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Reactive Precursor Particles as Synthetic Platform for the Generation of Functional Nanoparticles, Nanogels, and Microgels

*Alexandra Gruber, Lucila Navarro, and Daniel Klinger**

Precise control of the chemical functionality of polymer nanoparticles is a key requirement in tailoring their (dynamic) colloidal properties toward advanced applications. However, current synthetic techniques are still limited in the versatility of chemical design and preparation of such functional colloidal nanomaterials. Two major challenges remain: First, various particle preparation methods are restricted in their functional group tolerance, thus hindering certain combinations of polymer backbones with specific functional groups. Second, the preparation of particles with different functionalities requires the synthesis of different particle batches. But this often results in a simultaneous variation of colloidal features. As a result, the accurate determination of important structure–property relations is still hindered. To address these restrictions, postmodification of preformed reactive particles is gaining more attention. This technique has evolved from polymer synthesis, where postpolymerization functionalization enables the introduction of a plethora of functional groups without changing the degree of polymerization and the molecular weight distribution. Similarly, modifying precursor particles enables the introduction of functional groups into particles while reducing variations in colloidal features, e.g., particle size and size distribution. This powerful synthetic method complements established procedures for functionalization of particle surfaces, thereby enabling the facile preparation of (multi-)functional particle libraries, which will allow precise investigations of structure–property relations.

1. Introduction

Nanoparticles are a cornerstone of modern nanotechnology. Especially crosslinked polymer nanoparticles (e.g., nanogels, microgels, and crosslinked micelles) offer high structural stability and dynamic functionalities that enable advanced applications such as delivery of drugs and bioactive agents,[1–4] chemical sensing,[5,6] and catalysis.[6] A key requirement in tailoring the colloidal properties to such desired macroscopic functions is the adjustment of the particles' internal functionality, i.e., the introduction of functional groups into the polymer chains that build the 3D network. Through cooperative effects, these groups translate their molecular properties to the overall particles. This can be used to control particle characteristics such as mechanical hardness and chemical resistance but also dynamic and responsive properties such as swelling, degradation, and loading/release of functional cargoes. Thus, having control over the amount, type and local distribution of functional groups in the particle nanostructure is crucial to develop advanced materials with new complex functionalities.

1.1. The Need for New Methods That Enable the Synthesis of Comparable Functional Particles

Over the last decades, the required level of control has been approached by combining innovative methods from polymer synthesis and colloidal chemistry to create an increasing array of complex particle-based nanomaterials. Especially in the biomedical field, numerous multifunctional and stimuli-responsive nanoparticles and nanogels/microgels have emerged as highly specific materials where particle functionalities were precisely tailored to a particular challenge or application. However, up to now, the comparability between these different systems is often limited. As a result, fundamental structure–property relations that describe particle interactions with biological systems (e.g., particle pharmacokinetics) are only slowly emerging.[7] Similar considerations are valid in other areas of application, where the development of universal design criteria for functional polymer colloids is still limited. These open challenges can be attributed to the fact that the versatile introduction of specific functionalities into colloidal systems is often obstructed by the limited functional group tolerance of many particle preparation techniques. Consequently, the variation of internal/external chemical functionality often requires the synthesis of individual particle batches via different particle preparation methods. This, however, often leads to a simultaneous variation of the colloidal features, e.g.,

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particle size, size distribution, and morphology. As a result, it remains challenging to accurately distinguish between the influences of internal/external chemical structure and overall colloidal features that determine the particle properties.

1.2. Transferring Concepts from Polymer Synthesis to Colloidal Materials

To address these challenges, researchers were drawn to the field of polymer synthesis, where similar challenges are known. While advanced polymerization techniques allow the synthesis of highly functional polymers with precisely defined compositions, molecular weights, molecular weight distributions, and architectures, synthesizing different polymers with different chemical functionality but the same "chain features" is still challenging. This is because some functional groups can interfere with the polymerization process, leading to unwanted side products or even a complete inhibition of the reaction. To overcome this problem, postpolymerization modification of reactive polymers has emerged as a versatile approach. The concept was first introduced by Hermann Staudinger in the 1920s and later termed "polymer analogous reactions."[8] Postpolymerization modification is based on the polymerization of monomers that contain reactive groups, which remain active under the polymerization conditions. These groups can then be transformed into another functional group in a subsequent step without changing the degree of polymerization of the original macromolecule. Together with the advances in polymerization techniques and efficient functionalization reactions, this concept now offers an ever-growing toolbox to synthesize increasingly complex functional macromolecules. Besides the introduction of functional groups, postpolymerization modification is especially advantageous in the synthesis of functional polymer libraries that allow the comparison among them (for more information on the field of postpolymerization modification of polymers, the reader is referred to several excellent reviews[9–12]).

Applying this concept from polymer chemistry, the strategy of postpolymerization modification was transferred from free (linear) polymers to 3D crosslinked polymeric nanoparticles (**Figure 1**). With this method, a broad variety of functionalities can be homogenously integrated into the colloidal network, thus enabling the preparation of functional particles that are otherwise not accessible. Most importantly, through the secondary functionalization of "parent" reactive precursor particles, decoupling of the interior network functionality from the colloidal features can be obtained. Similar to the modification of linear prepolymers, where functionalization does not change the degree of polymerization and the molecular weight distribution, the modification of precursor particles leads to functional particles with identical scaffolds. Depending on the type of conjugation, this often results in only limited effects on the particle size and particle size distributions (Figure 1). However, this also strongly depends on the introduced functionality. For example, the introduction of charged species might have tremendous effects on the swelling of the nanostructures which can differ from the

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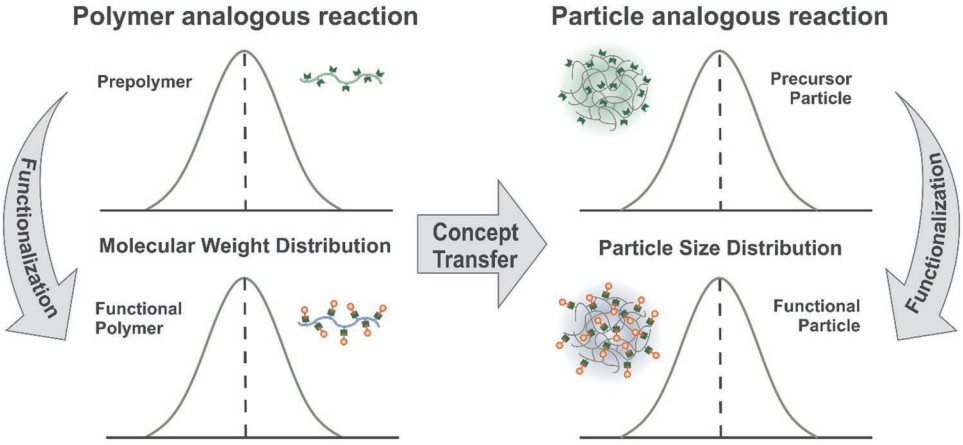

Figure 1. Transfer of the concept of postpolymerization modification from linear polymers to colloidal systems. The characteristics of the "parent" reactive precursor such as molecular weight distributions (polymers) or particle size distributions (particles) are translated to the newly formed functional system. This enables the preparation of functional libraries.

precursors. Nevertheless, in such cases the synthetic strategy still enables a high level of comparability that could allow the development of systematic structure–property relations, e.g., it could give the opportunity to investigate and compare the effect of different charged groups on the swelling of the colloids.

1.3. Influence of Precursor Particle Structure on Functionalization

The versatile concept of particle analogous reactions gives access to a wide variety of functional colloidal materials where the final properties can be tailored by the introduced chemical groups. However, the overall properties of a colloidal systems are, to a large extend, determined by the initial colloidal precursor structure since the location and accessibility of the reactive groups in the particle determine the spatial distribution of the functional moieties. Depending on the structure of the precursor particle, three main categories of structurally different modification routes can be defined: a) surface modification, b) complete modification, and c) interior modification (**Figure 2**).

So far, the concept of particle functionalization has been mainly applied to surface modification of nanoparticles, which has been realized by two central ways: On the one hand, reactive coatings, surfactants, or exposed groups are used on the surface of hard hydrophobic particles. On the other hand, micellar aggregates are used where only the hydrophilic outer polymer chains contain the reactive groups. Since this research area was already summarized in an excellent review by Barner-Kowollik and co-workers in 2012,[13] this article focuses on recent advances and select examples that highlight the similarities between colloidal precursor modifications and polymer analogous reactions. In contrast to selective surface functionalization, the discrete modification of the particle interior is much less explored. It is mainly achieved by the core modification of micelles or the network functionalization of nanogels that contain blocked surfaces, thus further demonstrating the importance of a careful selection of well-defined precursor architectures.

While these concepts demonstrate the selective functionalization of either surface or interior, a large variety of polymer nanoparticles does not allow this clear distinction. For precursor particles that carry reactive groups everywhere, in their interior and on their surface, the distinction between surface, interior and complete functionalization can be diffused. In such architectures, the diffusion of the functional reactants into the interior defines the spatial distribution of the corresponding functional groups.[14] This effect was demonstrated by Pich and co-workers, who functionalized microgels in their collapsed state, thereby hindering the diffusion of the reactant into the microgels. Thus, the functionalization preferably took place on the surface of the particles.[14] The access to the particle interior depends on the solubility of the particle-forming polymer and the functional reagents. A poor solvent for the polymer will hinder the swelling of the polymer network. Thus, the location of the functionalization will depend strongly on the interaction of the reagent with the polymer matrix. For reagents that cannot diffuse into the colloid (showing an unfavorable interaction with the polymer matrix), the functionalization is restricted to the surface. In contrast, reagents that show a good interaction with the polymer, can diffuse into the polymeric structure.[15] This is often used in order to install crosslinks of preassembled colloidal structures (such as micelles), where only partial functionalization is required.

A good solvent for polymer and reagents will enable swelling of the precursor architectures, thus leading to a complete, interior and exterior, modification.[16–19] This effect was shown by Walther and co-workers who conducted postmodification of reactive particles in a good solvent for the particle forming polymer. The swollen network allows easy access of the reactants to all reactive sites, thus resulting in a complete functionalization.[16,17] To enable such access to the particle interior, crosslinking is required. The covalent connection between polymers in the particle interior is needed to prevent a disintegration of the colloidal precursor structure during the modification reaction. Thus, careful selection of the crosslinking density and the solvent-dependent swelling of the reactive network can be used to control the location of postparticle functionalization in a variety of

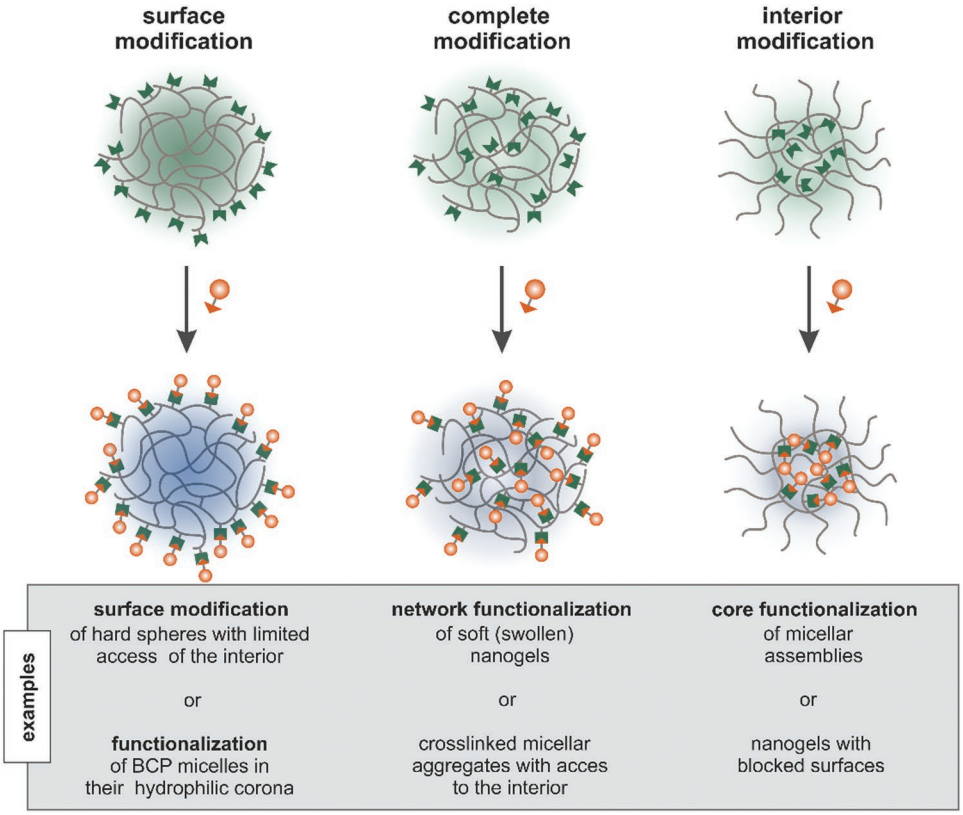

Figure 2. Schematic representation of the influence of the precursor particle structure on the final functionality after modification. Different localization of functional moieties in the particles and different particle properties lead to three main modification routes that govern the spatial distribution of functional groups: a) surface functionalization, b) complete functionalization and c) functionalization of the interior.

different precursor structures.[16] Among these different colloidal architectures, crosslinked micelles, nanogels/microgels, and crosslinked hydrophobic particles are the most important examples. Since each structure has a unique influence on the particle modification procedure, the following section will give a brief overview over these architectures.

1.4. Functionalization of Reactive Polymeric Micelles

The classical polymeric micelle consists of amphiphilic block copolymers, which undergo self-assembly in an aqueous environment to higher ordered structures ranging from spheres and worms to sheets and vesicles.[20] While the driving force for micellization is the segregation of the hydrophobic core-forming blocks in aqueous medium, this process is still dynamic and strongly depends on the block copolymer (BCP) concentration. This represents a major disadvantage, since dilution below the critical micellar concentration, e.g., by injection into the blood stream, can result in disassembly of the micelles.[21,22] Micelles formed by self-assembly of random copolymers show an increased stability compared to conventional ones, but can also disassemble in higher dilution.[23] Therefore, crosslinking of either the hydrophilic shell or the hydrophobic core is a common strategy to covalently stabilize these systems and prevent disassembly.[21] For this, (block) copolymers containing reactive units are first assembled to colloidal systems. In a subsequent step, covalent stabilization of the colloidal structure can be achieved by reacting the reactive groups with (bi-)functional crosslinkers. This crosslinking step can be categorized as a postfunctionalization of reactive precursor polymers, thus highlighting the link between polymer analogous reactions and colloidal functionalization reactions.

The resulting stabilized polymer micelles can be functionalized either in a subsequent step or during the crosslinking process. Both strategies enable the introduction of a variety of functionalities that might not be compatible with the micelle preparation method. For example, the introducing of hydrophilic groups into the reactive core can be used to invert the core polarity, so that a completely hydrophilic nanostructure is obtained.[24] Since this would not be accessible from the respective purely hydrophilic block copolymer, new particle functions can be accessed by this strategy.

The differentiation between surface and interior functionalization of the initial core–shell structures is strongly dependent on the location of the reactive groups in the micelles (core forming block or shell). However, a strongly crosslinked shell or core can prevent internal functionalization by inhibiting the diffusion of the functional moieties to the interior of the micelle and thereby limiting the functionalization to the outer region of the micelle.

1.5. Functionalization of Reactive Nanogels or Microgels

The crosslinked micelles represent an interesting transition to another important class of crosslinked nanoparticles: nanogels

or microgels, i.e., hydrophilic or amphiphilic 3D polymer networks that can absorb high amounts of water.[25]

Among others, nanogels/microgels have evolved as promising candidates for delivery applications because they combine characteristics of colloidal dispersions with those of macroscopic hydrogels.[2,3,26] Due to their hydrophilic or amphiphilic network, nanogels/microgels exhibit a high water content, soft structure, and good biocompatibility. In addition, their 3D crosslinked network structure defines the mechanical and diffusion properties.[2] Therefore, controlling the (stimuli-responsive) swelling of the colloidal networks offers the ability to precisely tune loading and release profiles. Furthermore, degradable network polymers or crosslinks allow the controlled disintegration of the polymeric network as response to biological relevant triggers[1] such as enzymes,[27,28] reducing agents,[29,30] or changes in pH.[31,32]

Even though a variety of different functionalities has been realized in these nanostructures, the utilization of reactive precursor nanogels/microgels can enable completely new architectures. These architectures are not accessible via standard nanogel/microgel preparation methods as precipitation- or emulsion-based polymerizations or via crosslinking of preformed aggregates. For example, the introduction of functionalities with drastically different solubility or reactivity can result in different morphologies and inhomogeneous distribution of functional groups in such standard preparation methods. By addressing these issues with network postfunctionalization reactions, also new libraries can be established that might enable the accurate determination of new structure–property relations.

