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Nanocarriers with Multiple Cargo Load—A Comprehensive Preparation Guideline Using Orthogonal Strategies

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Multifunctional nanocarriers enhance the treatment efficacy for modern therapeutics and have gained increasing importance in biomedical research. Codelivery of multiple bioactive molecules enables synergistic therapies. Coencapsulation of cargo molecules into one nanocarrier system is challenging due to different physicochemical properties of the cargo molecules. Additionally, coencapsulation of multiple molecules simultaneously shall proceed with high control and efficiency. Orthogonal approaches for the preparation of nanocarriers are essential to encapsulate sensitive bioactive molecules while preserving their bioactivity. Preparation of nanocarriers by physical processes (i.e., self-assembly or coacervation) and chemical reactions (i.e., click reactions, polymerizations, etc.) are considered as orthogonal methods to most cargo molecules. This review shall act as a guideline to allow the reader to select a suitable preparation protocol for a desired nanocarrier system. This article helps to select for combinations of cargo molecules (hydrophilic-hydrophobic, small-macro, organic–inorganic) with nanocarrier material and synthesis protocols. The focus of this article lies on the coencapsulation of multiple cargo molecules into biocompatible and biodegradable nanocarriers prepared by orthogonal strategies. With this toolbox, the selection of a preparation method for a known set of cargo molecules to prepare the desired biodegradable and loaded nanocarrier shall be provided.

1. Introduction

Therapies with a single drug often lack the efficiency to treat complex diseases. A combination of multiple active agents can create multifunctional and/or synergistic systems and enhance the treatment efficiency. This review article will act as a guideline on how to prepare biodegradable nanocarrier systems by so-called "orthogonal" encapsulation strategies loaded with multiple cargo molecules. "Orthogonal" means in this case that the preparation of the nanocarrier structure does not hamper the activity of the cargo, e.g., does not react chemically or destroy them physically.

The codelivery of complex drug molecules is, for example, important in modern immunotherapy, as successful tumor vaccination needs a strong and durable immune response. Combinations of several adjuvants demonstrated a synergistic stimulation of immune cells with enhanced antitumor vaccination effect.[1] Codelivery of cargo molecules with high local concentrations at the target site is challenging with common soluble formulations due to systemic distribution and fast excretion of the cargo molecules. Encapsulation of the cargo molecules into nanocarriers poses the only way to enable multicomponent systems and achieve effective simultaneous codelivery. Furthermore, early tumor diagnostic is also an important step to successful tumor treatment. The next level in nanomedicine is the combination of therapy and diagnosis, so-called theranostics, in one multifunctional delivery system to enhance treatment efficacy. Especially the heterogenicity of tumors makes image-guided therapies necessary and multifunctional nanocarriers could be applied for imaging, diagnosis, and therapy simultaneously, enabling early-stage treatment.[2,3] Coencapsulation of cargo molecules with different physicochemical properties is challenging in terms of control, efficacy, and orthogonality of the encapsulation process. Facing the importance of bioactive compounds for medical therapy, orthogonal encapsulation strategies ensure preservation of the cargo's bioactivity and increase the efficacy of drug delivery systems. "Orthogonal" means for one using chemoselective reactions, which do not involve the cargo in the reaction during the preparation of nanocarriers. In the same sense, nanocarriers formed by physical approaches such as self-assembly or coacervation can also be considered orthogonal to the cargo, but also here the cargo should not be altered, e.g. by denaturation.[4] Since orthogonality is an important criterion in encapsulation of medical agents, we will focus on orthogonal pathways to encapsulate multiple cargo molecules into nanocarriers. Furthermore, the

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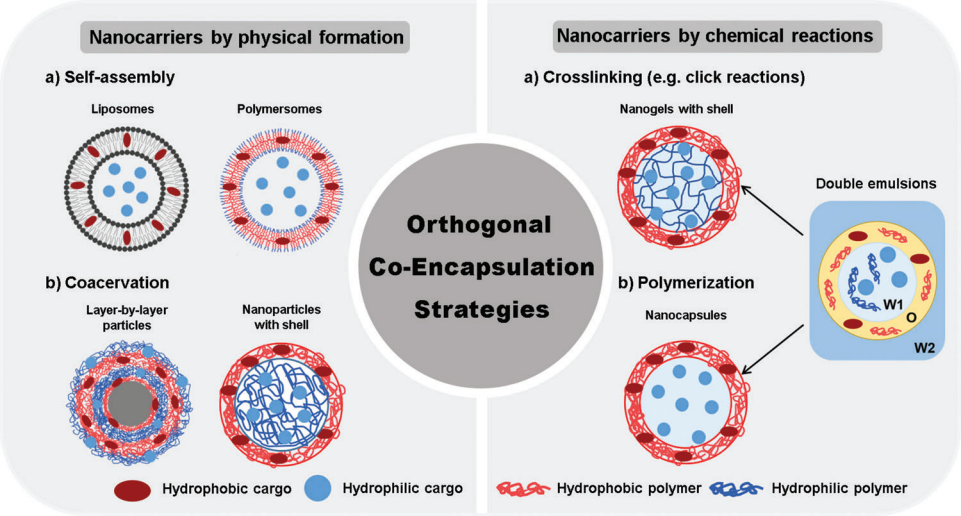

Figure 1. Orthogonal strategies for coencapsulation of hydrophobic and hydrophilic cargo molecules into nanocarriers. Created with Biorender.com.

physicochemical properties of the cargo molecules such as solubility and molecular weight influence the choice of encapsulation strategies. This becomes challenging for coencapsulation, when cargo molecules with different physicochemical properties like, e.g., hydrophobicity, solubility, or molecular weight need to be encapsulated simultaneously. Besides encapsulation, the release of the cargo molecules afterwards at the target site is also essential for the design of an efficient drug delivery system. Therefore, nanocarriers with tailored degradability or release mechanisms are required to ensure successful release of the cargo molecules.

The challenges faced are to provide high encapsulation efficiency, multicomponent encapsulation, and orthogonality as well as biocompatible and degradable nanocarrier materials in one nanocarrier system. This review shall act as a guideline to allow the reader to select a suitable preparation protocol for a desired nanocarrier system. This article helps to select for combinations of cargo molecules (hydrophilic-hydrophobic molecules, low molar mass compounds-macromolecules and organic–inorganic cargo molecules) with nanocarrier material and synthesis protocols. This article summarizes strategies for the coencapsulation of multiple cargo molecules into biocompatible and biodegradable nanocarriers for drug delivery prepared by orthogonal strategies (**Figure 1**). With this toolbox, the selection of a preparation method for a known set of cargo molecules to prepare the desired biodegradable and loaded nanocarrier shall be provided. We focus on biomedical applications, but the principle of coencapsulation by orthogonal approaches applies also for other fields, such as food or energy applications when multiple cargo molecules need to be protected or delivered simultaneously.

Low molar mass drugs (M_W < 1 kDa) can interact and diffuse rapidly through many biological barriers and membranes, theoretically reaching the target site with high effectivity. Their rapid diffusion through the vascular system enables fast systemic distribution but also causes fast excretion.[5,6] Concerning cancer treatment, high local concentrations of drugs lead to high anti-tumor effects and are more desired than systemic distribution. Furthermore, the high cytotoxicity of chemotherapeutics causes severe side effects for patients, when applied systemically. In order to protect the body from the toxic drugs and protect the drugs from fast excretion, encapsulation into nanocarriers is an efficient method to enhance pharmacokinetics of drugs.[7] Biomacromolecules such as antigens,[8] proteins,[9] or nucleic acids (RNA,[10] DNA[11]) have emerged as therapeutics for biomedical applications. The challenge with biobased macromolecular therapeutic agents (BTAs) is their low stability in many biological environments, in which they can degrade quickly. Therefore, nanocarriers as a platform to protect the BTAs during delivery are in major focus to develop novel treatments.[12] The challenge for delivery of macromolecules is their high molar mass, which requires a high degradation degree of the nanocarriers to ensure efficient release.[13] Additionally, to prevent the involvement of sensible BTAs in the nanocarriers preparation and to preserve their bioactivity, bio-orthogonal chemistries have to be chosen.

The solubility characteristics of the cargo molecules is one of the most important factors to be considered, when designing an optimal encapsulation strategy.[14] Hydrophilicity and hydrophobicity of the cargo mediate its solubility in the different phases, i.e., during the nanocarrier preparation, within the nanocarrier and afterwards in the surrounding environment. Therefore, choosing the suitable method with the right solvents influences the encapsulation efficiency during the nanocarrier preparation and entrapment efficiency during the delivery.

2. Encapsulation of Hydrophobic Cargo Molecules

Hydrophobic cargo molecules have a low water solubility (mostly below 1 mg mL^{-1}) and require organic solvents.[15] Many biocompatible and degradable synthetic polymers, such as polylactic acid (PLA), are soluble in organic solvents and thus optimal for the encapsulation of hydrophobic molecules into polymeric nanocarriers. The nanocarriers can be prepared either by physical formation, e.g., precipitation or self-assembly, or by chemical reactions, e.g., polymerization or crosslinking (**Table 1** gives an overview on different techniques to encapsulate hydrophobic cargo).

Table 1. Orthogonal strategies to encapsulate hydrophobic cargo molecules into degradable nanocarriers.

