν_{strech}(C-H); 2934 (m, ν_{strech}(C-H); 2862 (m, ν_{strech}(C-H); 1629 (s, ν_{strech}(C=O Amide); 1545 (s, $\nu_{bending}$(N-H Amide); 1463 (s); 1432 (s); 1314 (s); 1232 (m); 1191 (s); 1116 (m); 1027 (w); 945 (w); 818 (m).

2.2. HARD NANOMEDICINES

2.2.1. Multifunctional Gold Ganoparticles for Targeted Delivery of Radioisotopes and/or Drugs

One of the most conventional routes for AuNPs synthesis, developed by Turkevitch et al. in 1951, but still in use, consists on the reduction of Au(III) in $HAuCl_4$ by citrate in water. It is known as the citrate reduction method, which allows the formation of citrate stabilized AuNPs and a controlled size of the particles by varying the citrate/gold ratio.[40] This method provides nanoparticles that can be used as precursors in the synthesis of other AuNPs due to the non-covalent nature of the citrate-gold binding, which can be easily replaced with other molecules of interest, namely with thiol or even amine functional groups.

In 1994, Brust et al. introduced a new procedure for the efficient synthesis of stable gold nanoparticles with reduced dispersity and controlled size. This procedure is based on the use of thiolated ligands that strongly bind to gold due to the soft character of both Au and S. After addition of a reducing agent (*e.g.* $NaBH_4$), the Au(III) is reduced to Au(I) and the AuNPs are formed. This opened the opportunity to develop AuNPs using a great variety of thiolated ligands. This method allows the control of core nanoparticle size by shifting the ratio of thiol/Au in the reaction mixture; for instance, the use of larger thiol/Au ratios affords smaller core sizes with less polydispersity.[41] The obtained AuNPs can be repeatedly isolated and re-dissolved in common solvents without irreversible aggregation or decomposition. They also maintain the capability of being further functionalized with different thiolated molecules, since the Au of the nanoparticle surface is capable of reducing the thiol group and afford Au-S covalent bonding.

With this in mind, AuNPs can be functionalized with a variety of thiolated chelators, ranging from linear (e.g. DTPA)[42,43] to macrocyclic derivatives (e.g. DOTA),[44] affording stable radioisotope coordination for potential nuclear imaging or radiotherapy. Additionally, decoration of AuNP surface with different thiolated bioactive molecules (e.g. peptides, antibodies) can also be explored and afford nanoparticles with higher cancer cell affinity and tumor targeting.[44,45]

AuNPs can also be functionalized with thiolated chemotherapeutic drugs, based on known organic (e.g. doxorubicin)[46] or inorganic compounds (e.g. Pt-based (cisplatin)).[48] The release of these drugs from the nanoparticle structure in a controlled manner can be

achieved, typically through the usage of cleavable linkers, that are sensitive to either internal stimulus (triggered within a biologically controlled manner, such as pH or glutathione)[49] or external stimulus (triggered with the support of stimuli-generating processes, such as the application of light).[50]

2.2.2. Synthetic Routes for the Preparation of Iron Oxide Nanoparticles

Due to many desirable features such as biocompatibility, biodegradability, ease of synthesis and absence of hysteresis, the investigation of superparamagnetic iron oxide nanoparticles (IO NPs) has intensively increased in particular for their biomedical applications such as targeted drug delivery, magnetic resonance imaging (MRI), magnetic hyperthermia and thermoablation, tissue repair, biosensing and cellular therapy.[51] Characterized by iron and oxygen, there are eight known different iron oxides; among these, three are most valuable: hematite (α-Fe_2O_3), maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4).[51] In the past decades, a lot has been done to find a facile and flexible synthetic route able to produce IO NPs with the desired size and acceptable size distribution without aggregation. According to the state of art, the most practiced synthesis routes can be grouped in:

1. Wet chemical synthesis: co-precipitation, microemulsion, thermal decomposition, sol-gel processing, polyols, hydrothermal/solvothermal routes, sonochemical synthesis, micro-wave assisted synthesis;

2. Vapor/aerosol synthesis: spray pyrolysis, laser pyrolysis.

3. Biosynthesis

The main characteristics of each method with its advantages and drawbacks will be briefly described below.

Co-precipitation

Among the chemical methods of synthesis of IO NPs, the aqueous co-precipitation is the most commonly used.[52] It consists of mixing ferric and ferrous ions in a 1:2 molar ratio in very basic aqueous solution at room temperature; the size, shape and composition of the SPIONs are directly subject to the experimental parameters, such as the types of iron salts (chlorides, perchlorates, sulfates etc.), Fe(II)/Fe(III) ratio, pH value and ionic strength of the mediu.[53] This method offers the possibility to obtain spherical NPs.[53] However, the resulting NPs present problems of aggregation and large size distribution, which is common in aqueous routes, in addition to poor crystallinity and tendency to oxidize, thus compromising their magnetic properties.[52]