1.6. Functionalization of Crosslinked Hydrophobic Nanoparticles

Crosslinked hydrophobic nanoparticles are a special case of nanogels/microgels which do not swell in water, thus representing hard spheres. Among other, their hydrophobic nature makes them the perfect candidates for the encapsulation and transport of hydrophobic cargoes. However, for these applications it is extremely important to create hydrophilic surfaces. This not only increases colloidal stability and enables their processing into materials from aqueous dispersions but also varies their physiochemical properties in biomedical applications.[33] While the attachment of inert hydrophilic polymers can give stealth properties that prolong systemic circulation, specific targeting moieties can ensure precise localized interaction with biological systems.[34]

In these nanostructures, the difference between surface and interior functionalization is highly dependent on diffusion properties of the functional moieties into the reactive network. These diffusion properties are determined by the size of the functional moiety and the mesh-size of the network, that in turn is dependent on the crosslinking density as well as the swelling ability of the network polymer.[2,16] For details on diffusion of compounds in (hydro)gel networks, the reader is referred to the excellent work of Lustig and Peppas[35] and, Lin and Metters.[36] In analogy to the nanogel functionalization, postmodification of hydrophobic particles enables the introduction of new functionalities to the polymeric network, that cannot be incorporated via standard preparation methods. Furthermore, starting from the same parent precursor, particle libraries with different functionalities but similar colloidal features can be obtained.[18,19]

1.7. Chemical Reactions to Introduce Functionalities

Each of the different colloidal architectures for postparticle formation modification offers a broad variety of synthetic opportunities. Similar to postpolymerization modification of polymers there is an entire toolbox of different reactions that can be applied to install functionalities into colloidal systems (**Figure 3**).

Especially "click" chemistry is of high interest for particle functionalization. These reactions have gained an enormous popularity in the field of biomacromolecular conjugation, due to mild reaction conditions that enable biocompatible and bioorthogonal functionalization. The most common representative, the copper-catalyzed azide–alkyne cycloaddition reaction (CuAAC) shows several advantages as high orthogonality, quantitative yield, and mild reaction conditions, which makes it a promising candidate for particle functionalization.[37] However, the use of toxic metal catalyst represents a major disadvantage for applications in the biomedical field.[38,39] Though this toxicity issue can be overcome by using strain-promoted azide–alkyne cycloaddition (SPAAC). Another reaction belonging to the "click" family that avoids the use of metal catalysts is the radical thiol–ene reaction. Even though the application in the biomedical field is limited due to the required rather harsh reaction conditions of the radical formation, they also present excellent candidates for particle functionalization where more robust reagents can be employed. Other widely used postparticle formation functionalization reactions are based on active ester chemistry. The major advantage in the reaction of active ester bearing colloidal systems with amines is the resulting stable amide bonds. The disadvantage is that this amide bond formation occurs in a base-catalyzed substitution reaction, that requires the removal of the released alcohol afterwards. In addition, the solubility of the most frequently used active ester, the *N*-hydroxysuccinimide (NHS) bearing homopolymer is limited to dimethyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF). However, these solubility issues can be overcome by the copolymerization of these monomers or by the utilization of pentafluorophenyl (PFP)-based ester monomers.

It becomes obvious that there is no universal "one-fits-all" post-particle formation functionalization strategy. In fact, each of these reactions has its eligibility but can also show disadvantages in combination with certain functional groups. Thus, the selection of the best particle functionalization strategy strongly depends on the application and the respective requirements of the reaction conditions. In order to indicate the possibilities and limitations of the discussed functionalization strategies we aimed to evaluate them using the following criteria:

- availability of monomers/reagents
- ratio between the introduced functionality and the formed connecting points/spacers (e.g., triazoles from azide–alkyne reactions, maleimide-thiol adducts etc.)
- compatibility with the preparation method of the respective precursor particle (e.g., polymerization)
- field of application.

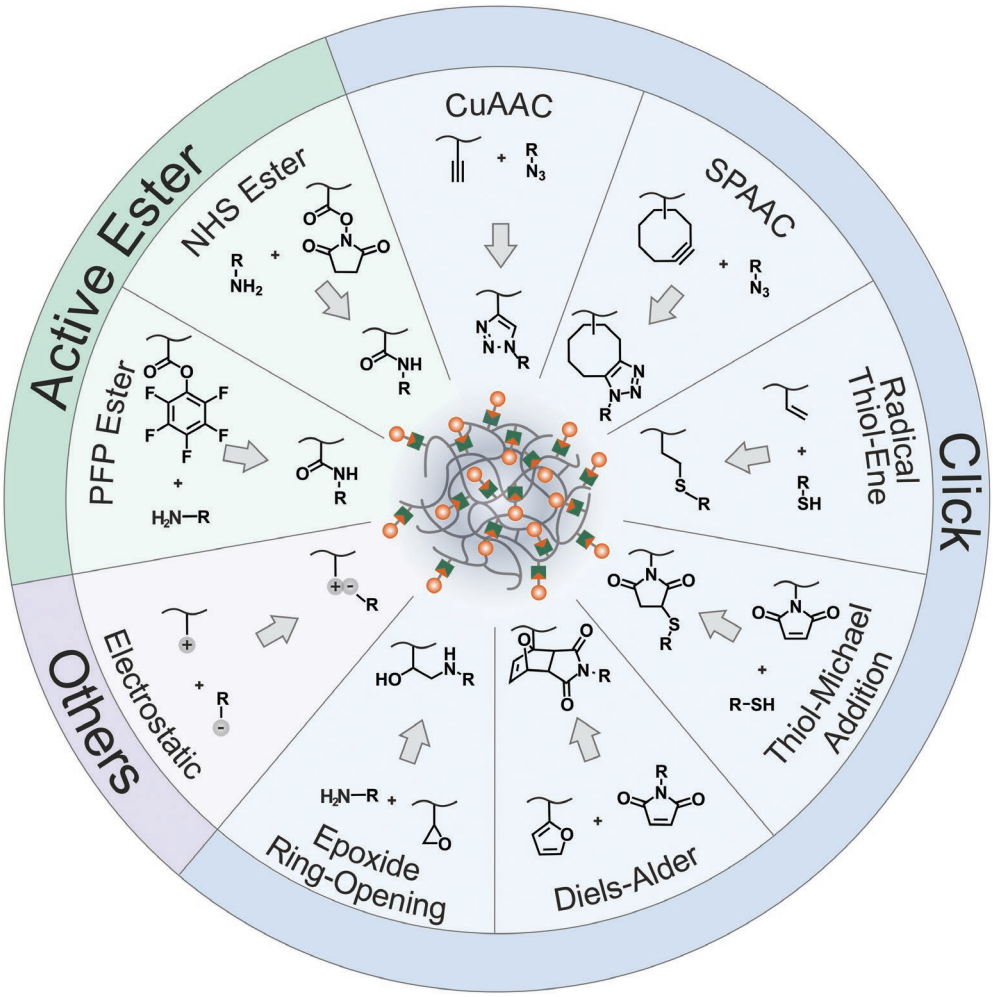

Figure 3. Schematic representation of the main classes of reactions for postparticle formation functionalization.

Due to the variety of different structures and functionalization possibilities, we structured this review by the type of chemical reaction used for the introduction of the functionalities. Within these sections, we classified the examples in the different structural modification routes, namely: surface modification, complete modification, and interior modification. As postpolymerization functionalization of colloids is still in its infancy the examples are limited. Thus, we included crosslinking of precursor particles as a special type of functionalization, highlighting the link between polymer analogues reactions and colloidal functionalization.

With this review, we want to communicate the basic concepts and advantages of postpolymerization functionalization of colloids. This synthetic strategy enables the synthesis of new complex, functional polymeric nanoparticles, that cannot be prepared by conventional preparation techniques. By presenting the milestones in the evolution of postpolymerization modification from linear polymers to colloidal systems, we hope to encourage and inspire new concepts for the design of functional nanoparticles libraries. Enabling structure–property investigations, this should pave the way to establish universal design criteria for colloidal systems and new applications, e.g., personalized nanomedicine.

2. Click Chemistry for Functionalization of Reactive Particles

The term "click chemistry" was first introduced by Sharpless and co-workers in 2001 and describes a set of reactions that are modular, wide in scope, stereospecific and occur under simple reaction conditions as in the presence of water and oxygen. In addition, these reactions are characterized by a high thermodynamic driving force, that induces the reactions to proceed rapidly and in high yields.[40] Initially designed to provide an effective conjugation technique in drug discovery,[41] "click" reactions have emerged as an attractive and versatile technique in a number of research areas, such as organic chemistry,[42] radiochemistry,[43] bioconjugation,[44] and polymer chemistry.[45]

The application of "click chemistry" to colloidal systems has led to a revolution in the field of biomacromolecular conjugation. This can be attributed to the fact, that these

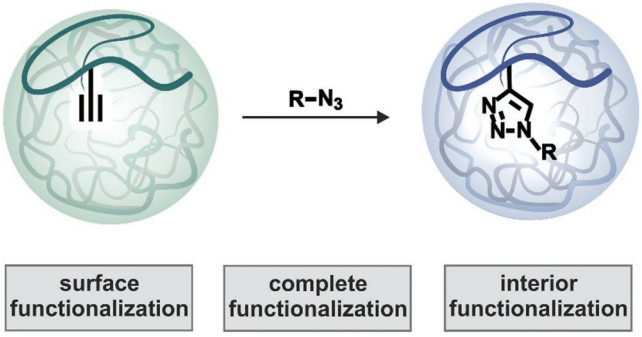

Figure 4. Schematic representation of the CuAAC. This chemistry has been applied in particle analogous reactions of the surface, the interior as well as the complete colloids.

reactions can occur in water under mild reaction conditions, thus enabling the preservation of the molecular structure of biological compounds.

2.1. Copper-Catalyzed Azide–Alkyne Cycloaddition

By far the most widely applied type of "click" reaction is the CuAAC, introduced by Sharpless and co-workers (**Figure 4**). He reported that the use of catalytic amounts of Cu(I) led to the formation of regio-specific 1,4-substituted triazoles under mild conditions while the thermally induced reaction rendered a mixture of 1,4- and 1,5-substituted products.[40] This highly regioselective reaction represented a revolutionary step in the development of new drug molecules. Later on, this reaction type also found significant applications in the field of polymer chemistry.[46] CuAAC often proceeds in quantitative yields in aqueous or organic media and is orthogonal with almost any other functional group. This high orthogonality and the absence of azide and alkyne groups in nature provide a unique advantage, that the reaction can even be applied in a complex and/or living environment.[47]

2.1.1. Surface Functionalization Using CuAAC

The synthetic strategy was quickly utilized in colloidal systems, where it has been used to create functionalities on the surface of nanoparticles. A special case of surface modification is the covalent stabilization of colloids via crosslinking on the surface. Wooley and co-workers presented shell-crosslinked nanoparticles, prepared by the crosslinking of preassembled block copolymer micelles, bearing alkyne groups in the corona with an azide-terminated dendrimer crosslinker.[48] The dendritic structure of the crosslinker allowed the incorporation of an excess of azide functionalities that were able to undergo the complementary reaction. As a proof of concept, the crosslinked particles were functionalized within the shell in a secondary "click" reaction with an alkyne-functionalized fluorescein.[48] This example nicely demonstrates how the architecture and the location of the reactive group within the colloid affect the spatial distribution of the newly introduced functionalities.

Another example for surface modification was reported by Bernard and co-workers who developed a series of crosslinked glyconanocapsules by interfacial step growth polymerization in an oil-in-water miniemulsion.[49] The aliphatic bifunctional alkyne (bis(propargyloxy)butan) was used to crosslink a carbohydrate that was previously modified with an azide group. After droplet formation, a catalyst system composed of sodium ascorbate/$CuSO_4$ was added to the aqueous phase. Since the crosslinker was designed to exhibit a partitioning coefficient toward the continuous phase, the formation of a polymeric membrane at the interface between the dispersed and the continuous phase was succeeded. Within this study, the authors also showed that the use of microwave irradiation can significantly reduce the time for the CuAAC reaction.[49]

A similar approach was later on used to synthesize biodegradable nanocapsules using azide-modified hyaluronic acid. Crosslinking via postfunctionalization with a redox responsive bifunctional alkyne led to nanocapsules that were able to release an encapsulated cargo upon cleavage by glutathione. Furthermore, the uptake and cleavage of the nanocapsules in dendritic cells were proven by confocal laser scanning microscopy.[50]

Besides the covalent stabilization of colloids, surface modification via CuAAC chemistry represents an easy way to obtain nanoparticles with specific ligands on the surface.[51–55] One strategy to synthesize the reactive precursor particles is to specifically introduce the azide/alkyne group to the surface of preformed particles.[56–59] Among others, this approach was reported by Paradossi and co-workers, who designed microgels by crosslinking azide and alkyne derivatives of poly(vinyl alcohol) (PVA) in inverse emulsion droplets using CuAAC.[60] By using an excess of the alkyne derivative of PVA, microgels with unreacted alkynes were prepared. Thus, conjugation of azido-grafted hyaluronic acid to the surface was enabled.[60]

A similar approach was reported for bone targeting nanogels based on a dextran scaffold. Using an excess of a one of the functionalized polymers, either the azide modified or the alkyne modified dextran, resulted in free unreacted "clickable" groups for subsequent conjugation (**Figure 5**). Nanogels with an alkyne excess were functionalized with an azide-modified bisphosphonate ligand and nanogels with excess of azide functionalities were conjugated with a fluorescent dye. By controlling the crosslinking time, it was also possible to generate bifunctional nanogels with both functions by conjugation with the fluorophore and the bisphosphonate ligands.[61]

An alternative to the use of preformed azide or alkyne modified polymers is the preparation of colloidal systems using the functional monomers. This concept was followed to prepare microparticles by dispersion polymerization of 4-vinylbenzyl azide and styrene. The azide moieties within the colloid had two functions: first, they served as crosslinking points for photocrosslinking and second, remaining unreacted azides on the surface were used for postparticle formation modification. By controlling the exposure time of the photocrosslinking process, the authors were able to control the residual surface azide groups of the resulting crosslinked particle, thus controlling the degree of functionalization. CuAAC modification of the surface was performed using the alkyne-functional fluorescent dye, Rhodamine B propargyl ester. With this, the authors

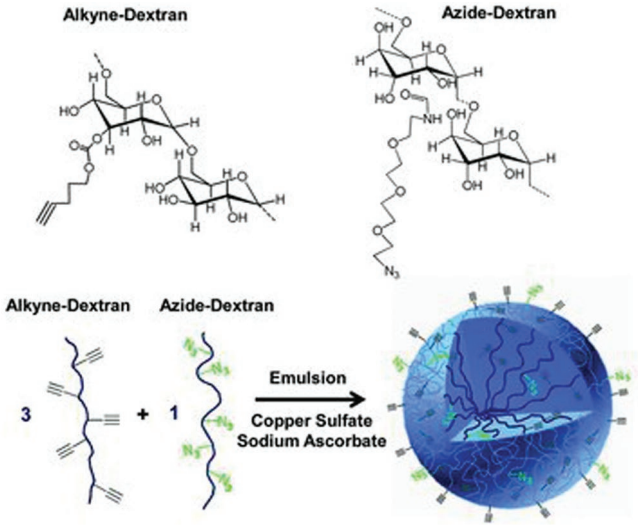

Figure 5. Preparation of reactive dextran-based precursor nanogels via click chemistry with remaining reactive groups for subsequent functionalization. Reproduced with permission.[61] Copyright 2013, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

proved the efficacy of the methodology for the preparation of fluorescent particles as probes for optical measurements.[62]

2.1.2. Complete and Interior Functionalization Using CuAAC

After having shown in the previous section that CuAAC facilitates the introduction of new functionalities to surfaces of nanoparticles, this section deals with the functionalization of the interior of nanoparticles. Crosslinking is not limited to the surface of nanoparticles, thus the reaction can also occur in the interior of colloids, utilizing self-assembly of alkyne bearing copolymers and crosslinking the core with a bifunctional azide linker.[63] In many examples, the location of the functionalization is determined by the site of the active groups in the precursor structure. For example, for block copolymer micelles with reactive groups in the core-forming block, the functionalization is restricted to the interior. In contrast, emulsion procedures often lead to a more homogenous distribution of the reactive functionalities. Thus, complete functionalization will be facilitated. One example is presented by Kang and co-workers who reported the preparation of fluorescent organometallic porphyrin complex nanogels via crosslinking of azide functionalized poly(ethylene glycol) (PEG) with a gallium–porphyrin complex as crosslinker in an inverse miniemulsion approach.[64]

The preparation of single-chain polymeric nanoparticles is another application of CuAAC.[65,66] Pomposo and co-workers presented a variety of single-chain polymeric nanoparticles by crosslinking of azide bearing copolymers with an alkyne-containing bifunctional crosslinker.[65] Instead of using a bifunctional crosslinker, single-chain nanoparticles can also be prepared by intramolecular CuAAC of copolymers bearing both functional groups, azides and alkynes. Polymers containing an excess of azide groups over alkyne moieties led to nanoparticles with unreacted azide moieties, that were used for further modification with propargyl glycine.[66]

Another crosslinking-approach that is not limited to the surface, is the preparation of dendritic polyglycerol based nanomaterials.[67–69] The preparation of biodegradable dendritic polyglycerol nanogels for encapsulation and release of pharmaceutical biomacromolecules represents one example of this approach. The respective nanogels were prepared via surfactant free, inverse nanoprecipitation and in situ crosslinking of alkyne and azide functionalized polyglycerol macromonomers. This technique allowed the encapsulation of labile biomacromolecules, including the therapeutic relevant enzyme asparaginase, into the polyglycerol network.[69]

Having proven the efficacy of CuAAC reactions for crosslinking purpose, recent attempts concentrate on complete functionalization of particles. The key to success of complete and internal functionalization relies on the accessibility of the reactive moiety within the particle structure by the CuAAC reagent. To overcome limitations in accessibility, Du Prez and co-workers designed highly microporous particles of glycidyl methacrylate (GMA) with ethylene glycol dimethacrylate as crosslinker.[70] The epoxide group allowed a two-step "click" process with sodium azide to introduce the azide functionality, followed by CuCAAC with phenyl acetylene. The large microporous cellular structure of the particles enabled the accessibility of the functional groups located inside the particles.[70] A similar procedure was reported using polystyrene particles with divinylbenzene and a porogen as additive. After polymerization, residual double bonds were converted into azide or alkyne functionalities, which allowed "click" chemistry conjugation with the CuAAC counterpart ligand.[71] In general, this procedure creates a micro- or macroporous structure, enabling the inner and outer functionalization of the respective particles. With this, "clickable" platforms can be generated for the conjugation of multiple molecules such as catalyst, biomacromolecules, or therapeutics.