Physical formation	Materials	*d* [nm]	Refs.	Chemical reaction	Materials	*d* [nm]	Refs.
Nanoparticles							
Desolvation	Natural polymers: albumin, dextran, gelatin, chitosan, alginate Synthetic polymers: PLA, PCL, PLGA, PBCA	50–500	[16,19,20,22–26]	Emulsion polymerization: radical, anionic, cationic, ROMP, polyaddition, polycondensation	PS, PMMA, PBCA, PLA, PCL, PLGA, PI	20–500	[54–61]
Self-assembly		50–300	[27–31]				
Solvent evaporation		150–400	[32,33]				
Salting-out		100–400	[34,35]	Crosslinking in or at interface of O/W emulsion	Albumin, dextran, gelatin, chitosan, alginate	150–500	[52,61–66,69]
Polymeric micelles							
Self-assembly of amphiphilic polymers	Hydrophobic block: PLA, PCL, PLGA, PACA Hydrophilic block: PEG, POx, dextran, chitosan, PVP	20–80	[43,44,47–51]	Core-crosslinked	Click crosslinking reactions: CuAAC, SPAAC, IEDDA, thiol-ene	20–100	[52,53]
				Shell-crosslinked			
				Interlayer-crosslinked			

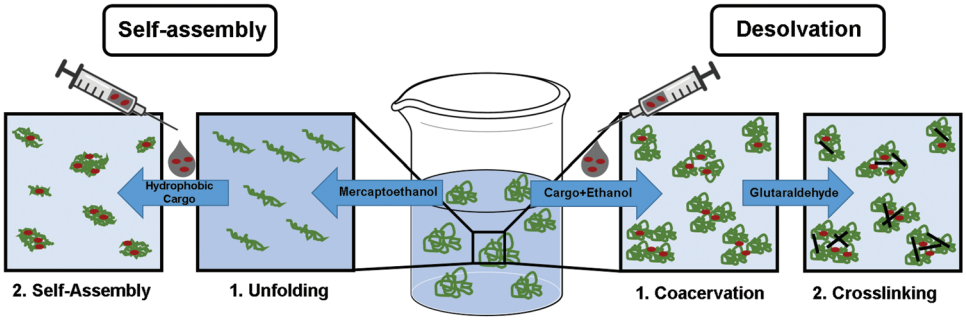

Figure 2. Encapsulation of hydrophobic cargo molecules during the preparation of nanocarriers by self-assembly or desolvation method.

2.1. Nanocarriers by Physical Formation

Nanoparticles are a common class of nanocarriers for the delivery of cargo molecules. Hydrophobic molecules are encapsulated into nanoparticle's matrix during the physical formation of the nanocarriers by precipitation or self-assembly.[16–18] For example in the desolvation method, water-soluble polymers are used as nanocarrier materials and the nanocarriers are formed by coacervation of the water-soluble polymers upon addition of a desolvating agent, i.e., an organic solvent (**Figure 2**). The hydrophobic drug can be dissolved in the desolvating agent, e.g., ethanol or acetone. Upon addition of the drug-containing desolvating agent to an aqueous solution of the nanocarrier material, precipitation of the polymer is induced to form nanoparticles. The drug is entrapped during the precipitation of the polymer.[16,19] The desolvation method is easy and cheap, but often lack high control over size and loading efficiency.[20] The size of nanoparticles is an important parameter for drug delivery, since it may influence their cellular fate, uptake, and clearance.[21] Usually, a size up to 200 nm are considered optimal for drug delivery systems for tumor therapy.[21] With the desolvation methods, nanoparticles with a size range of 50–500 nm can be obtained. The desolvating agent has a great effect on the size and size distribution of the resulting nanoparticles. For example, while methanol enables small nanoparticles with a size below 100 nm, acetone achieves sizes of 300 nm.[22,23] Other process parameters such as stirring rate, dropping speed, pH and temperature influence the particle size as well.[24] Crosslinking of the nanocarriers can improve their stability and encapsulation efficiency. For example, in albumin nanoparticles, glutaraldehyde[25] or carbodiimides[26] react with the primary amines to crosslink the proteins.[17] However, depending on the cargo, targeting amines as crosslinkable groups may not be orthogonal to the cargo (especially when biomolecules are loaded) and other encapsulation strategies need to be considered. A similar method that uses solubility change to form nanoparticles is the self-assembly method.[27,28] Human serum albumin (HSA) nanoparticles were loaded with hydrophobic paclitaxel due to the interaction of the drug with the hydrophobic domains of HSA.[29,30] Using β-mercaptoethanol to break the disulfide bonds in the protein exposes the hydrophobic domains of the protein, which self-assemble upon addition of hydrophobic drugs in aqueous media.[31] Induction of protein self-assembly was also achieved by modifying the primary amine groups with octane aldehyde, increasing the hydrophobicity of the protein.[28] The self-assembly method demonstrated higher drug-loading compared to most desolvation methods.[19] A similar size range of 50–300 nm can be obtained with the self-assembly approach as well depending strongly on the desolvating agent.[27]

Precipitation in emulsions offers the encapsulation of hydrophobic cargo molecules into polymeric nanoparticles with higher control over size and loading efficiency. The hydrophobic

cargo molecule and the polymer (mostly synthetic) are dissolved in the oil phase. The oil-phase is dispersed in an aqueous continuous phase to form an oil-in-water (O/W) emulsion. The nanoparticles are formed by precipitating the polymer inside of the oil droplet through solvent evaporation.[32,33] The hydrophobic cargo molecule, dissolved in the oil droplet, is entrapped in the polymer matrix during the precipitation leading to high encapsulation efficiencies. Since the size with this method can be controlled by the droplet size of the emulsion, nanoparticles with asize range of 150–400 nm can be obtained.[33]

In the salting-out approach, the polymer and hydrophobic cargo molecules are dissolved in a water-miscible organic solvent (e.g., acetone). The addition of salts (e.g., MgCl, CaCl) to the aqueous solution first impedes the miscibility of the solvents, forming an O/W emulsion. Dilution of the emulsion reverses the salting-out effect, leading to diffusion of the organic solvent to the aqueous phase. The polymer precipitates and entraps the cargo molecules within its matrix. Compared to the conventional emulsion diffusion method, the initial formation of an emulsion by salts enables higher control over the preparation of nanocarriers with smaller size, narrow size distributions, and high encapsulation efficiencies.[34] Depending on the salting-out agent and stabilizer nanoparticles between 100 and 400 nm can be prepared.[35]

Synthetic polymers are convenient nanocarrier materials, as they can be synthesized in high purity, controlled sequence and can be tailored to desired properties. Polylactic acid (PLA),[36,37] poly-ϵ-caprolactone (PCL), and poly-D,L-lactide-*co*-glycolide (PLGA)[38] are the most common biodegradable synthetic polymers used for nanoparticles by emulsion techniques.[32,34] Besides these polyesters, also several natural polymers have been applied as matrix for nanocarriers due to their biocompatibility and biodegradability. Since typically hydrophilic polysaccharides (e.g., chitosan,[39] alginate,[40] and gelatin[41,32]) or proteins (e.g., albumins) are used in biomedical applications, the formation of nanocarriers proceeds through desolvation or self-assembly. Owing to their natural role in binding hydrophobic molecules, such as steroid hormones or fatty acids, albumins have been widely utilized as non-immunogenic and non-toxic nanocarrier materials.[19,42]

Self-assembled micelles of amphiphilic block copolymers have also been used to encapsulate hydrophobic drugs in their hydrophobic core.[43,44] The driving force of the self-assembly process is the noncovalent interaction between the hydrophobic blocks in aqueous solution, forming a hydrophobic core and hydrophilic shell. Polymeric micelles have sizes below 100 nm, usually ranging from 20 to 80 nm depending on the length of corresponding block copolymers.[44] The hydrophilic shell allows dispersion of polymeric micelles in water, often poly(ethylene glycol) (PEG) or PEG-alternatives, such as poly(2-oxazoline)s (POx) have been used as the hydrophilic block. Both polymers are highly water-soluble, biocompatible and prolong blood circulation times by the so-called stealth effect.[45,46] Bio-based chitosan,[47] dextran,[48] or poly(vinylpyrrolidone) (PVP)[49] have also been applied as materials for polymeric micelles. Regarding the hydrophobic block, there are various synthetic polymers available, but in context of biodegradability and biocompatibility, PLA, PCL,[49] PLGA,[50] and polyalkylcyanoacrylates (PACA),[51] have received considerable attention.

2.2. Nanocarriers by Chemical Reactions

Polymeric micelles can also be crosslinked at the core, shell, or interlayer depending on the site of the crosslinkable group to increase their stability.[52] For example, chemical groups allowing click chemistry (**Figure 3**A) are attached to the amphiphilic polymers, mostly at the hydrophobic core-forming block, resulting in core-crosslinked micelles. The sizes of micelles range between 20 and 100 nm and can change compared to noncrosslinked micelles depending on the crosslinker.[52] Instead of using an external reagent to crosslink the polymeric nanocarriers, the cargo itself can act as a crosslinker. For example, Liu et al. designed core-crosslinked polymer micelles by phenol-yne click chemistry (Figure 3B).[53] The amphiphilic block copolymer poly(ethylene glycol)-*b*-poly(2-hydroxyethyl methacrylate (mPEG-PHEMA) was modified with alkyne groups by esterification with 5-hexynoic acid (HA). Since the cargo curcumin naturally possesses phenolic groups, the core was crosslinked by a phenol-yne click reaction upon self-assembly and curcumin entrapment. Resulting pH-responsive curcumin-loaded micelles proved higher stability during dilution in water, higher encapsulation efficiency, and thus a more efficient drug delivery compared to non-crosslinked micelles. Owing to the formed vinyl ether bond, the micelles were pH-responsive and rapidly hydrolyzed in intracellular acidic conditions (pH = 5), releasing curcumin in its natural form with preserved bioactivity.

Emulsion polymerization is a common strategy to prepare polymeric nanocarriers and entrap cargo molecules during a chemical transformation.[54] While conventional emulsion polymerization is limited with regards to encapsulation due to diffusion processes, in contrast the microemulsion and miniemulsion techniques allow high encapsulation and offer a versatile chemistry.[55,56] Polymeric nanocarriers prepared by polymerization in emulsions mostly include non-degradable latexes such as polystyrene (PS) and polyacrylates (e.g., poly(methyl methacrylate), PMMA) but also degradable polymers such as PBCA, PLA and PCL or crosslinked biopolymers.