Microemulsion

This system combines a stable isotropic mixture of oil, water and a surfactant (usually with additional co-surfactant).[51-53] The main advantage of utilizing this methodology to prepare IO NPs is that their size can be controlled by tuning the size of the micelles, formed by the surfactant. However, it requires a large amount of organic solvents, causes difficulties related to the residual surfactants on the NPs surface and the impossibility to reach high temperatures, which means NPs with low crystallinity and in low yield.[54]

Thermal decomposition

One of the best methods to obtain high control over the shape and size of the IO NPs, narrow size distribution and good crystallinity is the thermal decomposition of organometallic or iron precursors in organic solvents. This procedure displays superior properties (high quality in term of size, dispersion and crystallinity thanks to high temperature and surfactant) to those obtained with co-precipitation and microemulsion since nucleation can be separated from the growth phase and complex hydrolysis reactions can be avoided. On the other hand, the use of toxic chemicals, such as chloroform or hexane, makes this approach not environmentally friendly. Moreover, because of the hydrophobic coating, an additional step is needed to make water-dispersible and biocompatible NPs, which are only dissolved in non-polar solvents.[51,54,55]

Sol-gel and polyol process

The sol-gel reaction is a wet-chemical method in which iron alkoxides and salts (e.g., chlorides, nitrates and acetates) undergo reactions of condensation and hydrolysis,[2] to obtain a 3D metal oxide network (wet gel). Factors such as pH, temperature and concentration of the reagents can strongly influence the final crystallinity of the product. This process provides a simple method to synthesize monodispersed and large-sized NPs at ambient conditions. Still, post-synthesis heating treatment is needed to reach the desired crystallinity and, in addition, a purification step is required because of product contaminations.[51,54] The polyol method is understood as an inversed sol-gel method (the sol-gel method uses an oxidation reaction but polyol synthesis uses a reduction reaction).[51] The polyol is heated to its boiling point, and acts as a solvent and reducing agent in a medium with iron precursors, controlling the growth of the particles.[52] The main advantages of sol-gel methods are easy dispersion of IO NPs in water, high crystallinity, and high saturation magnetization. The disadvantages are the relatively high cost of the metal alkoxides and the release of large amounts of alcohol during the calcination step, requiring safety considerations during the sol-gel process.[51]

Hydrothermal routes

Called also solvothermal, when organic solvent is used in a non-aqueous system, the hydrothermal process is particularly appropriate for the growth of IO NPs with a good control over their composition and shape.[51] Synthesis takes place in a sealed container from the

high-temperature solution under high vapor pressure. The process is very adaptable, it grants the possibility to create crystalline phases which are not stable at the melting point. Despite being so versatile, it is characterized by a slow reaction kinetics at any given temperature.[51,53,54]

Sonochemical Synthesis

This procedure uses the effects of ultrasound deriving from acoustic cavitation. Briefly, it consists of creation, growth and implosive collapse of bubbles in liquid: this localized implosion creates high temperature and high pressure, which are perfect conditions for the preparation of bare or functionalized IO NPs from aqueous ferrous salts. Synthesis is fast, very simple and allows to have biocompatible NPs with excellent colloidal stability However, it not useful to realize the fabrication of IO NPs with controllable shapes or narrow size sistibution.[51,54]

Microwave-Assisted Synthesis

Is a relatively simple and recent method, in which a mixture containing the iron precursors is exposed to microwave electromagnetic radiation, causing molecule reorientation, and strong and homogeneous internal heating.[2] Due to the instantaneous 'in core' heating of materials in a homogenous and selective manner, different from the classical ones, this method has several advantages such as low-cost, reduced time of reaction and narrow size- and shape-control of the IONPs.[52]

Spray and Laser Pyrolysis

Spray and laser pyrolysis are the main aerosol technologies for fabricating magnetic IO NPs, which allow continuous production of IO NPs. In spray pyrolysis, a solution of ferric salts is sprayed into the reactor in the presence of a reducing agent. The solute condenses while the solvent evaporates. The dried residue contains NPs. It is a useful method to prepare colloidal aggregates of NPs.[53] Maghemite particles from 5 nm to 60 nm with diverse shapes have been generated using different iron precursor.[56] For reducing the reaction volume, laser became the energy resource and heated a gaseous mixture of iron precursor and a flowing mixture of gas producing small, narrow size, and non-aggregated NPs in the pyrolysis process. However, the final IO NPs made by this process had a very broad size distribution due to the difficulty of obtaining a uniform size of initial droplets or gaseous mixture.[51]

Biosynthesis

The microbial enzymes, bacteria or the plant phytochemicals with anti-oxidant or reducing properties are responsible for the reduction of salts and formation of NPs. [51] Generally, the biosynthesis method is a green chemical and eco-friendly route, and the obtained products

exhibit good biocompatibility. Currently, how to control the size and shape of IO NPs and the exact reaction mechanism is still now fully understood. [51]

Summary

In terms of simplicity of synthesis, classical co-precipitation is the preferred route. In terms of size and morphology control of IO NPs, thermal decomposition seems the best method to develop IO NPs smaller than 20 nm, and the hydrothermal or solvothermal method seems to be the most suitable for producing IO NPs larger than 20 nm. [51]