However, in order to also assure accessibility for reagents and enable complete postfunctionalization for soft nanostructures, other approaches must be taken. One strategy is based on the control of the crosslinking degree to provide flexibility to the networks. Thus, the networks can swell in a suitable solvent that contains the counterpart CuAAC reagent.

A perfect example of this, was reported by Walther and co-workers, who performed an emulsion polymerization of the alkyne bearing monomer propargyl acrylate (PA) with ethylene glycol dimethacrylate as crosslinker.[16] Postmodification involved the addition of pH-responsive cationic moieties by CuAAC reaction using 2-azido-*N*,*N*-dimethylethylamine. As this last step is limited by the diffusion of the azide-reagent to the alkyne moieties located in the interior of the particle, the key for a successful modification relies on the perfect tuning of the crosslinking density. This was achieved by the variation of the crosslinker proportion in the feed. Using this approach, the authors described an efficient diffusion of the reactants within the particle, thus controlling the degree of functionalization by adjusting the crosslinking density (**Figure 6**). With this, the authors presented the proof of concept for this synthetic approach, which is suitable for the preparation of functional particle libraries.[16] It is also an excellent example

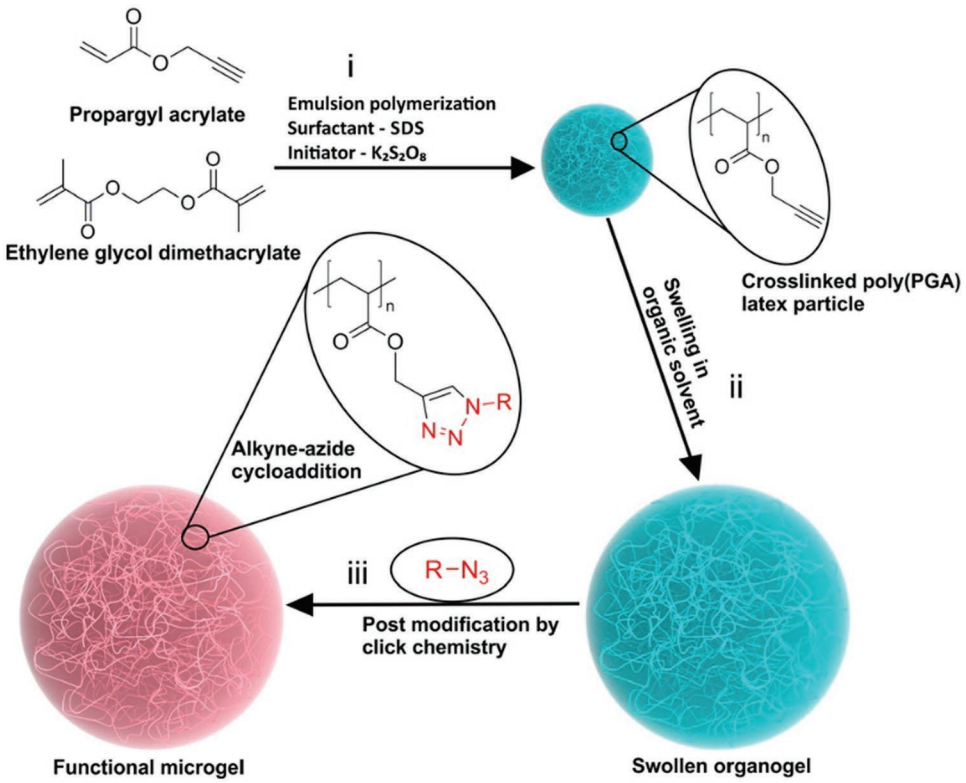

Figure 6. Uniform reactive latex particles enable the generation functional microgels via click postparticle formation modification. Reproduced with permission.[16] Copyright 2014, American Chemical Society.

where postfunctionalization of the well-defined "parent" latex particles enable the preparation of "children" particles with similar colloidal features, thereby allowing structure–property investigations.

A similar technique for complete particle functionalization was reported by Farley and Saunders, using microgels synthesized by copolymerization of PA with different monomers and crosslinkers.[72] These reactive microgel particles were then placed in DMF and the alkyne groups of the PA were reacted with 2-azido-1-ethylamine to achieve pH-responsive behavior. The limiting factor controlling the functionalization was the PA content in the respective precursor particles which was dependent on the type of comonomer and its concentration.[72]

These examples demonstrate that the suitability of CuAAC chemistry for particle functionalization depends on a variety of different factors as mentioned in the introduction. First, a careful selection of the starting materials and a suitable particle structure must be taken into consideration. In order to proceed, the reactions require the colocalization of the hydrophilic or hydrophobic functional reagent and the hydrophilic copper catalyst at the active site. However, this can cause potential hydrophobic/hydrophilic incompatibility between the system and the catalyst. As a result, a lack of colocalization and a reduction of the functionalization degree can be observed depending on the swelling of the colloids and their hydrophilicity/hydrophobicity. One possibility to avoid this effect is to include a complexation ligand in order to adapt the catalyst's hydrophilicity and control the localization of the resulting copper–ligand complex.[73] However, this approach adds unnecessary complexity to the system as well as additional synthetical steps.

Second, the versatility of CuAAC chemistry to introduce functionalities to colloidal systems depends on the availability of the reagents. Several commercial derivatives are available and currently increasing in number; however, additional experimental steps are often necessary. For instance, CuAAC applied for the conjugation of biomacromolecules requires the introduction of the reactive group to the protein/enzyme. This is often performed using heterofunctional linkers bearing NHS or maleimide groups that can react with amine or thiol groups present in the targeting biomolecule, thus deteriorating the "functionality-to-spacer-ratio."[44] In general, careful selection of the appropriate linker for the introduction of an azide/alkyne to a system must be considered, as it will play a key role in the final properties of the system. In addition, the solubility and stability of the linker, as well as the potential effect of the resulting triazole in (biological) particle environments are critical parameters for the design of a suitable functionalization strategy.[74]

Third, the exponential application of CuAAC reaction rapidly exposed some disadvantages especially regarding the use of copper. The active catalytic species necessary for the reaction is Cu (I); however, oxidation to the more stable species, Cu (II), can easily proceed in solution. The generated Cu (II) species is able to catalyze the oxidative coupling of terminal alkynes to generate diynes as byproducts.[75] This is the reason why Cu (I) is produced in situ and in the presence of an excess of stabilizer or reducing agent.[42,75,76] However, the selection of the reducing

agent could also have an impact on the final properties of the system. For example, sodium ascorbate is the most common employed reducing agent, but it also induces the oxidation of cysteine, methionine, and histidine through the reaction with the superoxide radical anion generated by the ascorbate.[77] Therefore, specific copper-stabilizing ligands must be used to avoid the degradation of specific moieties and to accelerate the reaction rate. Another drawback that must be considered when using copper catalysts is the extra purification as residual copper is considered highly toxic thereby limiting the application in living tissue. It has been proven that copper ions can produce reactive oxygen species (ROS) that might lead to structural damage of biomacromolecules such as proteins, nucleic acid, and lipids.[38,39] Removing copper ions using a complexation agent as EDTA represents a possible approach, however, reactions without metal catalyst are best suited for biomedical applications. In this context, the development of copper-free "click" chemistry such as SPAAC, radical mediated thiol–ene chemistry and Diels-Alder (DA) reaction offers the possibility to obtain nanoparticles without the need of potentially toxic catalysts.

2.2. Strain-Promoted Azide–Alkyne Cycloaddition

In 2004, the introduction of metal free cycloadditions of cyclooctynes with azides emerged as an interesting alternative to CuAAC in order to obtain stable 1,2,3-triazoles without the need of any potentially toxic catalyst (**Figure 7**).[78] SPAAC reactions are catalyst-free and proceed at room temperature in a very efficient way even in a complex environment. Due to its bioorthogonality, SPAAC reactions were initially developed for labeling of proteins and lipids in living cells, for oligonucleotide and protein modification as well as for tissue reengineering.[78,79] Nevertheless, this alternative type of "click" chemistry quickly entered the polymer and material science, too.

2.2.1. Surface Functionalization Using SPAAC

SPAAC proved to be especially useful for the conjugation and incorporation of specific moieties on the surface of colloidal systems. One example was presented by Calderón and co-workers who developed a nanogel precursor by

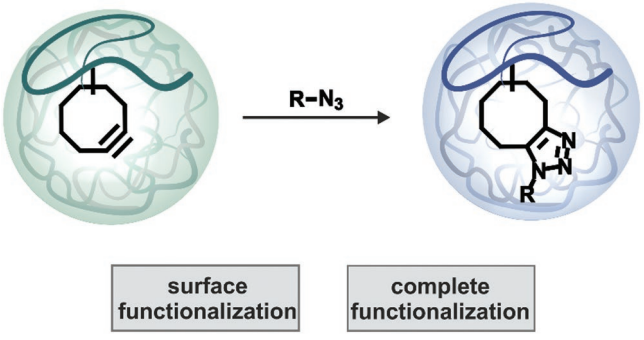

Figure 7. Schematic representation of the SPAAC. This chemistry has been applied to modify the surface or functionalize complete colloids.

thermonanoprecipitation.[80] The precursor particles were prepared via crosslinking of azide modified linear thermoresponsive polyglycerol with dendritic dPG-(1R,8S,9s)-bicyclo[6.1.0] non-4-yn-9-ylmethyl (4-nitrophenyl) carbonates (dPG-BCN) that act as a crosslinker. In a second step, unreacted BCN groups available on the surface of the nanogels were used for the decoration with β-cyclodextrin (βCD) (**Figure 8**). This strategy allowed the introduction of βCD moieties without changing the colloidal properties of the carrier. βCDs gained interest since they are characterized to have a hydrophobic pocket, giving the possibility for the encapsulation of hydrophobic drugs into a hydrophilic system.[80] Even though reactive groups are available in the interior as well as on the surface of the nanogels, the authors describe a surface decoration only. This is probably due to the size of the βCD moieties, making the diffusion of the βCDs into the network unlikely, thus limiting the site of functionalization to the surface. The Calderón group used a similar strategy to prepare magnetic transferrin decorated nanogels for targeting circulating tumor cells. The authors modified magnetic particles with BCN serving for both crosslinking and conjugation purposes. The modification of the reactive precursor with a thermoresponsive polymer and transferrin resulted in a magnetic nanogel, decorated with the desired protein.[81,82]

A main advantage of "click" reactions is the high orthogonality that allows the introduction of a number of different moieties into systems that already carry other functionalities without interfering or inducing any cross-reactions. As an example, De Geest and co-workers recently reported the synthesis of a nanogel by self-assembly and crosslinking of a block copolymer synthesized by reversible addition–fragmentation chain transfer (RAFT) polymerization of methoxy triethylene glycol methacrylate and pentafluorophenyl methacrylate (PFPMA) with an azide-functional chain transfer agent.[83] This copolymer was then used to prepare micelles by self-assembly in DMSO, driven by the hydrophobic nature of the PPFPMA block. The resulting micelles were subjected to two different modification reactions. On the one hand, active ester chemistry on the PPFPMA block was used to introduce a fluorescent probe and to core-crosslink the micelle through amide bond formation. On the other hand, the azide groups on the surface of the nanogels were utilized for a SPAAC reaction with a nanobody that was previously modified with cyclooctyne moieties.[83]

Barz and co-workers also took advantage of this high orthogonality of SPAAC in order to synthesize nanogels and add different functionalities in a single reaction step.[84] The authors used polysarcosine-*b*-poly(*S*-alkylsulfonyl)-L-cysteine block copolymers with three reactive, orthogonally addressable groups. This allowed the site-specific conversion of all units in a single step. Block copolymers were prepared having a combination of azides and amines as chain end groups and a thiol reactive *S*-alkylsulfonyl cysteine. The modification proceeded during the formation of core-crosslinked nanostructures through the thiol reactive moieties, and involved the simultaneous introduction of fluorescent dyes previously modified with dibenzyl cyclooctyne and NHS for reaction via SPAAC and activated esters chemistry, respectively.[84]

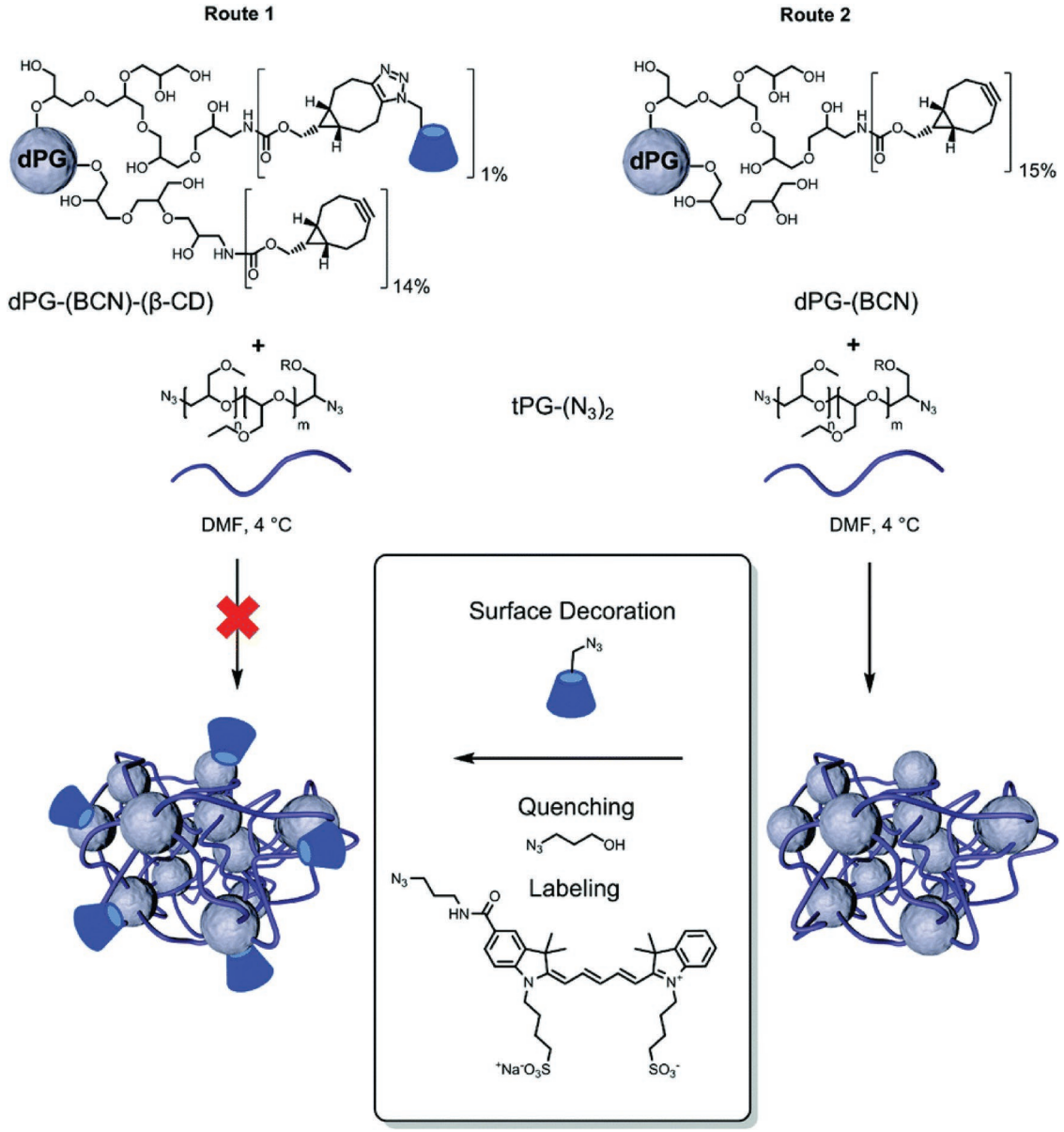

Figure 8. Synthesis route for the preparation of β-CD grafted thermoresponsive nanogels. Reproduced with permission.[80] Copyright 2018, Royal Society of Chemistry.

2.2.2. Complete Functionalization Using SPAAC

Besides surface functionalization, SPAAC is mainly used for the preparation and covalent stabilization of micro- or nanogels.[85,86] For example, by also taking advantage of the bioorthogonality of SPAAC, Haag and co-workers designed microgels using poly(ethylene glycol)-dicyclooctyne and dendritic polyglycerol (dPG)-polyazide for in situ encapsulation of living cells through a microfluidic device.[86] Azide conjugation was performed using different pH-cleavable linkers, enabling the release of the cells under acidic conditions, while maintaining cell viability.[86]

SPAAC chemistry emerged as a new technology to overcome the drawbacks of CuAAC reactions. The absence of a catalyst eliminated the need for colocalization of the catalyst and reagent and improved the biocompatibility prospect. In addition, the biorthogonality of SPAAC allows the reaction to proceed in a complex biological environment. Therefore, SPAAC is one of the few reactions that can occur in vivo.[87] However, there are only a few commercially available cyclooctyne derivatives on the market. In addition, similar to the CuAAC reaction, the functionalization with biomacromolecules requires the introduction of the cyclooctynes or azides prior to the conjugation. This is normally performed through a heterofunctional linker with an orthogonal reactive functionality such as NHS or maleimide.[47] The main challenges of SPAAC reactions remain the relatively large size and the high hydrophobicity of the cyclooctyne component. Since the cyclooctyne also gets introduced into the nanoparticles, it influences the colloidal properties. For example,

installing a small carboxylic acid functionality using SPAAC results in tremendous difference between the desired properties of the COOH functionality the introduced nonfunctional spacer from the reaction between the bulky cyclooctyne and the azide. Thus, an unfavorable "functionality-to-spacer-ratio" is the result. Therefore, such reactions are more of interest for the installation of high molecular weight functionalities.