A miniemulsion is formed from dispersing two immiscible solvents by high shear forces, e.g., ultrasonication or a microfluidizer, forming nanosized droplets in a size range of 50–500 nm. Nanoparticles prepared by miniemulsion polymerization have sizes ranging from 80 to 250 nm, influenced by the surfactant, osmotic pressure agent, and shear force.[57] Polymeric nanocarriers can be prepared in miniemulsion by a variety of reactions such as anionic/cationic polymerizations, ring-opening metathesis, polyaddition, and polycondensation.[55] For encapsulation of hydrophobic cargo, a direct oil-in-water miniemulsion is applied. The cargo is dissolved in the oil droplets and is entrapped in the polymer matrix upon polymerization. Huang et al. developed the synthesis of poly(*n*-butyl cyanoacrylate) nanoparticles via anionic miniemulsion polymerization.[58] They demonstrated that the miniemulsion polymerization achieved high loading of hydrophobic paclitaxel compared to conventional emulsion polymerization.

In contrast to miniemulsions, microemulsions form spontaneously and are thermodynamically stable. Their characteristic small sizes (<50 nm) enable unique possibilities due to a large interfacial area, small space domain, and optical transparency. The size of nanoparticles prepared by microemulsion

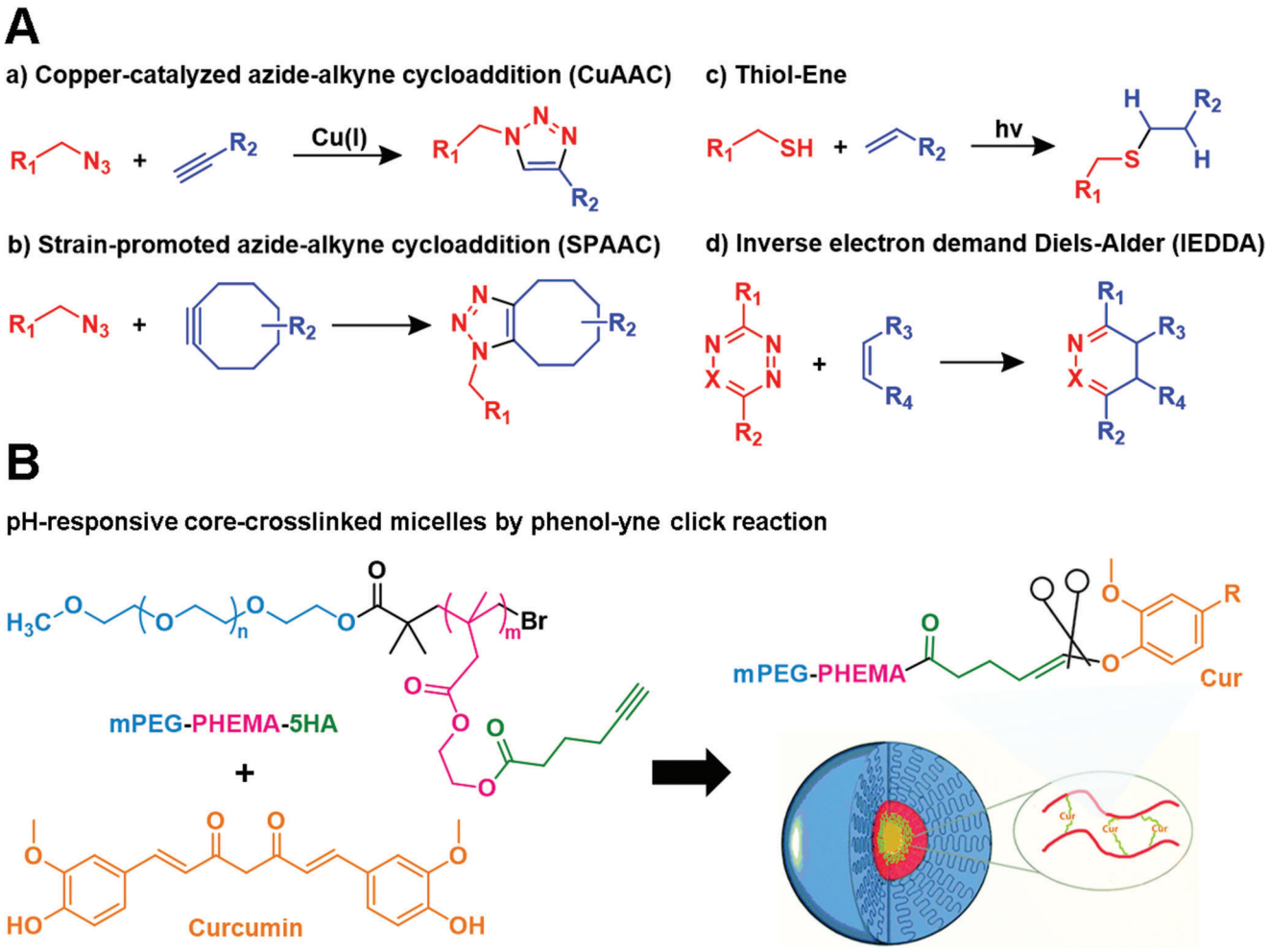

Figure 3. A) Examples of click reactions for orthogonal preparation of nanocarriers via crosslinking. B) pH-responsive reversibly core-crosslinked micelles by phenol-yne click reaction with curcumin. Adapted with permission.[53] Copyright 2019, Royal Society of Chemistry.

polymerization is sub-100 nm.[59] Various polymerizations similar to the miniemulsion techniques have been realized in either W/O, O/W, or bicontinuous microemulsions. However, high amounts of surfactant (and costabilizer) are needed to stabilize the droplets, limiting the use of microemulsion polymerization in spite of their easy preparation. With the development of polymerizable surfactants, the drawback of high amounts of free surfactant was optimized.[60]

Besides polymerizations, nanocarriers have also been prepared by crosslinking either after self-assembly within the dispersed phase or at the interface of the droplets.[61] Click chemistry is the most common orthogonal crosslinking reaction that enables chemoselective crosslinking of the polymer matrix, which needs chemical modification, without reaction with the desired cargo molecules.[62] The most applied click reactions in the biomedical field include azide-alkyne (copper-catalyzed (CuAAC)[63,64] or strain-promoted (SPAAC)),[65] thiol-ene and inverse-electron-demand Diels-Alder (IEDDA)[66] and more (Figure 3A).[67,68] Several nanocarrier morphologies were developed with crosslinking chemistry, for example,, nanoparticles, hollow nanocapsules, nanogels, or micelles.[52] For example, Zou et al. encapsulated the hydrophobic drug paclitaxel into degradable PLGA nanocarriers by CuAAC in miniemulsion. The alkyne-functionalized polymer was crosslinked by a diazide-modified paclitaxel, which served as both the drug and crosslinker, leading to higher encapsulation efficiencies compared to the physically encapsulated drug.[69]

3. Encapsulation of Hydrophilic Cargo Molecules

With increasing interest in biological therapeutics, such as peptides, proteins, or nucleic acids, as well as the extensive development of small, hydrophilic drugs, encapsulation strategies for the delivery of hydrophilic components emerged rapidly.[70] Owing to their high water-solubility, hydrophilic molecules exhibit a rapid clearance, leading to low biodistribution and intracellular absorption.[71] Encapsulation of hydrophilic drugs into nanocarriers improves their pharmacokinetics and delivery efficacy. Orthogonal encapsulation approaches for hydrophilic molecules—similar to hydrophobic cargo—include self-assembly systems or chemical reactions at the interface of—in this case—inverse emulsions (**Table 2**).

Table 2. Orthogonal strategies to encapsulate hydrophilic cargo molecules into degradable nanocarriers.

Physical formation	Materials	*d* [nm]	Refs.	Chemical reaction	Materials	*d* [nm]	Refs.
Nanoparticles/nanocapsules							
Solid lipid nanoparticles: Solvent evaporation in emulsion	Lipids, e.g., fatty acids, fatty alcohols, esters	50–1000	[77–83]	Inverse emulsions: Crosslinking (TDI, TET, TAD, copper-free AAC)	Proteins (albumins), carbohydrates (dextran, hyaluronic acid)	200–500	[54,84–86,90,100]
Spray-drying,	Synthetic polymers: PLA, PCL, PLGA, PBCA	300–5000	[16,74,75]	Inverse emulsions: polymerization (RAFT, anionic, polyaddition, polycondensation)	PBCA, PLA, PCL, PLA-co-PCL, PLGA,	50–500	[60,99]
Desolvation		300–500	[70,72,73]				
Nanogels							
Gelation (temperature, pH, ionic strength)	Natural polymers: albumin, dextran, gelatin, chitosan, alginate	100–500	[19,26,76]	Click crosslinking reactions: CuAAC, SPAAC, IEDDA, thiol-ene	Natural polymers, e.g., hyaluronic acid, dextran	20–250	[101–104]

3.1. Nanocarriers by Physical Formation

Similar to hydrophobic cargo molecules, hydrophilic compounds can be encapsulated into nanocarriers by physical processes such as precipitation and self-assembly.[70] In the desolvation method, the hydrophilic cargo molecule is dissolved in the aqueous media alongside the hydrophilic polymer. The cargo is entrapped into the nanocarriers upon the addition of the desolvating agent and precipitation of the polymers. Biopolymers such as proteins,[17] gelatin,[41] alginate,[40] and other (poly)saccharides[18] but also synthetic polymers such as PLA, PLGA, and PCL have been used to encapsulate hydrophilic molecules by desolvation or salting out.[34] Resulting degradable nanoparticles have sizes around 300–500 nm.[72,73]

A spray drying process can be used to prepare nanocarriers as a dry powder.[16] Spray droplets are generated from a solution of the nanocarrier material and the cargo. The spray droplets are air-dried to form cargo-loaded nanocarriers, which are separated from the drying air as a dry powder. The spray drying method is a convenient process to form nanocarriers in a continuous and scalable single-step approach. The size of the nanocarriers prepared by spray drying can be controlled by the atomization, mesh size, and solvent evaporation. While most spray-dried nanocarriers have sizes in the micron range, particles of nanometer size below 300 nm could also be achieved by this method.[74,75]