2.2.3. Laponite®-Based Nanomaterials

Laponite® is a synthetic clay that is composed of disk-shaped (about 25 nm in diameter and 0.92 nm in height) crystals with a chemical empirical formula of $Na^{+0.7}[(Si_8Mg_{5.5}Li_{0.3})O_{20}(OH)_4]^{-0.7}$.[57] An important feature of these nanodisks is that their faces possess a negative charge whereas their edges, due to exposed hydroxyl groups that can be protonated, depending on the pH, can show a positive charge.[58] Laponite® was shown to degrade, particularly under acidic conditions, giving rise to products such as aqueous silica ($Si(OH)_4$), and sodium, magnesium and lithium ions.[59] Laponite® XLG, a special grade having low heavy metals content, was found to be non-toxic towards human mesenchymal stem cells (hMSCs) when using concentrations lower than 1 mg/mL.[60] So, based on all these characteristics, as such or in combination with other materials, this clay may be an interesting platform in the design of nanomaterials for biomedical applications, including in cancer treatment[61-63] and bioimaging.[64,65]

In order to prepare Laponite®-based nanomaterials, the clay disks can be functionalized by covalent and non-covalent means.[66] The silanol groups present in Laponite® edges, for example, can be used for covalent functionalization by reaction with alkoxysilanes (silanization) that have additional reactive groups, like primary amines, methacrylates, benzophenones, and tertiary bromines, thus acting as intermediates in the formation of inorganic/organic hybrid nanomaterials.[67] Non-covalent functionalization of Laponite®, on the other hand, is mainly based on electrostatic interactions. Here, the interactions are established between the polar molecules or molecular ions present in the environment with either the negative charges at the clay surfaces or the positive charges at the clay edges.[68] Importantly, non-covalent functionalization of Laponite® with poly(ethylene glycol) gives rise to stable colloids in physiological media.[69]

2.3. ORNAMENTATION OF NANOMEDICINES WITH BIOMOLECULES

2.3.1. Inclusion of Nucleic Acids into Nanoconstructions

First carbosilane dendrimers were independently reported by two research groups. Van der Made and van Leeuwen applied the catalytic hydrosilylation and allylation steps to grow the carbosilane dendrimers up to the fifth generation using tetraallylsilane as a core.[70,71] Roovers et al. performed a similar synthetic route using tetravinylsilane as the core molecule and methyldichlorosilane in the hydrosilylation step.[72]

Carbosilane dendrimers represent a subgroup of the dendrimer family with a silicon atom as a branching point in an exclusively carbon-silicon framework. Therefore, carbosilane structures are inert, non-polar, neutral and chemically, hydrolytically and thermally stable. The absence of polar bonds allows to modify the dendrimer surface using a wide range of organic reactions. A strong physical-chemical contrast between the inner and outer layers of carbosilane dendrimers enables a formation of, for example, monomolecular micelles. In addition, the carbosilane domain can be only a part of the dendritic structure incorporated in the distinct parts of macromolecules.[73] The non-toxic carbosilane framework offers interesting opportunities for the further investigation in the field of biomedical research.[74,75]

The synthesis of carbosilane dendrimers is almost exlusively divergent process from the core to the interior generations and to the periphery.[76] The divergent synthesis of carbosilane dendrimers comprises a repetitive sequence of two clean, high-yield reactions: (a) a catalytic hydrosilylation and (b) a nucleophilic displacement using Grignard or organolithium reagents. The hydrosilylation reaction introduces branching point, therefore, creates the next generation, while the subsequent nucleophilic substitution forms the branches of the dendrimer. By varying the multiplicity of the branching points the length of the branches, new architectures of the carbosilane dendrimers can be designed. The synthesis of carbosilane dendrimers is very flexible - diverse architectures can be constructed by varying the length of spacers and the branching multiplicity.

2.3.2. Conjugation of Monoclonal Antibodies to Metal Nanoparticles

Gold nanoparticles (AuNPs) with unique properties, such as small size, high biocompatibility, low toxicity, and versatility due to the ease of surface functionalization are very promising candidates for potential clinical ways of drug delivery.[77-79] Traditional cancer therapies often cause significant damage in normal cells, whereas conjugation of therapeutic agents to vectors improves the selectivity and enhances the cytotoxicity. One of the most commonly used receptor-targeting agents in the treatment of patients diagnosed with breast cancer is monoclonal antibody – trastuzumab (exhibits affinity to HER2-positive tumour cells).

Conjugation of Trastuzumab to AuNPs

Firstly, in order to avoid agglomeration and uptake by mononuclear phagocytes system resulted in increasing circulation time in blood, the surface of synthesised nanoparticles (5 nm)[80] was modified by polyethylene glycol (PEG linker comprising the disulfide bridge with carboxyl groups at each end). Next, a mixture of 2-fold excess molar amounts of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) was added to the solution of pegylated gold nanoparticles to form an amide bond with the amine group of lysine. After an additional 4h, appropriate amounts of trastuzumab was attached to the AuNPs-PEG-NHS. The bioconjugate solution was alkalinized to pH 9.0 and left under stirring for 24h at room temperature in an inert gas atmosphere. The final product was purified in dialysis cassettes with a membrane of 30 kDa.[81]

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