The poor water solubility of some cyclooctyne reactants generates the need solvent mixtures such as acetonitrile/H_2O. However, the use of organic solvents, even in a mixture, could result in cytotoxicity, reducing the application of SPAAC in biological systems.[88] In this matter, among the cyclooctynes reagents available, BCN provides a good balance in terms of reactivity and hydrophilicity.[89]

2.3. Radical Thiol Addition (Thiol–Ene)

The origin of reactions between thiols and carbon–carbon double bonds or "enes" dates back to 1905 (**Figure 9**).[90] Later on, two types of thiol–ene reactions have been distinguished as emerging strategies for versatile and efficient functionalization: thiol–ene free radical addition and the catalyzed thiol Michael addition (in Section 2.4). Thiol–ene reactions have experienced a renaissance due to the recognition of its "click" characteristics. As many of the other "click" reactions, thiol–ene reactions present outstanding properties including quantitative yield and fast and efficient reaction that can progress in an aqueous medium. The radical thiol–ene reaction can be initiated by various strategies including traditional thermal conditions with common azo initiators such as 2,2′-azobis(isobutyronitrile) or via photochemical methods, with or without the addition of a photoinitiator.[91] In addition, the availability of a wide variety of both thiols and alkenes makes thiol–ene a favorable synthetic approach.

2.3.1. Surface Functionalization Using Radical Thiol–Ene Reaction

"Click" chemistry represents an efficient and flexible approach for the conjugation of biomacromolecules to the surface of colloids. As mentioned above, azide or alkyne groups can be introduced easily to a particle by using an azide-containing monomer in various polymerization processes. This is possible since the reactive groups do not interact with the polymerization process. However, this does not hold true for introducing alkenes since they may participate or interfere with free radical polymerization techniques. Therefore, other alternatives had to be created. One possibility to introduce reactive groups was reported by Xu and co-workers who synthesized crosslinked polystyrene microspheres by a two-stage dispersion polymerization of styrene with divinylbenzene or ethylene glycol dimethacrylate as crosslinkers.[92] This provided free unreacted vinyl groups on the microspheres surface. These groups were then utilized to covalently attach glucose to the microspheres via thiol–ene reaction using a radical initiator. However, the number of residual alkenes on the surface was dependent on the amount of crosslinker added in the two-stage dispersion polymerization. It could be shown that an increase of the crosslinkers over 3 wt% resulted in a higher polydispersity of the particles. To assure monodisperse particles the crosslinker concentration therefore is restricted to these 3 wt%, thus limiting the number of free alkene groups on the surface for further functionalization.[92]

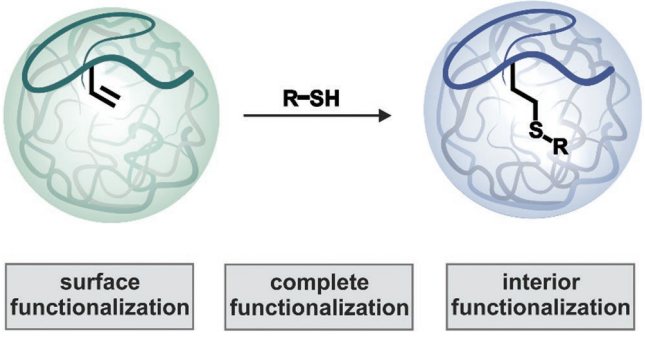

Figure 9. Schematic representation of radical thiol addition. This chemistry has been applied in particle analogous reactions of the surface, the interior as well as the complete colloids.

Zeng and co-workers have recently proposed another alternative to introduce alkenyl groups to the surface of particle.[93] They reported a RAFT-assisted dispersion photopolymerization of methyl methacrylate using a cycloalkenyl-containing macro-RAFT agent that was introduced as an ene-bearing moiety. This procedure led to microspheres with low dispersity and "clickable" double bonds on the surface. The content of vinyl groups on the resulting reactive particles could easily be regulated by the amount of the RAFT agent present in the dispersion polymerization. As a proof of concept thiolated biotin was conjugated to the surface by photo induced radical thiol–ene reaction (**Figure 10**).[93]

Another approach to introduce functional groups is to employ already functionalized polymers that bear the specific moieties for the preparation of the colloidal system. This strategy was recently followed by Auzély-Velty and co-workers who reported the synthesis of biodegradable polysaccharide nanogels. For this, two different polysaccharides were modified with pentenoic anhydride in order to introduce alkene groups on the polymer backbone. Taking advantage of these alkene groups, a thermoresponsive ethylene glycol-based copolymer was grafted on the backbone via thiol–ene chemistry. Temperature-induced self-assembly of the resulting polymers led to the formation of nanogels that were covalently stabilized with a bifunctional thiol linker via subsequent reaction with remaining alkenes of the shell-forming polysaccharide.[94]

Elbert and co-workers exploited the concept of separate, sequential "click" reactions for the preparation and attachment of PEG nanogels to glass surfaces in order to prepare protein resistant coatings.[95] The PEG nanogels were prepared via CuAAC of multiarmed PEG-azide and PEG-alkyne monomers. The addition of a copper-chelating agent prevented the bulk gelation. Then, radical thiol–yne reaction was used to attach the nanogels to mercaptosilanated glass. Additional surface crosslinking of the nanogel coating was performed via CuAAC of residual end groups after attachment to the glass substrate.[95]

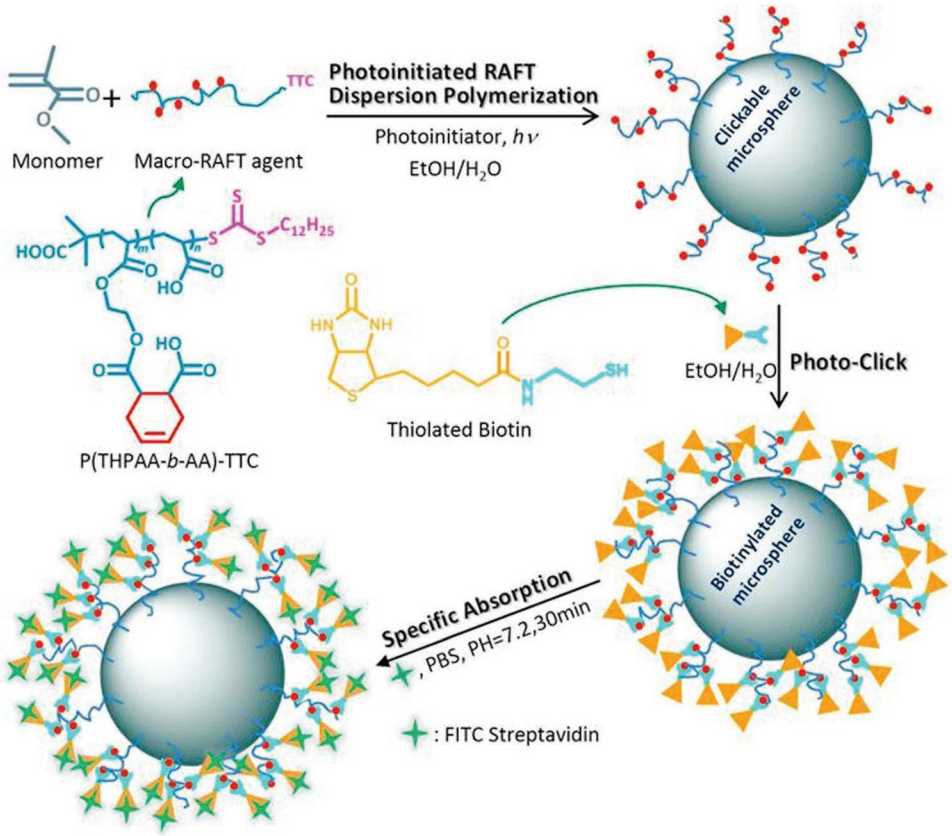

Figure 10. Schematic representation for the synthesis of surface functional PMMA microspheres and the subsequent modification via thiol–ene click reaction. Reproduced with permission.[93] Copyright 2018, Elsevier Ltd.

2.3.2. Complete and Interior Functionalization Using Radical Thiol–Ene Reaction

Radical thiol–ene reactions are not limited to the surface but are also used to introduce functional moieties to the inner of colloids. One special case of complete functionalization of colloids is the covalent stabilization of nanoparticles.[96,97] For example, norbornene-derived chitosan microgels, prepared in a water-in-oil templating method, were crosslinked in the presence of a thiolated crosslinker. Residual norbornene groups of the microgels were used for further postparticle formation modification through tetrazine ligation, which was demonstrated by grafting a self-quenching fluorescent tetrazine probe to the microgels.[98]

Overall, internal modification of particles, using radical thiol–ene reaction is less explored in comparison to other "click" reactions. A new approach in this area was presented by Klinger and co-workers who developed a facile strategy to obtain reactive microgels that can be functionalized via subsequent thiol–ene chemistry.[18] As shown in **Figure 11**, precursor particles can be obtained by miniemulsion formation and crosslinking of poly(allyl glycidyl ether) (PAGE) via thiol–ene reaction with pentaerythritol-tetrakis (3-mercaptopropionate) (PTMP) as a degradable crosslinker. The use of an amphiphilic block copolymer consisting of PEG and PAGE as nonionic reactive surfactant, led to a covalently attached PEG corona on the resulting microgels. This synthetic pathway allowed a postparticle formation functionalization of unreacted allyl glycidyl ether units in a subsequent reaction with different pH responsive moieties. With this, a functional library of microgels with comparable size, size distribution, surface functionality and crosslinking density was prepared.[18] This example highlights the recent trend in particle library preparation that facilitates the comparability of colloids with different functionalities by keeping the colloidal features as similar as possible.

The radical thiol–ene reaction has a tremendous potential to be as popular as the CuAAC reaction, as the introduction of alkene and thiol groups is straightforward and the coupling can be performed in absence of a toxic catalyst by exposure to UV light or elevated temperatures. In this matter, one special aspect to consider is that photoinitiators are often required to accelerate the reaction rate. Synthetic attempts have been made to increase the range of wavelengths available for photopolymerization in water and thereby broaden the application range of thiol–ene radical reactions.[99] However, the commercial availability is reduced to a few known initiators. Moreover, the reaction time is also a parameter to be considered since long reaction times under UV–vis light might limit the application of this reaction, e.g., biomolecules are prone to degrade under these conditions.

A huge advantage of this functionalization strategy is the wide commercial availability of thiols. Furthermore, in comparison to CuAAC and SPAAC, the radical thiol–ene reaction

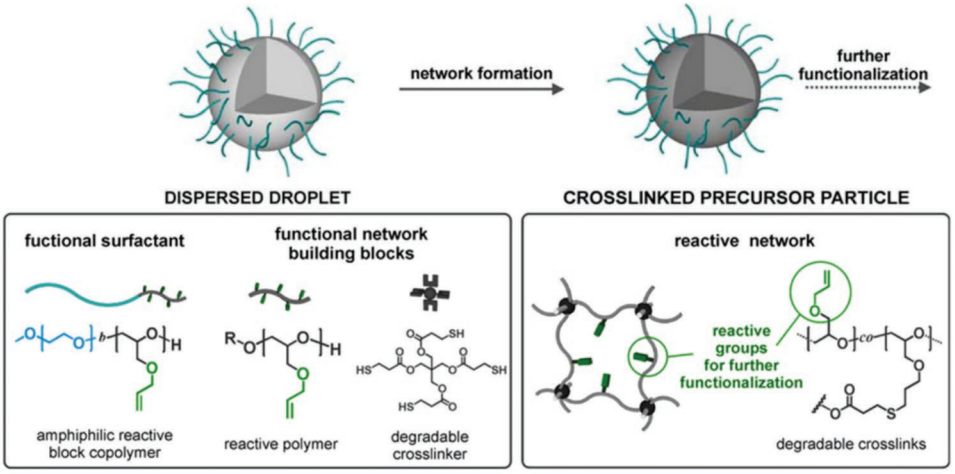

Figure 11. Preparation of reactive microgels via crosslinking of PAGE and PAGE-*b*-PEG using light-induced thiol–ene reaction with PTMP as crosslinker in a miniemulsion approach. Reactive groups of the microgels can be used for further functionalization via thiol–ene reaction. Reproduced with permission.[18] Copyright 2015, Royal Society of Chemistry.

benefits from a good "functionality-to-spacer-ratio." Meaning, that the introduction of a new functionality leads to less "dead" spacer material. While CuAAC and SPAAC are interesting for the functionalization with high molecular weight functionalities, the radical thiol–ene reaction is also valuable for the installation of small functions.

A major disadvantage of the radical thiol–ene reaction is that alkene bearing monomers cannot be polymerized by free radical polymerization processes. This results in a limited versatility in precursor particle accessibility, as for example conventional radical emulsion polymerization of the corresponding monomers is not feasible. Thiol–ene reactions are typically carried out under conditions that minimize side reactions; however, disulfide bond formation can still occur in an non-negligent amount by the reaction of two thiols.[100] Moreover, the reactivity of the alkene moiety is crucial for the efficiency of the reaction. Electron rich and strained alkenes (vinyl ethers and norbornenes) react rapidly with the thiyl radicals while electron poor alkenes (acrylates and maleimides) react slowly.[101] Acrylate groups are one of the most used species as it can react with thiols to form stable thioether linkages in the presence of radicals. However, under these conditions, the vinyl groups can also react with each other, rendering in a reduced efficiency and a mix mode of thiol–ene and ene–ene reactions.[102] Another drawback is that the use of radical thiol–ene is limited in the biomedical field, as the radical formation can be harmful for living tissue. Moreover, side reactions can occur with thiol groups present of cysteine containing biomolecules. Although in principle the thiols of the biomacromolecules could be used for bioconjugation purposes, the radical reaction conditions can induce the degradation of the target protein/enzyme. Therefore, the radical thiol addition is particularly suitable for applications in material science, taking advantage of the high degrees of functionalization. Whereas for the conjugation of biomacromolecules non-radical methods such as thiol-Michael addition, are better suited due to the mild reaction conditions.

2.4. Thiol-Michael Addition

The Michael addition reaction is a simple reaction between enolate-type nucleophiles and an α, β-unsaturated carbonyl in the presence of a catalyst. The Michael addition reaction toolbox includes the carbon-Michael reactions, oxa-Michael reactions, aza-Michael reactions, and the thiol-Michael reactions.[103] However, the reaction of thiols to form carbon–sulfur bonds outstands as an important reaction in the synthesis of biological active compounds.[104] Specifically, the thiol-Michael addition refers to a reaction between a thiol and an electron-deficient alkene (**Figure 12**). This type of reaction was first introduced in 1964 by Allen et al.[105] Proceeding in an aqueous system with the use of mild catalysts, the thiol-Michael addition earned a position as a modular "click" reaction with the ability to produce highly stereo- and regiospecific products.[106] The thiol-Michael addition reaction was mostly applied in organic synthesis, however, over the years it has also grown into material science, where it has first been used for polymer synthesis and/or functionalization and, more recently, into the field of colloidal systems. Acrylates and maleimides are the most common electron deficient alkenes, used for applications in nanoparticle functionalization.

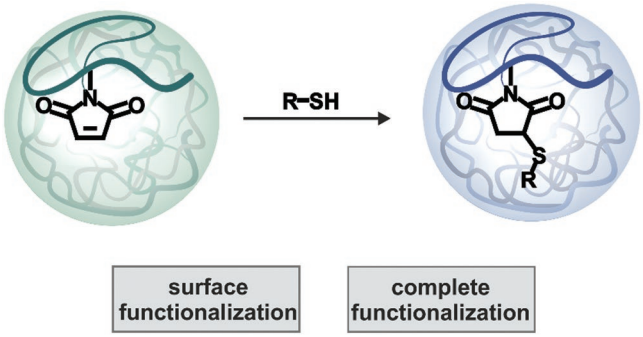

Figure 12. Schematic representation of thiol-Michael addition. This chemistry has been applied to modify the surface or functionalize complete colloids.

2.4.1. Thiol–Ene Michael Addition

Both, the thiol-ene Michael addition and the radical thiol–ene reaction generate thio-ether products; however, the characteristics of the thiol-Michael addition offer some advantages over those of the radical thiol–ene reaction. These are mainly attributed to the absence of radicals and the great selectivity among various vinyl groups, which is especially favorable in the biomedical field.