Nanosized hydrogels are convenient to encapsulate hydrophilic molecules by an inverse emulsion technique. When using proteins or carbohydrates, nanogels can be formed by gelation upon change in temperature, pH value, or ionic strength with sizes from 100 to 500 nm.[26,76] For example when hydrophilic cargo is dissolved together with proteins, the heat-induced denaturation results in the formation of nanogels, held together by hydrogen bonds, electrostatic or hydrophobic interactions.[19]

Lipids are an optimal nanocarrier material due to their high biocompatibility, biodegradability, and broad availability with the majority of nanocarriers in clinical use are based on lipidic formulations.[77,78] Similar to polymeric micelles, lipidic nanocarriers self-assemble from amphiphilic lipids. Solid lipid nanoparticles are prepared similar to polymeric nanoparticles via precipitation or emulsion techniques, such as solvent evaporation or solvent diffusion.[79] Obtained solid lipid nanocarriers have a size range from 50 to 1000 nm depending on used lipid, droplet size of the emulsion, temperature, and solvent.[80,81] Commonly, lipids such as fatty acids, fatty alcohols, or glycerol esters are used for preparing lipid nanocarriers.[82] Charged lipids such as cationic lipids, which have permanently charged heads (e.g., 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA)), or ionizable lipids, which are protonated at low pH values (e.g., DC-cholesterol), as well form solid lipid nanocarriers. Solid lipid nanoparticles have proven to be excellent nanocarriers for encapsulating and delivering small molecular drugs[82] as well as hydrophilic macromolecules such as mRNA[83] due to ionic interactions of the gene with the charged lipid material.

3.2. Nanocarriers by Chemical Reactions

Hydrophilic molecules can be encapsulated via chemical reactions in inverse W/O emulsions. As nanocarrier material, water-soluble biopolymers can be used, for example, proteins[84] or carbohydrates,[18,85] but also degradable polymers, such as PLA, PCL, PLA-*co*-PCL, PLGA, and others have been used.[26,34] In an inverse emulsion, the cargo is dissolved in an aqueous aqueous solution and dispersed in an organic solvent to prepare a W/O emulsion.[54,86] The cargo is entrapped in the nanocarriers either upon polymerization or crosslinking of polymers,[57] allowing the encapsulation of BTAs such as dsDNA, siRNA, oligonucleotides, or proteins with high efficiency.[12,87] While designing nanocarriers for hydrophilic drugs, the density of the nanocarriers'matrix is extremely important to prevent premature leakage of the hydrophilic cargo from the inside into the surrounding by osmotic pressure difference. Crosslinking of the polymer matrix decreases permeability for small cargo molecules: a crosslinking reagent is added to the inverse emulsion to crosslink the nanocarrier material either i) in the dispersed phase (a hydrophilic crosslinker) or ii) in the continuous phase (a hydrophobic crosslinker that reacts at the interface). In the first case,

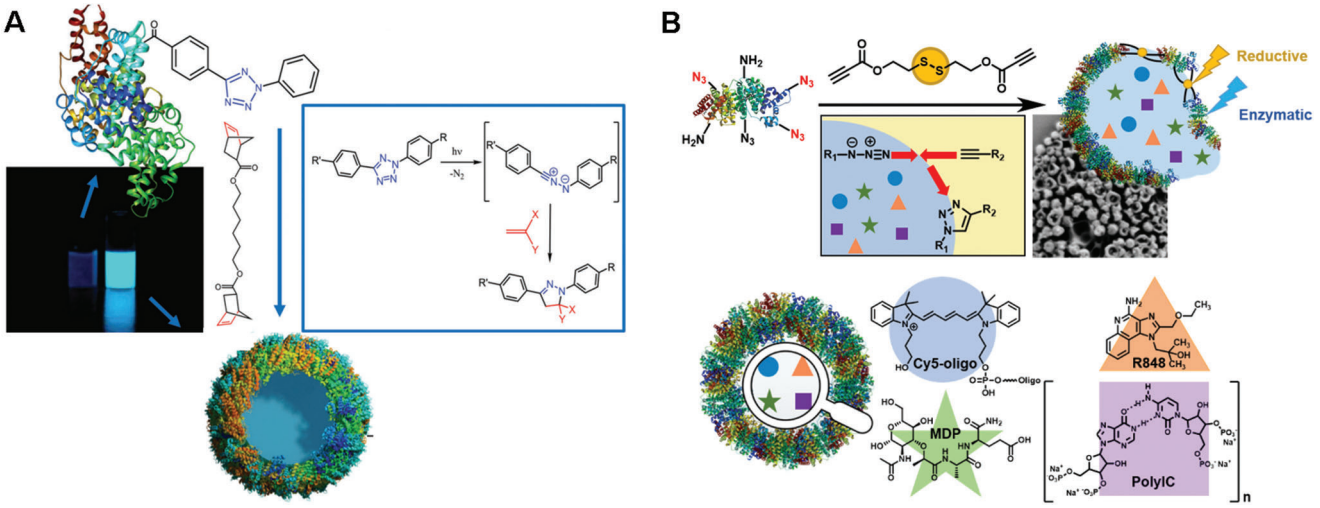

Figure 4. Preparation of protein nanocarriers (PNCs). A) Nonfluorescent protein–TET conjugates were cross-linked by dinorbornene in inverse miniemulsion to obtain self-fluorescent protein nanocarriers; reaction mechanism of the bioorthogonal UV-light induced 1,3 dipolar tetrazole–ene cycloaddition. Reproduced under the term of CC-BY 3.0 license.[95] Copyright 2017, The Authors, published by Royal Society of Chemistry. B) Multicomponent encapsulation into protein nanocarriers through interfacial azide–alkyne crosslinking with hexane-diol-dipropiolate in inverse miniemulsion. Chemical structures of Cy5-oligo dye and adjuvants R848, MDP and Poly(I:C) encapsulated into human serum albumin nanocarriers. Reproduced under the term of CC-BY 3.0 license.[96] Copyright 2022, The Authors, published by Royal Society of Chemistry.

solid nanocarriers are formed, while in the latter, nanocapsules with a solid shell and liquid core are generated, both in the size range of 200–500 nm depending on the droplet size of the emulsion.[88,89] Interfacial crosslinking requires less nanocarrier material and offers high encapsulation efficiency and concentrations of cargo molecules.[61,88] Furthermore, when a chemoselective reaction is applied, the hydrophilic cargo can be encapsulated without their involvement, preserving their activity. Various orthogonal crosslinking types were developed for the interfacial reaction in the inverse miniemulsion to prevent the involvement of the cargo, preserving the bioactivity.[61] If the cargo does not possess nucleophilic groups, such as amines or alcohols, polyaddition with 1,4-toluene diisocyanate (TDI) has been used for various matrix polymers, such as hydroxyethyl starch,[90,91] proteins (e.g., ovalbumin,[84] horse radish peroxidase,[92] or hepatitis c virus protein[93]). However, a major side reaction of TDI-crosslinking is the hydrolysis of the isocyanate groups, which leads first to the formation of amines and eventually urea linkages, which can hamper the biodegradation of the nanocarriers. In spite of this side reaction and possible reaction with cargo molecules, also the straightforward isocyanate chemistry was used to encapsulate and release hydrophilic cargo molecules (e.g., dyes,[84] adjuvants,[94] or dsDNA[90]). However, the content of covalent modification of the cargo molecules remained unclear in these cases. With the development of orthogonal crosslinking reactions, Piradashvili et al. demonstrated the preparation of fully degradable protein nanocarriers using the metal-free tetrazole-ene cycloaddition (TET-click) in inverse miniemulsion (**Figure 4**).[95] The crosslinking reaction between a TET-modified protein with a difunctional strained norbornene was induced by irradiation with UV-light (254 nm). The resulting protein nanocarriers demonstrated a prominent core–shell morphology and self-fluorescence due to the formed pyrazoline cycloadduct by TET-click. A therapeutic cargo, resiquimod (R848), could successfully be encapsulated into protein nanocarriers with high efficiency. Due to the high enzymatic degradability of the protein nanocarriers, the delivery of R848 to dendritic cells followed by intracellular release of the cargo lead to an efficient immune response. In comparison to protein nanocarriers prepared by crosslinking with TDI, nanocarriers by TET-click reaction demonstrated higher immune response, underlining the importance of an orthogonal crosslinking reaction to preserve the cargo's activity. The disadvantage of a TET-click reaction is the need of UV-light to induce the crosslinking process. UV-light or other harsh initiators, such as metal catalysts, can affect sensitive cargo molecules and decrease their bioactivity. Hüppe et al. developed the preparation of protein nanocarriers without the addition of initiators by using a metal-free azide-alkyne click reaction.[96] Azide-modified human serum albumin was crosslinked with hexanediol dipropiolate (HDDP) at the interface of an inverse miniemulsion. A crosslinker such as HDDP with an activated dialkyne reacts under mild conditions without the need of an initiator. With inserting a disulfide bond into the crosslinker, the protein nanocarriers are degradable by enzymatic as well as reductive degradation. By this approach, a triple combination of adjuvants, i.e., Resiquimod (R848), muramyl dipeptide (MDP) and polyinosinic-polycytidylic acid (Poly(I:C)) and dye Cy5-Oligo, could be encapsulated into fully-degradable protein nanocarriers with high control over the encapsulation efficiency. The multi-loaded nanocarriers demonstrated an additive immune activation of dendritic cells, exceeding the single-loaded nanocarriers.

In a similar approach, protein nanocarriers were formed via a Diels-Alder reaction of the tryptophan moieties of proteins with triazolinedione (TAD) in an inverse miniemulsion.[97] In this paper, the authors found that different crosslinking chemistries affected the protein conformation and hence carrier–protein and carrier–cell interaction. Other encapsulation strategies via inverse miniemulsion include carbohydrates as carrier

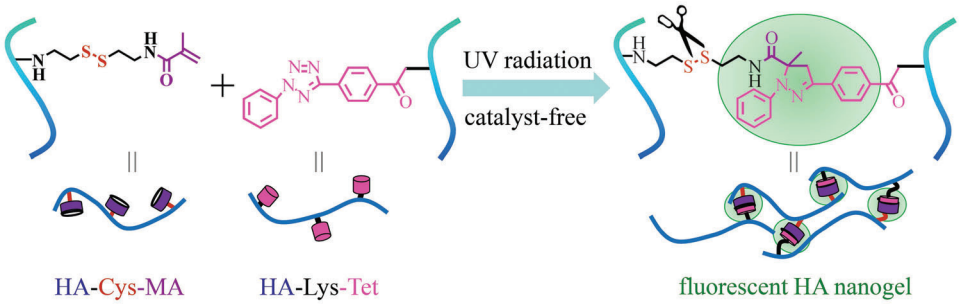

Figure 5. Illustration of nanogel formation from HA-Cys-MA and HA-Lys-Tet via catalyst-free and bioorthogonal tetrazol-alkene photoclick reaction. Reproduced with permission.[103] Copyright 2016, American Chemical Society.

materials, where for example azide-modified hyaluronic acid[85] was crosslinked through a copper-free azide–alkyne click chemistry with hexanediol dipropionate and dextran-based polyaldehydes through polycondensation with polyhydrazides to pH-responsive hydrazones.[98]

Sun et al. designed a novel interfacial reversible addition-fragmentation transfer (RAFT) polymerization in inverse miniemulsion to encapsulate methyl orange and bovine serum albumin) with high efficiency (≈90%).[99] An amphiphilic RAFT agent was synthesized with the desired hydrophilic-lipophilic balance (HLB), acting as the emulsifier as well as RAFT chain transfer agent. The disperse phase contained of the zwitterionic monomer, poly(ethylene glycol) diacrylate as the crosslinker and the initiator 2,2′-azobis(2-methylpropionitrile) (AIBN), resulting in nanocapsules with a well-defined core-shell structure. The zwitterionic nanocapsules (ZNCs) demonstrated dual-responsive swelling dynamics triggered by salt or temperature and cargo was released rapidly.

W/O microemulsions can as well be applied for the preparation of nanocarriers with similar strategies as introduced for miniemulsion. Compared to miniemulsion, microemulsion droplets have a size below 50 nm, presenting excellent templates for the preparation of super small nanocarriers. Nanoparticle sizes prepared by polymerization in inverse emulsions can range from 50 to 500 nm.[54,59] A broad of biodegradable water-soluble polymers were used for the preparation of nanocarriers in reverse microemulsions, including several proteins and polysaccharides.[60] The polymer, dissolved in the water droplet, can be crosslinked ex situ (addition of crosslinker to the aqueous phase before emulsification) or in situ (addition of crosslinker after emulsification). Craparo et al. prepared biodegradable polyaspartamid-nanocarriers by photo-initiated crosslinking in inverse microemulsion.[100] Functionalization of α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) with glycidyl methacrylate (GMA) introduced reactive vinyl and ester groups into the polymer. The double bonds enabled UV-induced crosslinking of the polymers and the ester groups enable hydrolytic degradability of the polymeric nanocarrier. Water-soluble cytarabine could successfully be encapsulated into PHEA-GMA nanocarriers and released upon enzymatic or chemical hydrolysis.

Nanogels are versatile drug delivery systems as they are biocompatible, degradable, and tunable in permeability.[101,102] As emerging nanocarriers for delivery of hydrophilic molecules, especially BTAs, orthogonal approaches were developed, such as metal-free strain-promoted azide–alkyne cycloaddition, thiol-ene, tetrazole-ene, or Diels-Alder reactions.[62,63,101] Click-crosslinked nanogels can be prepared in the size range of 20–250 nm, affected by used material and method.[101,102] Chen et al. reported bioorthogonal encapsulation of intracellular protein drugs, cytochrome c (CC) and granzyme B (GrB), respectively, into hyaluronic acid (HA)-nanogels via catalyst-free tetrazole-alkene photo-click reaction (**Figure 5**).[103] Biocompatible and biodegradable HA-nanogels were prepared from a redox-responsive cystamine methacrylate and lysine-tetrazole derivatives. The protein-loaded HA-nanogels were successfully taken up by cancer cells and protein could be released by reductive degradation of the disulfide bond inside the cancer cells, exhibiting high redox potentials. The protein-loaded HA-nanogels demonstrated high antitumor effects even at low doses. This proves the preserved bioactivity of the protein due to the bioorthogonal reaction.

A similar HA-based approach was developed by Famili and Rajagopal, who crosslinked HA-tetrazine with PEG-norbornene to prepare HA-hydrogels.[104] The catalyst-free inverse-demand Diels-Alder reaction allowed a bioorthogonal in situ encapsulation of Fab1-antibody fragments without altering the cargo. The intact antibody fragments were completely released from the hydrogel matrix over a period of several weeks in vitro and maintained their full antigen-binding capacity. Other nanogels based on biopolymers were prepared by tetrazine-norbornene reaction, such as alginate and gelatin.[101]

4. Coencapsulation of Hydrophilic and Hydrophobic Cargo Molecules

Coencapsulation of multiple cargo molecules with different physicochemical properties is important to create multifunctional nanocarriers with enhanced therapeutic efficacy due to a synergistic effect of multiple therapeutics.[87] Learning from the developed orthogonal encapsulation strategies of hydrophobic and hydrophilic cargo molecules, respectively, drug delivery systems for coencapsulation of both molecules were developed with similar approaches by physical entrapment or crosslinked nanocarriers. Moreover, the codelivery of small molecules and macromolecules requires nanocarriers with, on one hand, low permeability to keep small cargo molecules entrapped, and, on the other hand, an efficient degradability to release large cargo molecules. Depending on the small cargo, hydrophilic or hydrophobic, different approaches have to be taken for coencapsulation of small molecules with macromolecules (**Table 3**).

Table 3. Orthogonal strategies to co-encapsulate hydrophobic and hydrophilic cargo molecules into degradable nanocarriers.

Physical formation	Material/cargo	*d* [nm]	Refs.	Chemical reaction	Material /cargo	*d* [nm]	Refs.
Nanoparticles/nanocapsules							
Layer-by-layer	Anionic: poly(sodium)styrene sulfonate, Cationic: poly(allylamine)*HCl; degradable: PCL-PAA, PArg-PLGA, chitosan, proteins	50-250	[124–134]	Double emulsions: solvent evaporation+ crosslinking	DOX-**erlotinib** , verteporfin-cisplatin	200–400	[32,73,135–146]
Liposomes							
Thin-layer hydration Freeze-thaw cycles dehydration-rehydration, reverse-phase evaporation	Material: charged lipids: DOTMS, DC-cholesterol Cargo: doxorubicin-combretastatin, tariquidar-paclitaxel, cytarabine-daunorubicin	20-10^5	[77,78,105,109–117]				
Polymersomes							
Electroformation, microfluidics, DED	Rhodamine B-camptothecin	20-10^5	[109,118–123]				

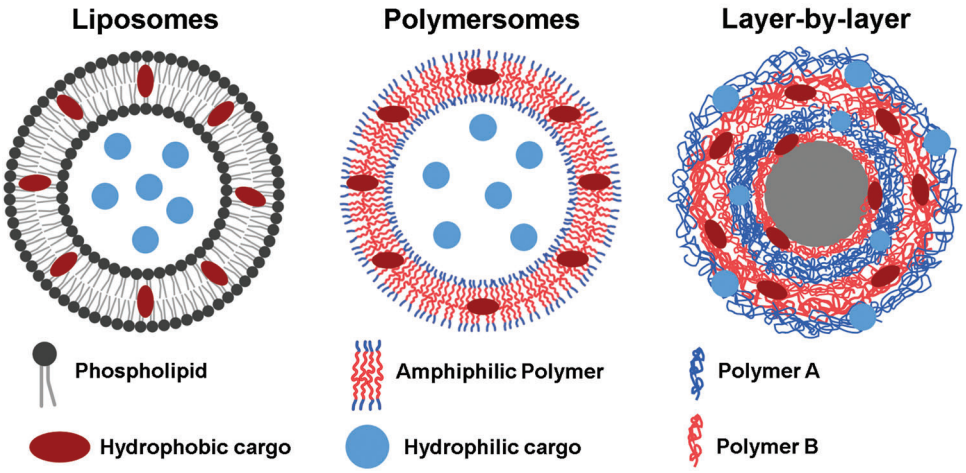

Figure 6. Coencapsulation of hydrophilic and hydrophobic cargo molecules into liposomes, polymersomes, and layer-by-layer nanocarriers. Created with Biorender.com.