Being part of the "click" chemistry family, the thiol–ene Michael addition presents a lot of similarities to CuAAC or SPAAC reactions, however one main difference, worth to mention, is the availability of the reactive groups in nature. As mentioned before, azides and alkynes are not present in native biomolecules presenting the advantage that CuAAC can be used to functionalize particles in a complex and living environment. However, if the purpose is to conjugate or functionalize colloids with biomacromolecules, such as proteins, thiol–ene chemistry renders favorable, due to thiols present in cysteine-containing macromolecules. Although cysteine is one of the least abundant amino acids, it is frequently found as a highly conserved residue in most catalytic sites in enzymes.[107]

Surface Functionalization Using Thiol–Ene Michael Addition: Tan and co-workers used thiol–ene Michael addition for bioconjugation of aptamers and enzymes to the surface of nanoparticles.[108] The thiol–ene Michael addition was enabled by functionalizing hydrophobic nanoparticles with alkene groups on the surface. In a subsequent step, these alkene groups were subject to thiol–ene Michael addition with different macromolecules such as thiol–PEG, a thiolated aptamer and cysteine-containing horseradish peroxidase. The resulting nanoconjugates demonstrated excellent target binding ability and enzymatic activity.[108] Thus, thiol–ene Michael reaction can be considered as a rising star for bioconjugation with broad applications in biosensing, bioanalysis, bioimaging, drug delivery, and theranostics.

Another example for surface functionalization was reported by Zhuang and co-workers who proposed a one-pot strategy for the development of glucose sensitive core–shell nanogels.[109] The nanogels were synthesized by one-pot copolymerization of poly(ethylene glycol) diacrylate (PEGDA), poly(ethylene glycol) acrylate (PEGA), poly(3-acrylamidophenyl boronic acid) (AAPBA), and pentaerythritol pentaerythritol-tetrakis (3-mercaptopropionate) (PTMP) through thiol–ene reaction between the thiols of PTMP and the double bonds. This reaction mixture led to the formation of a core, mainly consisting of the tetra functional PTMP and the bifunctional PEGDA. Free mercapto groups of PTMP were furthermore able to react with PEGA and the sugar-binding AAPBA to obtain a pegylated nanogel, sensitive to glucose.[109]

Complete Functionalization Using Thiol–Ene Michael Addition: The thiol–ene Michael reaction has been extensively used for the preparation of hydrogels by radical photopolymerization.[110] However, the radicals formed during this synthesis can be harmful to cells and living tissue and thereby reducing the width of application. Taking advantage of the radical-free approach that the thiol–ene Michael reaction offers, Haag and co-workers used a dithiolated PEG macro-crosslinker and acrylated hyperbranched polyglycerol for the synthesis of novel microgels for cell encapsulation.[111] Droplet-based microfluidics of the polymers in presence of the cells resulted in cell-loaded microgels with low polydispersity. The advantage of using these prefabricated macromolecular precursors for the microgel synthesis is that it allows a systematic variation of the macromonomer properties. This provides insights into the relations between the microgel properties (crosslinking degree) and the viabilities of the encapsulated cells.[111] The Haag group furthermore prepared pH-sensitive cationic nanogels for siRNA delivery via thiol-Michael nanoprecipitation of thiolated dPG and acrylated polyethyleneimine in the presence of siRNA (**Figure 13**).[112]

Thiol–ene Michael addition can be further used for the preparation of single-chain nanoparticles. Paulusse and co-workers reported well-defined nanoparticles via intramolecular thiol-Michael addition between thiol bearing styrene-, acrylate, and methacrylate-based polymers and bifunctional acrylates.[113]

Another example of thiol–ene Michael addition for postfunctionalization of the surface as well as the interior of a colloid was reported by De Geest and co-workers.[114] They reported core-crosslinked micelles with cysteine reactive vinyl sulfone moieties that can be used for protein ligation. The micelles were formed by self-assembly of an amphiphilic RAFT block copolymer of PFMA and methoxy triethylene glycol methacrylate. The PFP active ester groups were used for crosslinking with a bifunctional diamine as well as for the introduction of free thiols that were subsequently transformed into vinyl sulfone groups. The remaining RAFT end groups on the hydrophilic block, present on the surface of the micelles, provided thiol groups, that as well were converted to vinyl sulfone moieties. These vinyl sulfone moieties both in the interior as and on the surface of the micelle were used for protein ligation via thiol–ene Michael addition with the cysteine groups of the protein bovine serum albumin (BSA). Despite the sterical constraints, the vinyl sulfone moieties in the interior remained well accessible for protein conjugation.[114] This highlights how the location of the active groups dictates the position of the introduced functionality.

In general, the thiol–ene Michael addition benefits from the wide availability of commercial and synthetic thiols. As already mentioned above, the lack of bioorthogonality of the thiol–ene Michael addition could be seen as disadvantage since cross-reactions with cysteine containing biomacromolecules can occur. However, especially the reactivity toward thiols and thiol containing biomacromolecules represent an advantage for protein/enzyme conjugation over other functionalization strategies, since no modification of the biomacromolecules is needed prior to the conjugation reaction. The thiol–ene Michael addition avoids the use of toxic catalyst and is therefore more suitable for applications in the biological field than for example CuAAC. Furthermore, thiol–ene Michael reaction shows a good "functionality-to-spacer-ratio" therefore is also suitable for the introduction of small functionalities. The thiol–ene Michael addition is conducted using a base or nucleophile as catalyst, thereby preventing radical formation which can lead to the degradation of proteins. Therefore, the thiol–ene Michael addition is more suitable for the conjugation of biomacromolecule, while the radical approach is more appropriate in material science. Additionally, the formation of side products is also reduced compared to the radical thiol–ene reaction. However, the

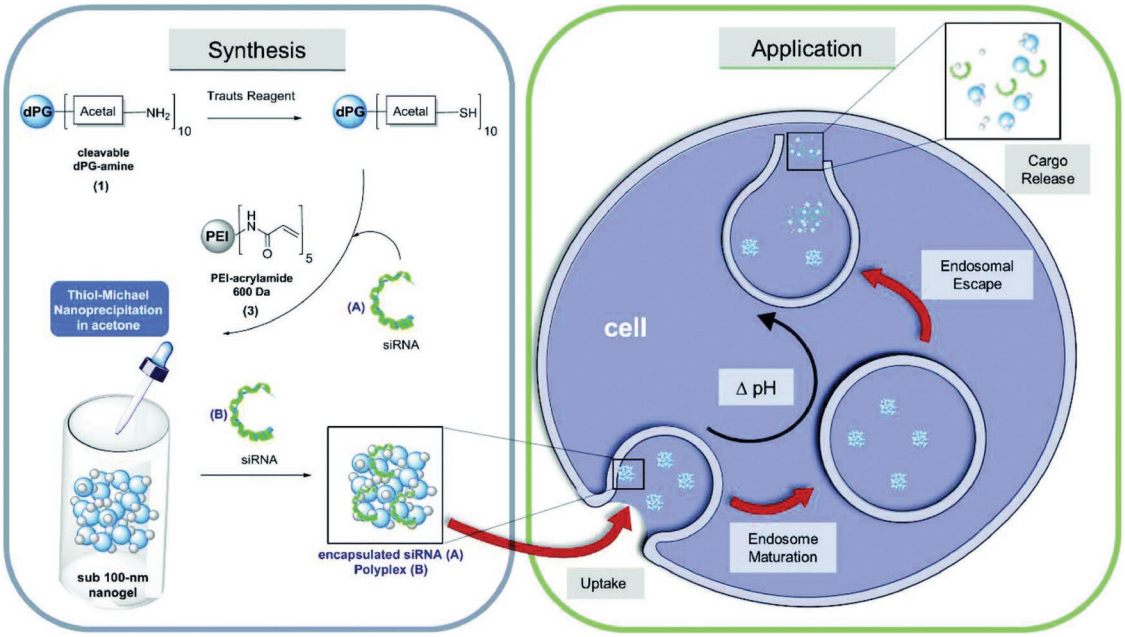

Figure 13. Synthetic route for the preparation of pH-sensitive nanogels via thiol–ene Michael nanoprecipitation and its application as gene delivery platform. Reproduced with permission.[112] Copyright 2017, Royal Society of Chemistry.

selection of the catalyst is depended on the application and the environment. Here, special attention must be paid to the steric accessibility of the thiol groups, solvent polarity, and pH.[115] The use of certain nucleophiles, such as phosphines, can also result in side reactions by reacting with the vinyl groups. This is because in the nucleophile-mediated pathway, the nucleophile reacts with the alkene to generate a strong base and catalyze the reaction.[116,117] In contrast, the base-catalyzed reaction proceeds with a much slower rate resulting in less quantitative conversion. For further insights on the effect of the catalyst and the environment on the reaction rate, we strongly recommend the review of Long and co-workers on this subject.[115]

2.4.2. Thiol–Maleimide Michael Addition

Reactions between thiols and maleimides have been recognized as some of the most efficient Michael-type additions. The main driving forces of the reaction are the withdrawing effect of two coupled activating carbonyls and the release of ring strain upon product formation. The reaction between maleimides and biological molecules bearing thiols has been used since 1949,[118] but it was not until 1980 that thiol–maleimide reactions got attention as a potential toolbox for the functionalization of nanocarriers.[119]

Surface Functionalization Using Thiol–Maleimide Michael Addition: The maleimide-thiol reaction is frequently applied in functionalization protocols because of the high reactivity of maleimides under mild conditions, the selectivity toward thiol groups at physiological pH and the stability of the thioether bond under most conditions. Therefore, thiol–maleimide reaction find among others application in the field of surface functionalization.[120,121] Nie and co-workers designed a multifunctional nanogel conjugated to the tumor targeting peptide, iRGD, in a multistep procedure.[122] An initial free radical polymerization was performed to obtained the plain nanogels of poly(*N*-isopropylacrylamide-*co*-acrylic acid) via a precipitation methodology. This was followed by the introduction of positively charged doxorubicin into the negatively charged swollen nanogel particles at pH 7.4. Fluorescent BSA encapsulated gold nanoclusters were then attached to the surface by activation of the acid group of PAA moieties. And finally, BSA was modified with a maleimide-bearing linker, which was used as an anchor point for the iRGD-SH in thiol–maleimide Michael conjugation reaction.[122] The complexity of this multifunctional drug delivery system combines the features of drug delivery, diagnosis, and therapeutic applications in a single platform.

Complete Functionalization Using Thiol–Maleimide Michael Addition: Due to the high orthogonality owned by the "click" chemistry reactions, it is possible to employ the same precursor nanoparticle for several purposes. Sanyal and co-workers reported a "clickable" nanogel on the basis of a poly(ethylene glycol)-methacrylate-based maleimide-bearing copolymer.[123] The nanogels were prepared by thermally induced self-assembly of the copolymers and subsequent crosslinking, using a dithiol crosslinker. Residual maleimide groups on the polymer backbone were used to conjugate a thiol-bearing peptide-based targeting group, while residual thiol groups from crosslinking were used to introduce a maleimide-bearing fluorescent dye.[123] The Sanyal group went even further and added another reactive group to the precursor nanoparticle. By using reactive copolymers containing PEG, maleimide, and pendant hydroxyl groups as side chains, nanogels were formed by self-assembly and crosslinked with a bifunctional thiol. The hydroxyl groups were later modified with NHS to obtain a nanogel with three potential functional groups: a maleimide, a thiol, and an activated carbonate. This allowed the functionalization with a

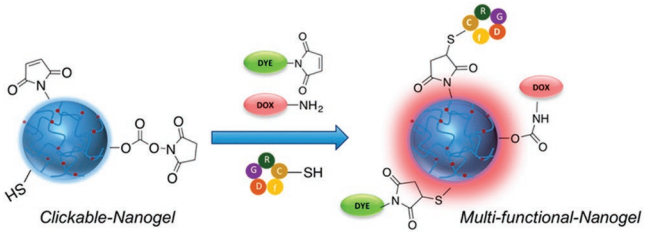

Figure 14. Schematic representation reactive precursor nanogels for multifunctionalization. Reproduced with permission.[124] Copyright 2018, American Chemical Society.

thiol-containing cancer cell targeting peptide, a maleimide-containing fluorescent indocyanine Cy5 dye, and the anticancer drug doxorubicin, respectively (**Figure 14**).[124] This proves that the combination of different reactive groups given by the range of "click" chemistry reactions into the same colloidal systems opens the possibility to target different applications at the same time.

The thiol–maleimide reaction is part of the thiol-Michael additions, therefore the stated common advantages and disadvantages for thiol–ene Michael addition apply here as well. However, the use of maleimides introduces a nonfunctional spacer between the colloid and the installed functionality. Thus, these reactions are rather used for the introduction of larger functionalities, since here the ratio between spacer and functionality is shifted toward the functional moiety, e.g., proteins. As a result, the thiol–maleimide Michael addition is commonly used for bioconjugation purposes to introduce a biomacromolecule to a reactive particle by reaction of the maleimides with the thiol group of cysteines.

One drawback, worth mentioning, is the possible hydrolysis of the maleimide groups under aqueous conditions, yielding maleamic acid which prevents the reaction with thiols.[100] Therefore, this side reaction can significantly affect the functionalization degree and affect the overall properties of the resulting system. In addition, as in most functionalization methods, purification steps are necessary to remove unreacted reagents, as potential cytotoxicity could compromise the application of the system.[125] However, significantly fewer compounds need to be removed compared to functionalization strategies that liberate equal molar ratios of leaving groups upon reaction, as for example active esters.

2.5. Diels–Alder Reaction

The Diels–Alder (DA) reaction is a [4 + 2] cycloaddition between an electron-rich diene (e.g., furan and its derivatives) and an electron-poor substituted alkene, also referred to as dienophile (e.g., maleic acid and its derivatives, vinyl ketone, etc.) (**Figure 15**). During the reaction a cyclic, six-membered product is formed. Invented in 1928 by Otto Diels and Kurt Alder, the DA reaction evolved to one of the most common reactions used in organic chemistry. For their discovery Diels and Alder were awarded with the Nobel prize in chemistry in 1950.[126] In the 1990s the DA reaction also emerged in the field of postpolymerization modification.[127] Especially over the last decade, increasing attention has been dedicated to DA as these reactions have been adapted to meet the criteria of the "click" chemistry: efficiency, versatility, and selectivity.[40] An additional attractive feature of

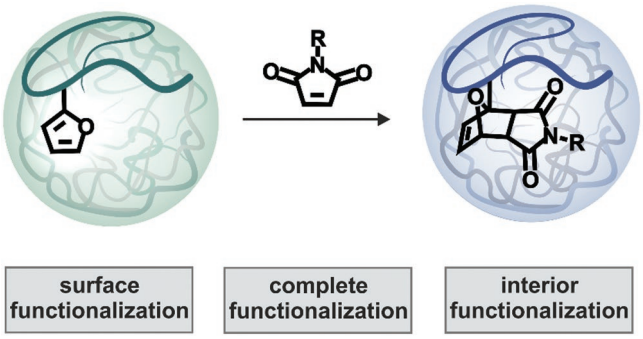

Figure 15. Schematic representation of DA reaction. This chemistry has been applied to postfunctionalize the surface, the interior as well as the complete colloids.

many DA reactions is their thermal reversibility. The cyclic DA adducts can decompose into the starting diene and dienophile at elevated temperatures compared to the forward reaction.[127,128] Already in the early stages of postpolymerization modification of polymers, this characteristic has been taken advantage of in order to prepare thermoresponsive materials.[127,129] Therefore, it is not surprising that the DA reaction also finds it application in postpolymerization modification of colloidal systems.

2.5.1. Surface Functionalization Using DA Reaction

The DA reaction is often used to introduce sensitive molecules as antigens or antibodies to the surface of colloids. This strategy is used as a mild alternative to the conventional conjugation methods that often require either activation or coupling reagents, thereby increasing the possibility of side reactions and losing the moiety's binding activity. One example of this approach was reported by Shoichet and co-workers who prepared immune-polymeric nanoparticles by self-assembly of biodegradable, amphiphilic, furan-functionalized copolymers.[130] The furan groups attached on the termini of the PEG corona of the resulting micelles were reacted by DA cycloaddition with the breast cancer antibody (anti-HER2), which was previously modified with maleimide functional groups.[130] In a follow up study, the chemotherapeutic doxorubicin was also covalently attached to these anti-HER2 bearing micelles via DA reaction. The respective micelles were able to deliver doxorubicin intracellularly to HER2-overexpressing cells in vitro.[131] Furthermore, the authors used a similar strategy to prepare dual functionalized micelles containing furan as well as azide groups on the termini of the PEG corona that enabled the chemical modification via DA cycloaddition as well as CuAAC reaction with antibodies, peptides, and nucleic acids.[132,133] These examples perfectly illustrate the orthogonality of the DA reaction, enabling the conjugation of different targets at the same time.

2.5.2. Complete and Interior Functionalization Using DA Reaction

Besides surface modification, the [4 + 2] cycloaddition can also be used for complete and interior functionalization of colloids.