4.1. Nanocarriers by Physical Formation

Important for a successful coencapsulation is the presence of two different compartments in the nanocarriers: either a hydrophobic core with a hydrophilic shell or a hydrophobic shell with a hydrophilic core, both of which are able to encapsulate hydrophilic and hydrophobic compounds separately. Combining the encapsulation strategies for hydrophobic and hydrophilic molecules, both can be encapsulated simultaneously into liposomes,[105] polymersomes, or layered polymeric nanoparticles (**Figure 6**).[106–108]

Double-layered liposomes are formed by a self-assembly process of amphiphilic phospholipids in aqueous media, creating a lipid bilayer with a hydrophobic inner layer and a hydrophilic core.[77] Depending on the preparation method, liposomes could be obtained as small (SUVs, 20–100 nm), large (LUVs, 100 nm–1 µm) or giant unilamellar vesicles (GUVs, >1 µm), offering a broad size range for a variety of applications.[109] Phosphatidylcholine originated from soybean or egg yolk, and its hydrogenated derivatives are usually applied as the lipidic nanocarrier material for liposomes.[110] The thin-layer hydration method is a common method for the preparation of liposomes.[77] The lipid solution is reduced and upon the addition of an aqueous solution, the lipid film is hydrated to assemble into a lipid bilayer, forming liposomes. The encapsulation of drugs proceeds with dissolving the hydrophilic drug in the external aqueous phase and the drug is encapsulated with the assembly of the liposomes. Although this method is a convenient approach for the encapsulation into

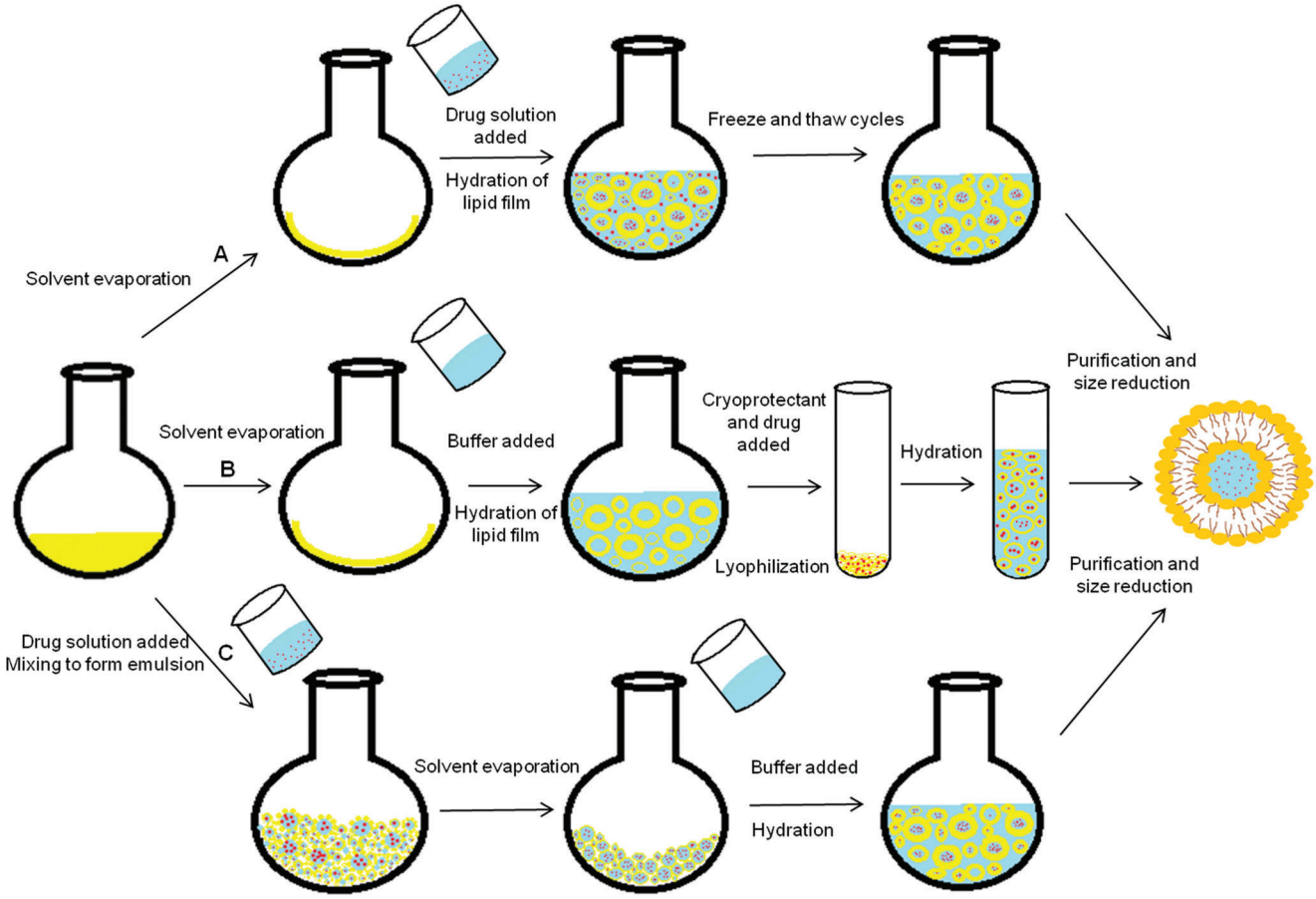

Figure 7. Preparation of liposomes as nanocarriers for encapsulation of hydrophilic cargoes by A) freeze-thaw cycles, B) dehydration-rehydration of preformed empty vesicles and C) reverse-phase evaporation methods. Reproduced with permission.[111] Copyright 2014, Elsevier.

liposomes, it lacks high encapsulation efficiency due to the large volume of the aqueous phase compared to the entrapped volume. If expensive cargoes like therapeutic drugs should be encapsulated, a low encapsulation efficiency is cost-intensive for recycling the drug and scale-up the process in clinical developments.[77] Therefore, other strategies were developed for the efficient loading of hydrophilic cargoes into liposomes. The liposomes are loaded with hydrophilic drugs either by passive loading, where the drug is encapsulated during the self-assembly, or active loading, where first the liposome is formed and the drug loaded afterwards by a transmembrane diffusion due to a pH value or ionic gradient.[111] In case of the freeze-thaw cycle method, the first step is identical to the thin-layer hydration, except as a next step the liposome dispersion undergoes several freeze-thaw cycles to increase the drug loading with each reassembly of the liposomes (**Figure 7**A).

Another strategy with high payload is the dehydration–rehydration approach, where first the liposomes are formed with the thin-layer method using only buffer as hydration solution (Figure 7B). Then the drug is added to the liposome dispersion and lyophilized. With rehydration of the lyophilized liposomes, the drug is encapsulated with high efficiency. A third method, the reverse-phase evaporation, uses an emulsion approach, where an aqueous drug solution is dispersed in the lipid-containing oil (Figure 7C). The liposomes form first with solvent evaporation followed by hydration and the lipid bilayer assembles around the aqueous drug droplets, resulting in high encapsulation efficiency. Besides high loading, the permeability of the lipid bilayer plays a crucial role to prevent the leakage of drugs. In general, the liquid crystalline phase of the bilayer is more permeable to hydrophilic cargoes compared to the gel state.[111] Therefore, the addition of cholesterol mediates the membrane organization and increases the stability of the membrane and entrapment efficiency of the cargoes.[112,113] Other factors influencing the drug loading and entrapment are the lipid concentration, the length of the overall lipid chain as well as the hydrophobic carbon tail length of the phospholipid and (electrostatic) interaction of the drug with the lipid bilayer.[114] Besides efficient drug loading and entrapment into the nanocarriers, the drug release plays a significant role for efficient drug delivery. Owing to the high availability and tailored synthetic approaches for lipidic materials, liposomes can be designed with a variety of release mechanisms with stimuli ranging from pH value, magnetic field, hyperthermia, light, and ultrasound.[115,116]

Upon liposome formation, hydrophobic drugs can be entrapped in the hydrophobic layer and the hydrophilic drug in the aqueous core. For example, hydrophilic doxorubicin (Dox) hydrochloride and hydrophobic dehydrochlorination Dox,[105]

Dox and combretastatin,[117] tariquidar and paclitaxel, cytarabine and daunorubicin and more combinations, were encapsulated into liposomes.[106] The encapsulation efficiency of hydrophobic drugs into the lipid bilayer depends on the solubility of the drug in the lipid bilayer, influenced as well by the membrane composition.[112] Regarding hydrophilic compounds, the membrane properties such as thickness, permeability, and polarity affect the encapsulation of hydrophilic cargo molecules as well.[111] Although liposomes have been widely used to encapsulate BTAs and small molecular drugs, such as DOX-siRNA or PTX-pDNA, loading of BTAs was difficult to control with such methods and ratios of, e.g., siRNAs in the carrier were rather low (≈3500 siRNA per liposome).[106]

Polymersomes present a similar strategy for orthogonal coencapsulation by owning hydrophilic and hydrophobic compartments.[118,119] Similar to liposomes, polymersomes are prepared through self-assembly of amphiphilic block copolymers, which allows size ranges from 20 nm to 200 μm depending on the preparation approach.[109,119] Polymersomes are obtained by solvent-free methods (film hydration, electroformation or gel-assisted hydration) and solvent-displacement methods (solvent injection, emulsion phase transfer, or microfluidics).[109] Often, preparation methods for polymersomes (e.g., electroformation or microfluidics) require special equipment and are time consuming. Houbrechts et al. developed a strategy to form giant polymersomes by a simple double emulsification approach, which allowed efficient encapsulation of large components, such as proteins.[120] Single double emulsion droplets (DED) were used as templates to form the polymersomes by self-assembly upon solvent displacement. Instead of using microfluidics to form the DEDs, a simple double emulsification approach is used with one low molecular weight block copolymer acting as the surfactant for both emulsions and the polymersome material itself. This allowed an easy and fast strategy to form polymersomes with basic laboratory equipment, suitable for clinical scale-up. Cargo molecules are encapsulated into polymersomes by passive entrapment in either the membrane or inner space. In comparison to liposomes, polymersomes have a lower lateral fluidity and transmembrane permeability, retaining cargo molecules with higher efficiency. However, low permeability can be problematic in terms of release. Fortunately, polymers can be synthesized with a variety of chemistry and tuned to desired permeability and responsivity.[121] Besides synthetic amphiphilic block copolymers (e.g., PCL-*b*-PEO), also amphiphilic biopolymers can be applied as nanocarrier material to encapsulate both hydrophilic and hydrophobic compounds into vesicles.[122] For example, Pramod et al. developed vesicles based on amphiphilic polysaccharides for dual-encapsulation of hydrophilic and hydrophobic compounds.[123] The amphiphilic polysaccharide was obtained from naturally available dextran as the hydrophilic backbone, modified with aliphatic chains from the cashew nut as hydrophobic tails. The amphiphilic dextran-based polymer self-assembled into vesicles of $d_h \approx 120$ nm, in which hydrophilic cargo molecules, such as rhodamine B, were encapsulated into the core and hydrophobic molecules, such as anticancer drug camptothecin, in the shell. By encapsulation, the stability of the hydrophobic drug against hydrolysis could be increased almost 10-fold compared to its isolated form. The developed dextran-based vesicles were degradable at the aliphatic ester linkage connected to the dextran-backbone by esterase and thus releasing both cargo molecules. The encapsulated anticancer drug formulation demonstrated a 1.5-fold higher activity in neutralizing fibroblasts than the free drug, underlining the importance of drug delivery systems to enhance therapeutic efficacy. This drug codelivery system is an elegant example for an orthogonal coencapsulation approach into biocompatible and biodegradable polymer vesicles.

In case of polymeric nanoparticles, when combining hydrophilic and hydrophobic polymers by layering them to a nanoparticle, hydrophilic and hydrophobic cargo molecules can be coencapsulated into the respective layer. Polymeric nanocarriers via layer-by-layer (LbL) assembly utilize electrostatic complexation of polyelectrolytes, forming multilayer assemblies of polymers.[124] This method is based on the alternating assembly of oppositely charged polymers around a preformed charged core particle. The LbL-assembly approach applied in aqueous media without the use of chemical reactions or modifications enables a mild encapsulation strategy of sensitive cargo molecules, such as genes, with preservation of their structure and activity. Solid nanocarriers (e.g., polystyrene, silica, or polycarbonate[125]) act as core templates for layering and can be removed by dissolution (e.g., with THF, HF, or HCl, respectively) to obtain hollow nanocarriers. Preformed cargo-loaded nanocarriers (e.g., liposomes, charged nanoparticles, crosslinked nanogels) can as well be applied as core templates to prepare core- and shell-loaded LbL-nanocarriers.[124,126] Depending on the core size and the number of layers as well as the layer material and incorporated cargo molecules, layer-by-layer nanoparticles can be prepared from 50 to 250 nm.[124,127] Usually, inorganic cores (e.g., gold nanoparticles) are around 10–30 nm, while cores from liposomes or organic nanoparticles have a size above 100 nm.[128,129] Since the properties (e.g., permeability) of the nanocarriers depend on the properties of the polyelectrolyte pairs, the majority of LbL-nanocarriers are prepared from synthetic polymers, mainly anionic poly(sodium styrene sulfonate) and cationic poly(allylamine) hydrochloride. However, with regard to biomedical applications, more biocompatible and degradable polyelectrolytes (e.g., poly(*N*-vinyl caprolactam)/poly(L-aspartic acid), poly(L-arginine)/poly(L-glutamic acid)) are of higher interest. For example, LbL nanocarriers were prepared from chitosan/alginate NPs and a combination of lapatinib and paclitaxel could be simultaneously encapsulated into respective layers.[130] The cargo itself can act as a polyelectrolyte: proteins and polypeptides are convenient polyelectrolytes for LbL-approaches due to their amphoteric character.[131,132] Nucleic acids, DNA,[133] and RNA,[129] can act as both cargo and polyelectrolyte: for example, siRNA was used as polyelectrolyte for the LbL-assembly of nanocarriers resulted in higher siRNA content compared to passive entrapment processes (e.g., liposomes).[134] Additionally, the layering can proceed onto a nanosized core, in which small molecular drugs have been encapsulated, providing an efficient strategy for co-encapsulation of drugs and BTAs. Deng et al. utilized the LbL approach to co-deliver siRNA with the low molar mass drug doxorubicin (DOX) to counteract drug resistance of triple-negative breast cancer (**Figure 8**).[134] The authors prepared the nanocarriers from a doxorubicin-loaded liposome (core) and alternately deposited poly-L-arginine and siRNA onto the core via ionic interactions. The resulting NCs effectively entrapped up to 3500 siRNA chains

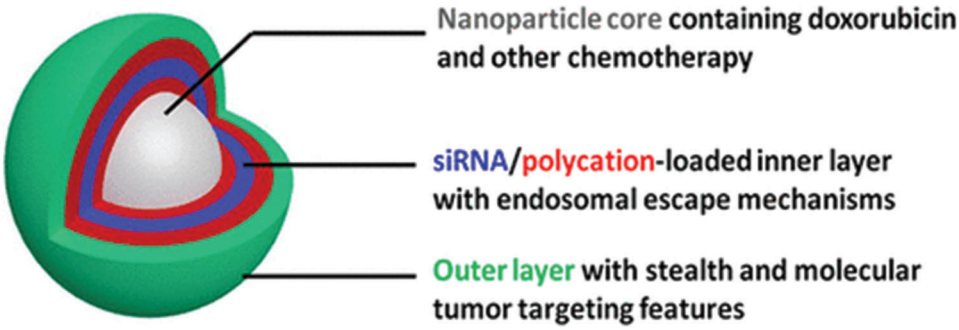

Figure 8. Coencapsulation of biomacromolecules and small molecular drugs into nanoparticles via layer-by-layer method. Reproduced with permission.[134] Copyright 2013, American Chemical Society.

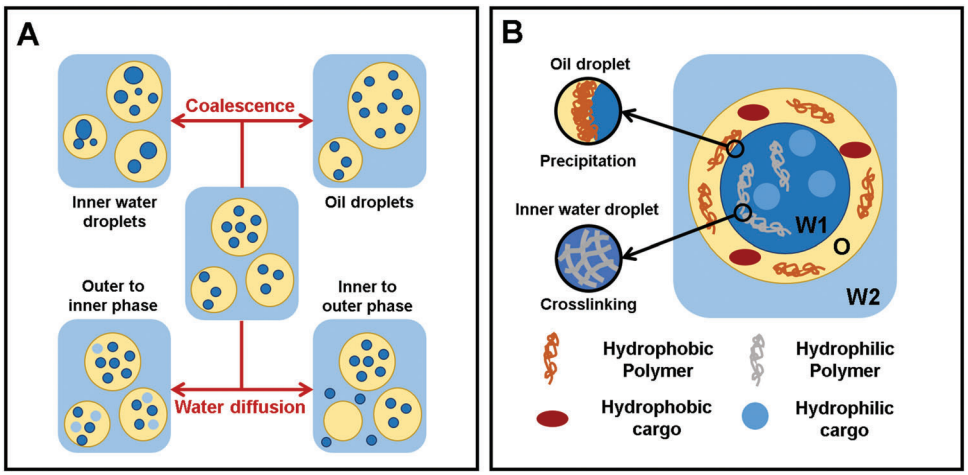

Figure 9. A) Schematic representation of possible instabilities occurring in W/O/W double emulsions. Adapted with permission[135] Copyright 2018, American Chemical Society. B) Coencapsulation of hydrophobic and hydrophilic compounds into nanocarriers by preparation in double emulsions. W1 = inner aqueous phase; O = inner oil phase; W2 = outer aqueous phase.

per layer—similar numbers were obtained in a whole liposome. The dual-loaded NCs demonstrated synergistic effects, with an eightfold decrease in tumor volume compared to the control treatment. The liposome core could load hydrophilic as well as hydrophobic drugs, allowing a broad application for coencapsulation of BTAs with low molar mass drugs. The developed NCs composed of a liposome with layered deposition could further be improved by deposition of an outer layer with stealth and targeting moieties, creating a multifunctional drug delivery system.

4.2. Nanocarriers by Chemical Reactions

Hydrophilic and hydrophobic cargo molecules can also be encapsulated simultaneously by double emulsion approaches.[135–138] Usually, as a first step the hydrophilic cargo is dissolved in the aqueous phase, either or not with a hydrophilic nanocarrier material, e.g., gelatin, which is then dispersed in an organic phase containing the hydrophobic cargo and a nanocarrier matrix material. Then, this W/O emulsion is dispersed in a second aqueous solution to generate the double W/O/W emulsion, which allows nanoparticles sizes around 200–400 nm.[138,139] W/O/W emulsions are prone to the coalescence of the internal water droplets and W/O emulsion droplets (**Figure 9**A). Furthermore, as water diffusion from the internal to the outer aqueous phase would dissolve the inner water droplets, several stabilizers have to be added to the double emulsion.[140] Surfactants with low HLB values, such as polyglycerol polyricinoleate (PGPR; HLB = 4), stabilize the first W/O emulsion to prevent coalescence of the internal water droplets.[141] The W/O emulsion is then stabilized toward the outer aqueous phase with another surfactant (high HLB), such as sodium dodecyl sulfate (SDS, HLB = 40), or via Pickering stabilization.[136,142] Afterwards, the core–shell nanoparticles are formed through solvent evaporation of the organic layer to encapsulate hydrophobic components in the polymeric shell and the hydrophilic components in the aqueous core (Figure 9B).[138] With the double emulsion method, various combinations of cargo molecules (e.g., DOX and erlotinib,[143] verteporfin and cisplatin,[73] siRNA,[20,32,136] or DNA and thiazoles[138]) were coencapsulated into degradable polymeric NPs based on PCL, PLA, or PLGA.[72,136]

Various crosslinking chemistries were applied to form core–shell nanocarriers with either a hydrophilic or a hydrophobic core. Lipid–polymer hybrid materials, i.e., "lipogels" were