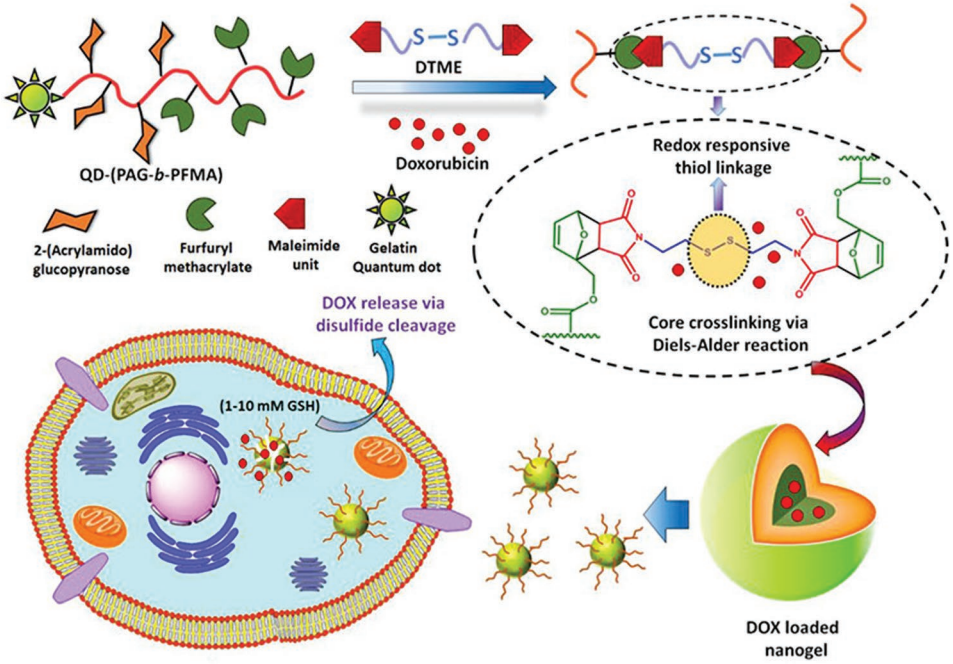

Figure 16. Schematic illustration of doxorubicin loading and core-crosslinking of micelles via DA reaction, resulting in redox responsive nanogels. Reproduced with permission.[135] Copyright 2019, American Chemical Society.

The main application in this section is the covalent stabilization of nanostructures. For example single-chain polymeric nanoparticles can be prepared, using intramolecular UV-light triggered DA crosslinking of a 2,5-dimethylbenzophenone and maleimides bearing copolymer.[134] Furthermore, Singha and co-workers presented redox responsive fluorescent glycopolymer based nanogels via DA core crosslinking of self-assembled micelles.[135] For this, a functional block copolymer containing activated PFP and furfuryl groups was synthesized followed by the polymer analogous reaction of the PFP groups with glucosamine to prepare the respective glycopolymer. In a subsequent step, the copolymer was furthermore modified at the terminal acid functionality with fluorescent gelatin quantum dots to generate fluorescent polymers. Self-assembly of these amphiphilic block copolymers and core crosslinking via DA cycloaddition with dithiobismaleimidoethane as crosslinker led to redox-responsive nanogels (**Figure 16**).[135] Another example for crosslinking via DA reaction is the generation of hyaluronic acid microgels for cell encapsulation and delivery. The microgels in the size range of 200 to 300 µm were prepared via high-throughput microfluidics of furan grafted hyaluronic acid molecules with dimaleimide PEG, that acts as crosslinker.[136]

Furthermore, the reversibility of the DA reaction has been used in order to produce thermosensitive micelles.[137,138] An example was presented by Sumerlin and co-workers, who prepared block copolymers of styrene and maleic anhydride via one-pot cascade polymerization.[137] Subsequent ring-opening of the anhydride groups by amidation with furfurylamine resulted in block copolymers with pendant furan groups. Self-assembly to polymeric micelles and subsequent core-crosslinking via DA reaction of the furan functionalities with a bismaleimide crosslinker, led to thermosensitive micelles. Due to the reversibility of the DA reaction these micelles have the ability to dissociate back into single polymer strains upon heating.[137]

In a similar approach, Lim and co-workers used redox-responsive dithio-containing bismaleimides as crosslinking agent via DA reaction, to prepare redox-responsive core-crosslinked micelles for doxorubicin loading.[139,140] In subsequent studies, the authors furthermore introduced a diselenide containing bismaleimide crosslinker to obtain a sensitive crosslinking agent in the presence of a reductive-oxidative medium.[141,142]

In general, the DA reaction is highly efficient, however at room temperature the DA reaction only exhibit a very low reaction rate.[143] This can be an issue for example for in situ crosslinking of nano- and microgels. Increasing the temperature or the addition of a catalyst could increase the reaction rate but limiting the biological applications. By comparing the availability of the monomers and reagents, it become obvious, that the DA approach cannot keep up with the functionalization strategies using amines or thiols that exhibit a great commercial and synthetical availability. Although the conjugation of biomacromolecules via DA reaction is possible, it requires the introduction of the reactive moiety prior to the conjugation. Another point to consider, is the possible side reactions, as maleimides are strong electrophiles and can undergo Michael-type addition and result in the formation of unwanted side products.

An additional disadvantage of the DA reaction is the formation of the relatively large cyclic DA adduct, adding unnecessary nonfunctional space between the functionality and the colloid. Therefore, DA reactions are of interest for the installation of high molecular weight functionalities. For the introduction of small functionalities, thiol–ene or active ester chemistry is more suitable. However, the formed DA adducts can decompose into the starting diene and dienophile at elevated temperatures

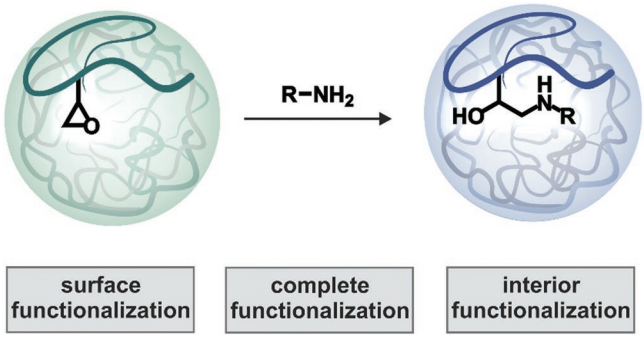

Figure 17. Schematic representation of epoxide ring-opening reaction. This chemistry has been applied in particle analogous reactions of the surface, the interior as well as the complete colloids.

compared to the forward reaction.[127,128] This represents a beneficial feature for the design of degradable materials and carries.

2.6. Epoxide Ring-Opening Reaction

Epoxide-functionalized polymers have a long history in post-polymerization modifications. This can be explained by their tolerance toward radical based polymerization techniques. The nucleophilic ring-opening reaction of the three-membered ring is stereospecific, often highly regioselective and nearly quantitative, and therefore meets the criteria of "click" reactions (**Figure 17**).[40] Already in the 1960s systematic studies on postpolymerization modification of polymers containing epoxide groups as poly(glycidyl methacrylate) and poly(glycidyl acrylate) were conducted, using secondary amines.[144–146] Epoxide containing polymers represent a versatile platform for modification with a variety of different nucleophiles such as amines, thiols, carboxylic acids and azides.[144,147–150] Similarly to the thiol-Michael additions, epoxide ring-opening reaction are favorable for the conjugation of biomacromolecules, since many of these contain accessible amines or thiols in their structures. However, postmodification with primary amines can be adverse since crosslinking can occur, due to reaction between the newly formed secondary amine and a still unreacted epoxide group.[151]

2.6.1. Surface Modification Using Epoxide Ring-Opening Reaction

The postpolymerization modification reactions via epoxide ring-opening by nucleophiles have also found their way to colloidal systems, where they have been used to introduce surface functionalities. One example was reported by Pich and co-workers who designed new polymeric carriers for antibodies.[152] For this, surfactant-free emulsion polymerization was applied to synthesize poly(styrene-*co*-glycidyl methacrylate) polymeric colloids that were stabilized by grafted PEG chains. It is assumed that the difference in hydrophilicity and reactivity of the two monomers led to core–shell structures, where the epoxide groups are mainly located in the outer region of the synthesized particles, thereby limiting the location of functionalization to the surface. The reactive epoxide groups on the surface were then used for immobilization of antibodies through covalent binding via epoxide ring-opening reaction.[152] In another study, aqueous microgels were synthesized from poly(*N*-vinylcaprolactam) and glycidyl methacrylate. In a subsequent, step epoxy groups on the surface were used for chemical coupling of a peptide via a thiol–epoxy reaction between the epoxy groups and thiols of the cysteine residue in the peptide sequence. The coupled peptide allows the selective site-specific immobilization of enzymes.[153]

Another example for surface functionalization was reported by the group of Gallei, who prepared colloidal core–shell architectures with reactive epoxide functionalities showing low dispersity.[154] The reactive shell of epoxide functionalities was formed using semicontinuous and stepwise emulsion polymerization. For this, poly(butyl acrylate) seeds were synthesized, followed by the continuous addition of styrene and butyl acrylate. In the following step, a mixture of butyl acrylate and vinylphenylglycidyl ether was added. In a subsequent step ring-opening of the epoxide groups of the reactive shell was performed with amine functionalized silica particles, yielding in raspberry like organic-inorganic hybrid particles with an organic core and smaller silica particles as shell.[154]

In contrast to the postfunctionalization of glycidyl bearing colloids, it is also possible to invert this reaction; thus, amine bearing nanoparticles are functionalized with the glycidyl bearing counterpart. One example of the inverse approach is the preparation of poly(*N*-isopropylacrylamide) PNIPAM coated magnetic nanoparticle clusters in the size range of 20–150 nm. For this, glycidyl-functionalized PNIPAM was grafted onto magnetic nanoparticles via ring-opening reaction between the glycidyl groups of the polymer and the amino groups on the surface of the magnetic nanoparticles.[155]

2.6.2. Complete and Interior Functionalization Using Epoxide Ring-Opening Reaction

Besides surface modification, epoxide ring-opening reaction with nucleophiles can be also applied for postfunctionalization of the internal of colloidal systems. For instance, poly(*N*-vinyllactams) with glycidyl side groups served as robust building blocks for protein conjugation via epoxide ring-opening reaction and the development of biohybrid nanogels with enhanced stability. In this sense, water-soluble copolymers of GMA and three different cyclic *N*-vinyllactam derivatives were synthesized and assembled into nanogels in a water-in-oil emulsion. Crosslinking was achieved by epoxide ring-opening reaction between glycidyl groups with bifunctional amine-terminated PEG as a model multifunctional crosslinker. This concept was then further advanced for the preparation of biohybrid nanogels by using proteins as crosslinker. Biomacromolecules typically contain several amine groups on the surface providing the ability to act as crosslinker. In the study, cellulase and enhanced green fluorescent protein were used as proof-of-concept for the preparation of biohybrid nanogels (**Figure 18**).[156]

Another example for interior functionalization was reported by Winnik and co-workers, who presented nanogels labeled with fluorescent europium-doped lanthanum fluoride (LaF_3:Eu) nanoparticles.[157] These nanogels in the size range of 250–350 nm

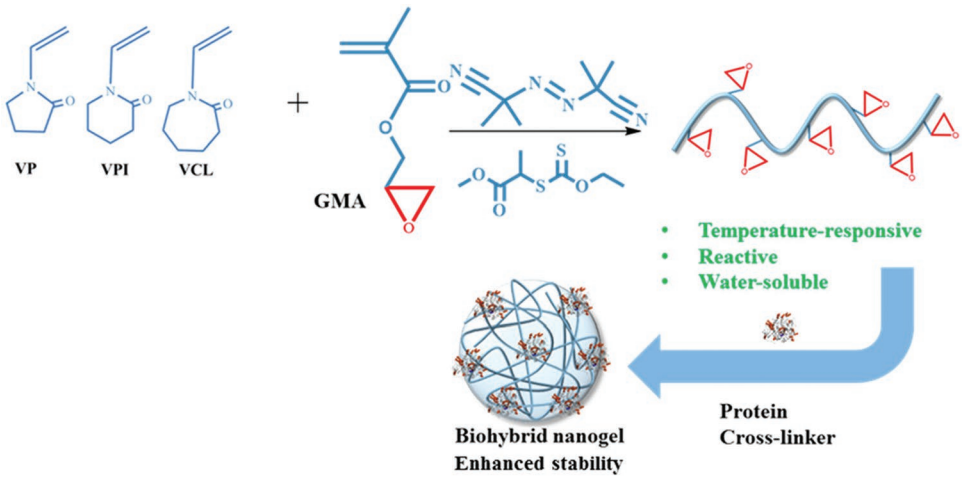

Figure 18. Synthetic route for the design of biohybrid nanogels by crosslinking of glycidyl-functionalized copolymers with proteins via epoxide ring opening reaction. Reproduced with permission.[156] Copyright 2019, American Chemical Society.

were prepared by surfactant-free heterophase polymerization of *N*-vinylcaprolactam (VCL) and GMA. A core–shell structure of the nanogels was achieved due to different reactivities of the monomers, leading to a compact GMA-rich core, which is surrounded by a soft VCL-rich shell. The nanogels were then modified in the interior with 2-aminoethylphosphate stabilized LaF_3:Eu nanoparticles by reacting the amino groups on the surface of the particles with the epoxide groups of the GMA units.[157]

In general, epoxide ring-opening reaction represents an upcoming functionalization strategy for colloids. Especially, the sensitivity toward amines and thiols proves to be beneficial for the conjugation of biomacromolecules to colloids without prior modification. Furthermore, this synthetic strategy benefits from the wide commercial availability of amines and thiols. The tolerance of epoxide functionalized monomers toward radical based polymerization techniques represents an additional advantage, enabling conventional particle preparation methods such as emulsion polymerization. Furthermore, the functionalization process does not require a toxic catalyst nor lead to the formation of large nonfunctional linkers/connecting moieties between the colloid and the newly introduced functionality. Thus, a reasonable "functionality-to-spacer-ratio" is the result. On the downside, postmodification with primary amines generates secondary amines that may react with another epoxide group, leading to additional crosslinking.[151] Furthermore, the ring opening of the epoxide leads to hydroxyl groups, which might generate hydrogen bonds or act as nucleophiles in other reactions. Depending on the application, this can represent a disadvantage of this synthetic strategy. In these cases, active ester chemistry might be an alternative, that are also sensitive to amines and exhibit a good "functionality-to-spacer-ratio."

In this chapter, several reaction types of the "click" family have been reviewed. These synthetic routes found broad application in particle analogous reactions because of their mild reaction conditions, their good biocompatibility and their high probability for bioorthogonal functionalization. Each of these reaction types has its eligibility, but also show some disadvantages for selected applications, such as toxic catalyst or radical formation hindering their use in living tissue. It becomes obvious that there is no universal reaction for postparticle formation functionalization; thus, the selection of the modification strategy is strongly dependent on the requirements of the respective application.

3. Active Esters in Particle Analogous Reactions

Even though over the last 20 years "click" chemistry, especially the CuAAC reaction, has emerged as one of the most popular reactions in the field of polymer functionalization, the concept of activated ester chemistry for postmodification is much older. Already in the 1970s the groups of Feré and co-workers[158] and Ringsdorf and co-workers[159] pioneered the synthesis and postpolymerization modification of reactive ester polymers. Since then a variety of different polymeric active esters have been made accessible by various polymerization techniques.[9] The most common reaction of the resulting polymeric active esters is the nucleophilic substitution with amines, leading to an amide bond formation. The amide bond is one of the most abundant chemical linkages in nature and is present in many organic molecules and biomolecules, due to its unique stability toward various reaction conditions, e.g., acidic, basic, oxidizing, or reducing environments.[160,161] Because of their good nucleophilicity amines react selectively with active esters, even in the presence of weaker nucleophiles such as alcohols. An additional advantage of active ester chemistry from the synthetical point of view is that many amines are accessible from natural or commercially sources, while azide or alkyne functionalized building blocks that are used in 1,3-dipolar cycloaddition reactions need to be synthesized beforehand. However, the basic conditions needed for the amide bond formation as well as for the alcohol released during the reaction represent disadvantages. Nevertheless, active ester chemistry remains popular for the introduction of new functionalities to polymeric systems. While active ester containing polymers were already extensively reviewed by Das and Théato,[162] this section will focus on postpolymerization modification of particles.

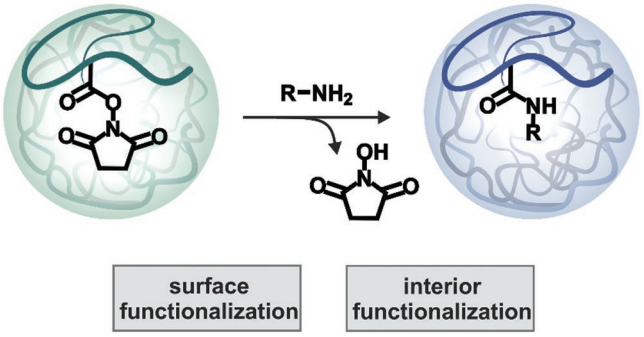

Figure 19. Schematic representation of amidation of NHS esters. This chemistry has been applied to modify the surface or functionalize complete colloids.

3.1. Reaction of *N*-Hydroxysuccinimide Esters

Already in 1972, Ringsdorf and co-workers[159] and Feré and co-workers[158] reported the direct polymerization of *N*-hydroxysuccinimide acrylate (NHSA) and methacrylate (NHSMA) and the subsequent modification of the NHS ester-bearing polymers with amine terminated functionalities (**Figure 19**). Therefore, NHS derivatives are the oldest and most frequently employed active esters for postpolymerization modification. Functional poly(meth)acrylamides that could not be prepared by direct polymerization became then accessible via nucleophilic aminolysis of NHS ester polymers with primary and secondary amines under mild reaction conditions. However, a major drawback of the homopolymers of NHSA and NHSMA is their limited solubility that is mainly restricted to DMF and DMSO. Thus, copolymerization of NHSA and NHSMA is a common solution to increase solubility of the resulting copolymers. With its long history in postpolymerization modification, NHS ester polymers also have found their application in the preparation of functional colloidal systems.

3.1.1. Surface Functionalization Using NHS Ester Chemistry

In the area of colloids, NHS ester chemistry has been mainly applied for the covalent stabilization of colloidal structures by reaction of the reactive groups with bifunctional crosslinkers. Wooley and co-workers extensively investigated shell-crosslinking of block-copolymer micelles via the NHS residues with diamines.[163–165] For example, they prepared an amphiphilic block copolymer of methyl acrylate with NHSA and *N*-acrylolylmorpholine by RAFT polymerization and assembled it in an aqueous solution. The resulting micelles were then covalently stabilized by reacting the NHS groups of the shell with diamine linkers. In addition, it was possible to introduce of a dye via NHS chemistry prior to crosslinking.[163]

Shell-crosslinking via NHS active ester chemistry with a peptide as crosslinking agent was furthermore reported by Cordovilla and Swager for the synthesis of photonic nanoprobes for protease sensing.[166]

Furthermore, NHS ester chemistry also found its application in the covalent surface stabilization of thermally induced self-assemblies.[167–171] For this, typically thermoresponsive block copolymers were used that form nanostructures upon heating above the lower critical solution temperature (LCST). The obtained nanostructures can then be fixed, preventing disassembly at temperatures below the LCST. For example McCormick and co-workers synthesized a thermal responsive ABC triblock copolymer (poly(ethyleneglycol)-*b*-poly(*N*,*N*-dimethylacrylamide-*s*-NHSA)-*b*-poly(*N*-isopropylacrylamide) that self-assembled into micelles upon heating above the LCST of the PNIPAM block.[170] This micellar structure was then stabilized by postpolymerization modification of the NHS ester moieties with bifunctional primary amines in aqueous solution, resulting in shell-crosslinked micelles.[170] In a follow up study, the authors introduced the redox-responsive crosslinker cystamine to the system, thereby enabling reversible cleavage and controlled release of bioactive agents.[171]

Instead of using NHS ester groups bearing polymers, Thayumanavan and co-workers presented a versatile polymer nanoparticle platform with primary amines for inversed functionalization.[172] For this amphiphilic random copolymers derived from 2-aminoethyl methacrylamide and 3-(9-methylcoumarinoxy)propyl methacrylamide were prepared and self-assembled to amphiphilic aggregates in aqueous media. This led to particles with hydrophobic coumarin moieties in the core (also used for crosslinking) while the hydrophilic amino moieties were mainly present on the surface of the aggregates, exposed to the aqueous phase. In a subsequent step, the amines were used to introduce new functional groups via complementary reactive moieties, as NHS ester, PFP ester, epoxides, and isocyanates. Since the amines were mainly present on the surface of the nanoaggregates the authors used this approach to introduce new surface functionalities.[172] However, it is possible that some of the amines are also present in the interior of the particles and therefore interior functionalization cannot be excluded completely. This is another excellent example that shows how the distribution of the active groups influences the location of the functionalization and that the distinction between surface and interior functionalization is diffuse.

In another study, a similar strategy was used to PEGylate a cationic dextran based nanogel by covalent attachment of NHS–PEG to reactive amine groups of the nanogels. Even though the amine groups are present all over the network, PEGylation will probably take place predominantly on the surface, due to the size of the functional PEG.[173]

Another example for the competition between functionalization on the surface and the interior is the preparation of a biohybrid microgels for nutrition delivery for plants. In this study, pH-responsive microgels were prepared via inverse miniemulsion of poly(allylamine) hydrochloride and *N*,*N*′-methylene bisacrylamide and subsequent crosslinking by Aza-Michael addition. The resulting microgels were functionalized with strong chelating ligands for Fe^{3+} ions by NHS coupling. In order to allow interaction of the hydrophilic microgels with hydrophobic surfaces of plant leaves, the microgels were postfunctionalized with an anchor peptide fused with a fluorescent protein PlnA–eGFP. This binding was achieved using a bifunctional NHS and maleimide bearing coupling agent. The peptide was connected to the coupling agent via thiol–ene reaction of the maleimide group to a free cysteine thiol group of the PlnA-eGFP. This modified coupling agent reacted via

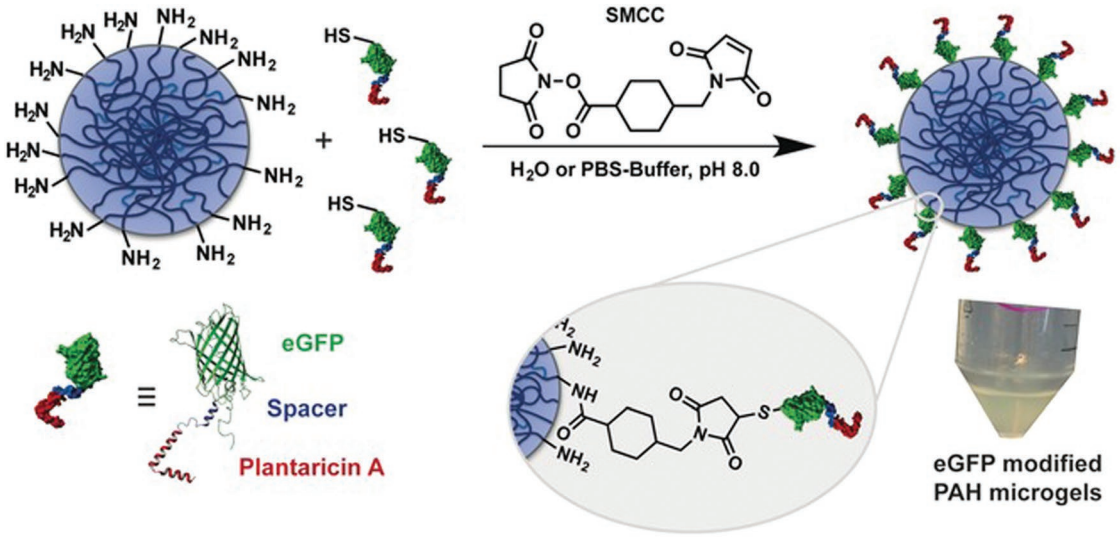

Figure 20. Postparticle formation functionalization of microgels with anchor-peptide fusion protein was achieved by amine containing microgels with thiol–ene and NHS-coupling. Reproduced with permission.[14] Copyright 2017, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

its NHS ester moiety with the residual amine groups of the microgel. Due to the basic conditions during the coupling reaction, the microgels were in a collapsed state and the diffusion of the functionalities into the polymer network was hindered. Therefore the reaction preferably took place on the surface of the particles (**Figure 20**).[14] The last examples illustrate that the distinction between surface and interior functionalization is often diffuse and solely depends on the accessibility of the functionalities.

The inversed functionalization is one possibility to work in an aqueous environment. Another one is based on the activation of carboxylic acids by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/NHS chemistry and the subsequent functionalization with amines. The additional activation step of the carboxylic acids by NHS is often quantitative and can occur under mild conditions in water at room temperature. Tsapias and co-workers reported the surface decoration of PEGylated polyester nanocapsules of perfluorooctyl bromide with a peptide (RGD). For this, nanocapsules were prepared by an emulsion-evaporation process. The carboxylic function of the PEG was activated with EDC/NHS before the peptide was coupled using its terminal primary amine.[174] Yang and co-workers prepared fluorescent nanogels, which were also functionalized on the surface with RGD peptides. The nanogels were prepared by free radical crosslinking of photoluminescent polymer (synthesized by polycondensation reaction of citric acid, maleic acid, L-cysteine and PEG) with acrylic acid. Surface carboxylic acid groups of the nanogels were then activated with EDC and sulfo-NHS and for conjugation with the peptide.[175]

3.1.2. Interior Functionalization Using NHS Ester Chemistry

Since some of the examples above already demonstrate the gray area between surface and interior functionalization, this section focuses on the interior functionalization only. Covalent stabilization of colloids cannot only occur on the surface, it is also possible to crosslink the interior of colloids.[176,177] Thermosensitive block copolymers consisting of a hydrophilic PEG and a random block consisting of PNIPAM and PNHSA formed micelles upon heating above the LCST of the PNIPAM block. Covalent stabilization of the micelles was achieved by coupling the NHS groups of the core forming block with ethylenediamine. The resulting nanogel was able to shuttle between a hydrophobic ionic liquid and an aqueous phase in response to temperature changes.[178]

Similarly, Liu and co-workers reported reversible core crosslinked PEG-*b*-P(NIPAM-*co*-NHSA) micelles with thermosensitive swelling ability by reacting the NHS functionalities with redox-responsive cystamine.[179] By including naphthalimide-based Hg^{2+}-reactive moieties in the copolymer, the forming micelles can be used as ratiometric fluorescent detectors for Hg^{2+} ions. Upon Hg^{2+} addition, micellar dispersion showed a colorimetric transition from yellowish to colorless and a fluorometric emission transition from green to bright blue.[180]

Besides crosslinking of the shell or the core, there is also the possibility to crosslink the nexus, the interface between the core and the shell of micelles. Stenzel and co-workers demonstrated nexus crosslinking of a self-assembled triblock copolymer consisting of a poly(ethylene glycol) methacrylate, NHSMA, and a cisplatinum carrying block via reaction of acid degradable ketal diamino crosslinker with pendant NHS groups.[181]

Furthermore, NHS ester chemistry was applied for the preparation of biohybrid nanogels with immobilized proteins. These nanogels were prepared by a water-in-oil emulsion of a water-soluble reactive copolymer consisting of poly(*N*-vinylpyrrolidone) and PNHSMA. Covalent crosslinking was achieved via reaction of amine groups present on the surface of the respective proteins and the NHS residues.[182]

Another approach that conjugated macromolecules to colloids was presented by Tan and co-workers who presented star polymer nanoparticles that enable positionally assembled enzyme cascade reactions. First *N*-adamantylacrylamide (ADAM) and NHSA were copolymerized with NIPAM via RAFT

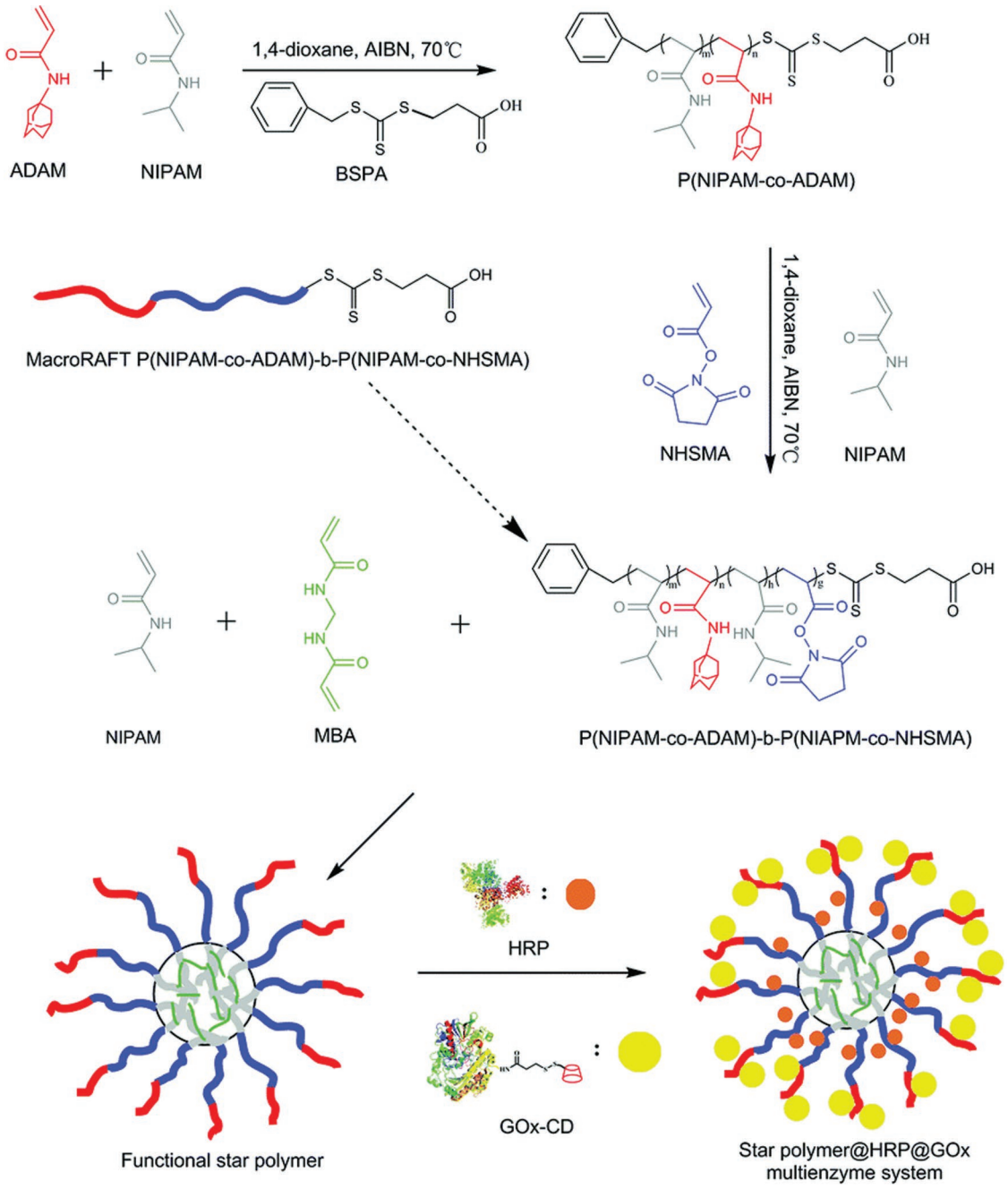

Figure 21. Synthetic strategy for the preparation of star polymer nanoparticles for the conjugation of enzymes via NHS ester chemistry and adamantane β-cyclodextrin host–guest recognition. Reproduced with permission.[183] Copyright 2019, The Royal Society of Chemistry.

polymerization to obtain the diblock copolymer (PNIPAM-*co*-PADAM)-*b*-(PNIPAM-*co*-NHSMA) as arm of the star polymer to be formed. This was achieved in a further polymerization with *N,N′*-methylene bisacrylamide and NIPAM. Two different enzymes were conjugated to the inner and outer layer of the star polymer. One enzyme was assembled in the outer layer of the star polymer through host–guest recognition between the adamantane moiety and the β-cyclodextrin-modified enzyme, while the other enzyme was conjugated to the inner segment by reacting the amine groups on the surface of the second enzyme with the NHS ester groups of the star polymer (**Figure 21**). An increase in the ratio of crosslinker/macroRAFT enhanced the amount of the incorporated arms, thus increasing the steric hindrance. This in turn hinders the diffusion of the enzyme into the core, leading to a decrease of the enzyme loading capacity.[183]

Summarizing, we can conclude that NHS ester chemistry is a favorable functionalization strategy for the introduction of new functionalities to polymeric nanoparticles. Active ester chemistry benefits from the large number of commercial and

natural available amines. In this context, NHS ester chemistry shows great potential for the conjugation of biomacromolecules to colloids due to free amine groups present in different amino acids such as lysin. However, the release of equal molar ratios of the corresponding alcohols and the associated purification steps represents a major disadvantage, even though purification of colloids is a standard procedure in polymer chemistry.

A general characteristic of active ester chemistry is that no additional spacer needs to be introduced/formed in order to establish the new functionality. The result is a good "functionality-to-spacer-ratio." Furthermore, the tolerance of active ester toward radical polymerization techniques is beneficial. Therefore, precursor particles can be easily prepared via radical polymerization of the respective monomers. Even though NHS esters are widely used in polymer and particle analogous reactions, there are two known side reactions during the functionalization step 1) ring opening of the succinimide moiety and 2) intramolecular attack of amides on neighboring activated esters leading to glutarimide formation.[184] Yet, these side reactions can be suppressed by increasing the reaction temperature, time, and equivalents of the respective amines.[185]

Nevertheless, the poor solubility of the homopolymers of NHSA and NHSMA in most organic solvents represents a major drawback. Thus, copolymerization with other monomers is a common tool to enhance the solubility of NHS ester containing polymers and to extend the scope for functionalization with amines of different polarity.[162] By performing an additional reaction step also functionalization in water can be executed. For this, carboxylic acids act as starting material and the new functionality is introduced via EDC/NHS coupling.

As an alternative for functionalization in aqueous conditions, sulfonated NHS-ester monomers have been proposed for polymer analogous reactions.[186,187] However, so far, these water soluble NHS derivative monomers have not found application in colloidal systems. This might be due to their short half-life and rapid hydrolysis.[186]

From this we can conclude that NHS ester chemistry is suitable for the introduction of small functionalities as well as conjugation of biomacromolecules. However, for applications that require the reaction to proceed in an aqueous environment additional steps must be taken, thus thiol–ene reactions might be alternatives.