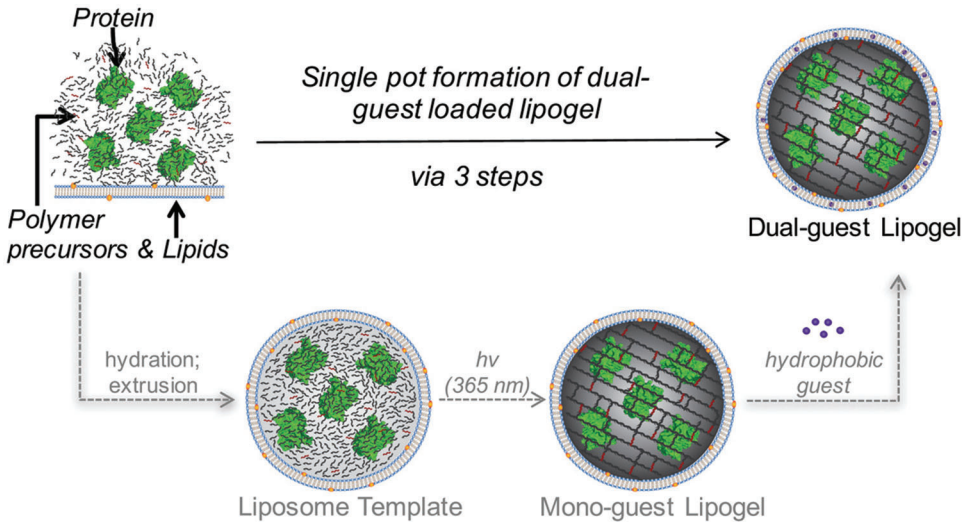

Figure 10. Single-pot method to prepare lipogels via liposomal templating and subsequent core cross-linking under UV irradiation for coencapsulation of hydrophilic compounds (here green-fluorescent protein) into the crosslinked gel-core and hydrophobic compounds (here DiI dye) into lipid-bilayer. Reproduced with permission.[145] Copyright 2017, American Chemical Society.

used to encapsulate hydrophilic and hydrophobic components simultaneously.[144] Homyak et al. reported lipogels for coencapsulation in a single-pot method with three formation steps (**Figure 10**).[145] The inner core contained of the hydrophilic cargo (green-fluorescent protein (GFP) and polymer precursors (2-(2-methoxy ethoxy)ethyl methacrylate (DEGM)). The nanogel-core (d = 100–200 nm) was coated with a lipid layer (≈10 nm) by self-assembly of the liposome upon hydration. The core was crosslinked by UV-induced polymerization and crosslinking with (*N,N′*-bis(methacryloyl)-L-cysteine (CDM). In the last step, the lipogel was loaded with the second hydrophobic cargo (dye) into the lipid bilayer shell. The technique for lipogel formation could be transferred to various combinations of lipidic and gel-like nanocarriers for co-delivery of hydrophilic and hydrophobic compounds.[144,146]

5. Organic and Inorganic Cargo Molecules

The combination of organic molecules, such as therapeutic drugs, with inorganic nanoparticles for imaging, creates multifunctional nanocarriers for theranostics.[2,3] Important imaging materials compose of inorganic nanoparticles (NPs), such as gold NPs, iron oxide NPs or quantum dots (QD), possessing unique optical, plasmonic, electric, or magnetic properties.[147] For example, the quantum mechanical effects in gold NPs makes them efficient contrast agents for photoacoustic imaging or computed tomography (CT).[148] Inorganic nanoparticles (INPs) can be stabilized with either hydrophilic or hydrophobic coatings, such as citrate- or oleic acid groups, to disperse them into water or organic solvents, respectively.[149] Therefore, codelivery of INPs with therapeutic agents is based on previously developed coencapsulation strategies. For example, self-assembled nanocarriers, such as micelles or liposomes, have as well demonstrated efficient coencapsulation properties of INPs with therapeutics.[150,151] Wang et al. combined anti-cancer drug quercetin with fluorescent polyacrylic acid-terminated silicon quantum dots (PAAc-SiQDs) by co-encapsulation into biodegradable PEG-*b*-PLA nanocarriers (**Figure 11**).[152] The nanocarriers were prepared by a double emulsion process using an internal emulsion of DMSO in DCM, which was emulsified and stabilized in an aqueous polyvinyl alcohol solution. The amphiphilic polymer (dissolved in DCM) encapsulated the quercetin and PAAc-SiQDs in DMSO during the self-assembly. The resulting dual-loaded nanocarriers (d ≈ 130 nm) exhibited a strong red fluorescence, which enabled monitoring of the drug. Encapsulation of quercetin resulted in higher anti-cancer efficiency compared to the free drug.

The double emulsion strategy combined with solvent evaporation was also used to coencapsulate INPs with both hydrophilic and hydrophobic drugs into nanocarriers of around 200 nm.[153] The hydrophilic drug was dissolved in the inner aqueous phase, entrapped by a polymeric shell, which again entraps Fe_3O_4-NPs and hydrophobic cargo upon solvent evaporation.[154] Several publications reported the coencapsulation of DOX with iron oxide nanoparticles into degradable PLGA nanocarriers by single emulsion and double emulsion solvent evaporation.[155] Anisotropic Janus particles have raised interest as drug delivery systems due to their unique structural asymmetry.[156] Lim et al. demonstrated the coencapsulation of INPs and small molecular drugs into biocompatible nanosized anisotropic Janus particles by a single-step solvent emulsion technique.[157] The hybrid Janus particles based on PLGA or PLA with PMMA could efficiently encapsulate oleic acid-coated iron oxide NPs and drugs DOX and PTX by selective encapsulation into the different "faces" of the Janus structure.

6. Conclusion and Outlook

Nanocarriers with multiple cargo load are promising multifunctional drug delivery systems to improve treatment efficiency. However, the coencapsulation of multiple molecules, especially with different physicochemical properties, simultaneously into

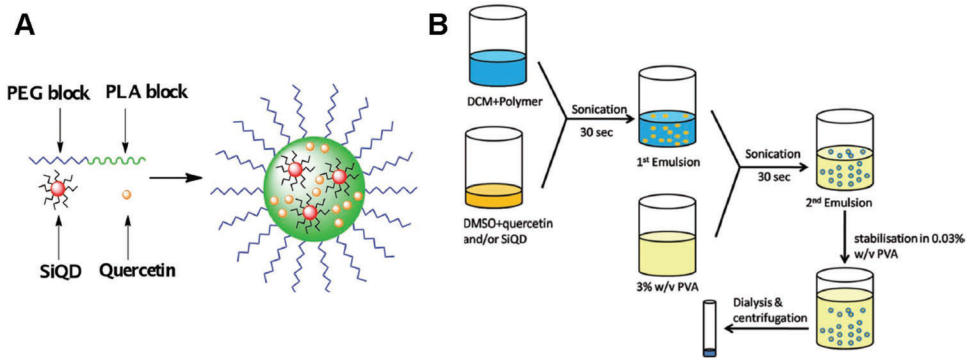

Figure 11. A) Coencapsulation of quercetin and polyacrylic acid-terminated silicon quantum dots into poly(ethylene glycol)-poly(lactic acid) nanoparticles. B) Schematic of coencapsulation into PEG-PLA nanocarriers by double emulsion method. Adapted with permission.[152] Copyright 2013, Wiley-VCH GmbH.

one nanocarrier system is challenging and requires a complex design of the nanocarrier preparation. In an optimal coencapsulation strategy, multiple cargo molecules can be encapsulated with high loading efficiency and without the cargo involving in the nanocarriers formation process. Orthogonal approaches are essential for the encapsulation of bioactive cargo molecules to keep them active—a chemical reaction might decrease their activity. Orthogonal strategies to prepare nanocarriers can be divided in two groups: 1) Nanocarriers by physical formation such as self-assembly into liposomes or polymeric micelles and 2) Nanocarriers by chemical reactions such as click crosslinking or polymerizations. For coencapsulation of hydrophobic and hydrophilic molecules, there are nanocarriers such as liposomes or layer-by-layer nanoparticles, which offer both hydrophobic and hydrophilic domains, in which cargo molecules can be loaded. With such multidomain nanocarriers, a broad range of cargo molecules from hydrophilic to hydrophobic, low molar mass or macromolecules and organic with inorganic can be coencapsulated and codelivered simultaneously. To date, codelivery systems focused on dual-loaded nanocarriers, but creating multifunctional systems with more than two active components could be promising to achieve synergistic properties for efficient multitherapy. Furthermore, drug delivery in nanocarriers especially in vivo depend on more effects, where issues of low circulation time, unspecific uptake into cells and fast liver excretion limit the clinical application of nanocarriers. To tackle these issues nanocarriers have to be equipped with not only multiple bioactive compounds inside but also multifunctional properties outside, such as stealth and targeting. Combining all those requirements in one nanocarrier system is of high demand to achieve the translation of nanocarriers as drug delivery systems into clinical therapy.

Multifunctional nanocarriers not only offer great potential in tumor therapy but other related field as well. A combination of therapeutic and diagnostic agents in one system, known as theranostics, can be applied as well for the treatment in noncarcinogenic diseases, for example in the liver, spleen, or brain.[158–161] In those cases, where the pathway to the tissue and its microenvironment differ from tumor tissue, the nanocarriers require different properties to promote optimal accumulation, degradation, and release of the multiple cargo components. Since up to now, a vastly broad range of therapeutic and diagnostic agents was developed, there are many combinations available. The challenge lies in finding the ideal nanocarrier for coencapsulating and accompanying both therapeutic and diagnostic agents, which may have very different chemical and physical properties. Furthermore, multiloaded nanocarriers can be useful in other applications, such as food or plant protection. Similar to biomedical agents, nanocarriers formulated with plant bioactive such as pesticides or fungicides, allow on-demand plant protection.[162–165] Instead of sprayed onto the plants, loaded nanocarriers are injected into the trunk and can release the bioactive upon degradation. This approach requires no spraying and less bioactives, which has high potential for more sustainable and environmental-friendly plant protection. For food applications, encapsulation of bioactive compounds into nanocarriers, increases their stability and biocompatibility.[166,167] Additionally, combining different components, for example, hydrophobic or hydrophilic supplements, neutraceuticals or aromatics, opening up a broad range of applications.[168] The multiple food bioactives could be encapsulated either for simultaneous transport or into layered responsive nanocarriers for release at different target sites. In conclusion, co-encapsulation into nanocarriers can be applied in a broad range of applications, where it is necessary to combine multiple components in one system. Moreover, coencapsulation of different cargo components has a great potential to improve the application of nanocarriers in the respective fields.

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Conflict of Interest

The authors declare no conflict of interest.

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