3.2. Reaction of Pentafluorophenyl Esters

PFP ester polymers are the second most exploited active ester polymers for postpolymerization modifications (**Figure 22**). Comparing NHS and PFP ester bearing polymers, PFP ester polymers demonstrate higher reactivity and greater hydrolytic stability and are soluble in a wider range of organic solvents.[188] First introduced in the early 1970s in peptide chemistry,[189,190] it took around 30 years until PFP esters were rediscovered for polymer synthesis.[191] In 2005 the group of Théato reported the first postpolymerization functionalization of polypentafluorophenyl acrylate (PPFPA) and methacrylate (PPFPMA) polymers.[188] This laid the foundation for a new postpolymerization modification method that quickly found its application

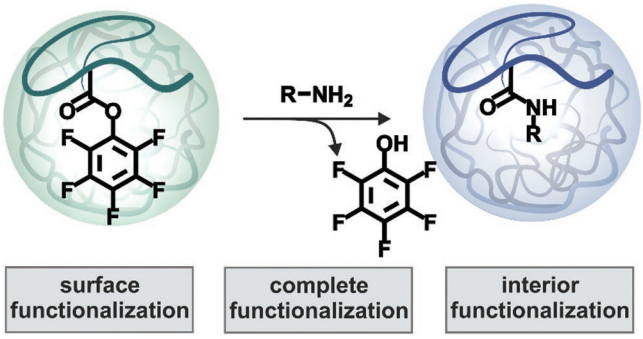

Figure 22. Schematic representation of amidation of PFP esters. This chemistry has been applied to postfunctionalize the surface, the interior as well as complete colloids.

in colloidal chemistry, too. For example, the nanoprecipitation of partially postpolymerization modified PPFPMA polymers with the model fluorescent dye dansylcadaverine yielded in well-defined fluorescent polymer nanoparticles.[192] Self-assembly of preformed polymers to nanostructures is another approach that gained popularity. For instance, Palmans and Meijer functionalized PPFPA precursor polymers with different hydrophilic and hydrophobic side groups and assembled them to single-chain polymeric nanoparticles or complex supramolecular structures.[193–195] Another example is the RAFT copolymerization of PFPMA and lauryl methacrylate. Subsequent postfunctionalization resulted in amphiphilic copolymers that self-assembled in aqueous solutions to polymeric micelles or micelle-like aggregates.[196,197]

3.2.1. Surface Functionalization Using PFP Ester Chemistry

Besides using postpolymerization modification of PFP-ester bearing polymers to prepare functional polymers, it is also possible to postfunctionalize PFP ester bearing colloids in order to introduce new surface functionalities. One example of postparticle formation modification on the surface was reported for glycopolymer nanocapsules to be used for the encapsulation of hydrophilic cancer drugs. These polymeric nanocapsules were synthesized in a one-pot process via inverse miniemulsion periphery RAFT polymerization of PFPMA and a redox responsive divinyl crosslinker, stabilized by an amphiphilic macro-RAFT block copolymer of poly(*N*-(2-hydroxypropyl methacrylamide) and PPFPMA. The decoration of the shell with sugar moieties was conducted via reaction with the highly active PFP functionalities of the shell.[29]

In another example, nanogels were prepared by the assembly and crosslinking of random copolymers consisting of PFPA and poly(ethylene glycol) methacrylate with a diamine crosslinker. After this nanogel formation step, remaining PFPA groups were used to incorporate further surface functionalities.[198] Even though the authors used this postparticle formation step in order to introduce surface functionalities, remaining PFPA groups are also located inside the nanogel. Thus, this is another example showing the difficulty for a clear categorization between surface and complete functionalization. The same often holds true for the differentiation between complete and interior functionalization.

3.2.2. Functionalization of the Interior Using PFP Ester Chemistry

Having seen that postpolymerization modification of PFP esters can be used to introduce functionalities to the surface of nanoparticles, this section deals with postmodification taking place only in the interior of the colloids. One of the major applications in this area is the crosslinking of micellar systems. As an example, the Zentel group prepared crosslinked cationic hydrogel particles in the nanometer range as siRNA carriers.[199–201] For this, well-defined amphiphilic reactive ester block copolymers were synthesized by RAFT polymerization of PFPMA and methoxy triethylene glycol methacrylate. In polar aprotic solvents, an aggregation tendency of the block copolymers was observed, leading to nanometer-sized polymeric superstructures. These polymer aggregates were then used as reactive precursors to generate covalently stabilized nanogel particles by crosslinking the hydrophobic inner core with amine-containing crosslinkers. The same reaction step could also be used to introduce further functionalities to the nanoparticles, such as fluorescent dyes.[199–201]

A similar strategy was used to prepare pH sensitive hydrazone-linked doxorubicin nanogels. Amphiphilic block copolymers composed of *N,N*-dimethylacrylamide and PFPA were assembled and crosslinked with a bifunctional amine crosslinker. Remaining active ester moieties after crosslinking were reacted with ethanolamine and hydrazine thereby inverting the core hydrophilicity and enabling the conjugation of doxorubicin through the formation of an acid-labile hydrazine bond.[24] To generate stimuli-responsive nanostructures, acid-sensitive ketal crosslinkers were introduced in related systems, resulting in the disintegration of the nanogels upon acidic conditions.[202–206]

The synthesis of pH degradable mannosylated nanogels for dendritic cell targeting was quite similar to the examples mentioned above. Glycosylated nanogels were prepared via core crosslinking of amphiphilic copolymers composed of an acetylated glycosylated block and a PFP activated ester block. A one-pot approach of self-assembly, pH-sensitive crosslinking, and removal of remaining PFP esters and protecting groups yielded in fully hydrated nanogels.[207] In a follow up study, De Geest and co-workers furthermore introduced cationic groups to the core forming block after crosslinking with an pH-responsive ketal crosslinker via PFP ester chemistry.[208] Upon pH triggered hydrolysis of the crosslinker, the nanogels disassembled into soluble unimers and exert potent membrane-destabilizing activity. This is an interesting feature in view of cytoplasmic delivery of membrane impermeable (macro)molecules.[208]

The concept of crosslinking of self-assembled block copolymers was further extended by O'Reilly and co-workers who used polymerization-induced self-assembly (PISA) via RAFT polymerization to prepare (PEG-*b*-PPFPMA) nanostructures.[209] These structures were then stabilized via postpolymerization modification of the active esters of the core forming block with nonresponsive or redox-responsive diamines.[209]

Besides crosslinking, interior functionalization of PFP esters can also be used to introduce new functional groups. Hoven and co-workers partially modified preformed block copolymers (PPFPA-*b*-PNIPAM) with light-responsive moieties of *ortho*-nitrobenzyl protected diamine and self-assembled them to micelles in water.[210] Upon UV irradiation the protected diamine groups were released and induced an in situ crosslinking by reaction with remaining PFP groups of the core forming block. Afterwards, 4-Nitro-7-piperazino-2,1,3-benzoxadiazole was covalently attached to the core of the crosslinked micelles to demonstrate the possibility for additional postpolymerization functionalization of residual PFPA moieties.[210] The same research group furthermore reported a cascade postpolymerization modification of a single PPFPMA homopolymer for the preparation of redox responsive nanogels. For that, PPFPMA was first subject to functionalization with oligo(ethylene glycol) methyl ether amine yielding in a random PPFPMA-*r*-PEGMA polymer. In the next step, the copolymer was self-assembled in water and crosslinked with the redox responsive dithiol crosslinker cystamine. The obtained nanogels were further postmodified with *N*-isopropylamine, resulting in non-toxic nanogels that were able to encapsulate and release nile red as hydrophobic model compound.[211]

3.2.3. Complete Functionalization Using PFP Ester Chemistry

Having seen examples where the functionalization is restricted either to the surface or the interior of the colloids the following examples deal with complete functionalization. One approach to generate nanogels for complete functionalization is based on the nanoprecipitation and subsequent photocrosslinking of preformed PPFPMA via coumarin groups. The coumarin groups were introduced before particle formation via partial substitution of PFP esters. It was demonstrated that the crosslinked nanoparticles can be used as platform to produce stimuli-responsive nanoparticles by postmodification of remaining ester groups with various functionalities including amine terminated PNIPAM (**Figure 23**).[212]

Another strategy to generate a synthetic platform based on the postpolymerization modification of PFP esters was reported by Walther and co-workers, who prepared PPFPA latex particles via emulsion polymerization.[17] As a proof of concept the precursor particles were postmodified with *N,N*-dimethylethylendiamine (DMEDA), resulting in pH-responsive nanogels.[17]

The Klinger group went one step further on the path to a synthesis platform for investigating structure–property relationship of amphiphilic nanogels. For this PPFPMA, reactive precursor particles were prepared via miniemulsion polymerization and postmodified in a second step with different hydrophilic and hydrophobic functional groups. Since all nanogels originated from the same precursor particles, amphiphilic nanogels with different degree of amphiphilicity but similar colloidal features were obtained and investigated with respect to their loading and release profile for the hydrophobic model compound nile red as well as to their protein corona formation (**Figure 24**).[19,213]

From this section, we can conclude that PFP ester chemistry is a powerful tool for the preparation of functional colloids. Similar to NHS active esters, PFP ester chemistry benefits from the wide variety of commercial and natural amines and exhibits a good "functionality-to-spacer-ratio." Furthermore, PFPA and PFPMA can be easily polymerized via radical polymerization processes and the polymers show a wider range of solubility and better hydrolytic stability than NHS ester polymers.[188]

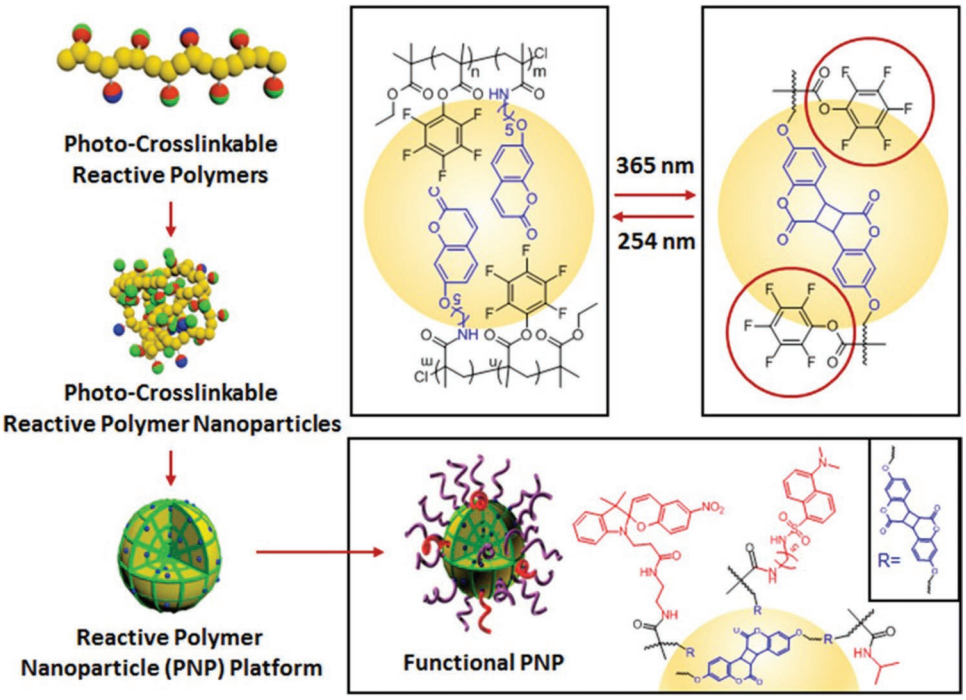

Figure 23. Schematic representation of the synthesis of reactive polymer nanoparticles for the preparation of a functional nanoparticle library via PFP ester chemistry. Reproduced with permission.[212] Copyright 2016, John Wiley & Sons, Inc.

PFP ester chemistry has been employed in various particle-formation techniques, ranging from emulsion to miniemulsion to crosslinking of micelles.

Even though the homopolymers are soluble in a wider solubility range of organic solvents than NHS ester homopolymers, they are still poorly soluble in water, which limits their utility in aqueous systems. For these application, thiol–ene reactions might be better suited. This together with the basic conditions needed for the aminolysis and the released alcohol during this reaction step represent the disadvantages of this functionalization strategy. However, the purification and removal of small molecules from prepared colloids is a standard procedure that

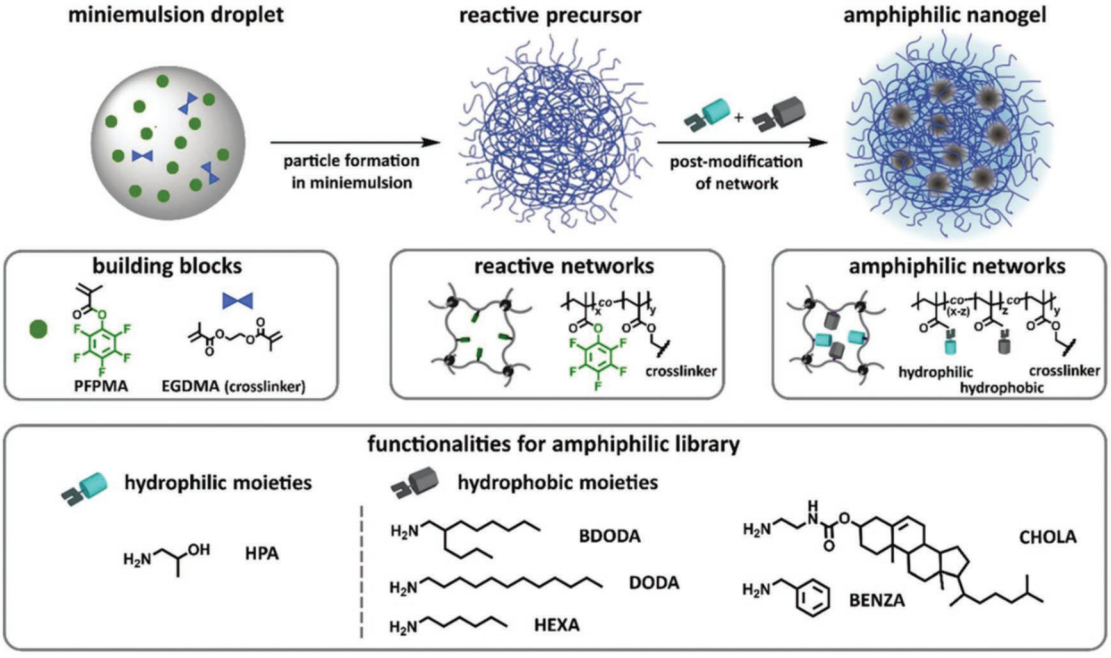

Figure 24. Schematic representation of a library preparation of amphiphilic nanogels with similar colloidal features based on polypentafluorophenyl methacrylate precursor particles. Reproduced with permission.[19] Copyright 2018, Royal Society of Chemistry.

is performed anyways. Besides these disadvantages, numerous discussed applications show the potential of PFP ester chemistry in particle analogous reactions. Furthermore, many of the examples presented in this section are showing the recent trend toward the preparation of functionalized colloidal libraries. This is an important development in better understanding the effect of certain structural features or functionalities and could pave the way to new fundamental design concepts for colloids.

4. Other Reactions for Particle Functionalization

The previous sections summarized the milestones in transferring postfunctionalization strategies from polymers to particles for "click" and active ester chemistry. In addition to these well-established postpolymerization modification reactions, there is a wide range of other synthetic tools that can be used for this purpose. In this section, a brief overview is given on several other chemistries used for postmodification of colloidal systems.

4.1. Thiol–*Para*-Fluoro Substitution Reaction

One possibility for particle functionalization is the thiol–*para*-fluoro substitution reaction. This chemistry enables the modification of PFP groups bearing polymers via quantitative substitution of the *para*-fluoro position with thiols. Roth and co-workers presented polymorphic nanoparticles prepared via PISA that were crosslinked through thiol–*para*-fluoro substitution reaction.[214] Chain extension of poly(poly(ethylene glycol) methacrylate) with 2,3,4,5,6-pentafluorobenzyl methacrylate via PISA led to nanoparticles in the full range of morphologies (spheres, worms, and vesicles). Upon heating, worm phases were able to undergo thermo-reversible delegation and morphological transition into spheres. The authors were able to covalently stabilize these morphologies by crosslinking the core forming block via thiol–*para*-fluoro postfunctionalization with a dithiol.[214] Although PFP groups containing polymers and particles can be easily prepared via radical polymerization methods and numerous thiols are commercially or synthetically available, this functionalization strategy has not attracted much attention in particle analogous reactions. One reason might be the overshadowing success of the PFP ester chemistry. Furthermore, depending on the PFP moiety, PFP active ester chemistry can be a competing reaction, e.g., PFPMA units are sensitive to thiol–*para*-fluoro substitution as well as aminolysis.[215] Additionally, since the PFP group is not a leaving group in this functionalization strategy, it remains in the colloidal system. The result is a large fluorinated spacer upon introduction of a new functionality and an unfavorable "functionality-to-spacer-ration." Furthermore, the properties of the introduced functionality might be influenced by the fluorinated linker.

4.2. Thiol–Disulfide Exchange Reaction

Another strategy utilizing thiols is the thiol–disulfide exchange reaction. This reaction is well known from biology, where among other things, it plays an important role in protein folding.[216] Disulfides are attractive moieties for postfunctionalization since one sulfur in the disulfide bond can be easily replaced by an thiol compound of interest. An advantage of this thiol–disulfide exchange reaction is its reversibility. The newly formed bond can be cleaved, either via reduction or exchange with another thiol. Thayumanavan and co-workers used this chemistry for the crosslinking and surface modification of several nanostructures.[217–221] For example, they designed programmable nanoassemblies from nonassembling homopolymers through the introduction of electrostatic interactions. Once constructed, the vesicle-like structures were covalently stabilized by self-crosslinking induced by the addition of dithiothreitol (DTT) via dithiol–disulfide exchange reaction of the side chains. Moreover, the same chemistry was applied to introduce a fluorescent dye to the surface. While this functionalization reaction is reversible, the authors furthermore modified the surface with isothiocyanate bearing rhodamine through reaction with accessible amine groups. With this, the authors presented a synthetic platform that facilitate the generation of programmable nanoassemblies with tunable properties.[219]

Furthermore, the Thayumanavan group applied the thiol–disulfide exchange reaction for postfunctionalization of non-cationic RNA–polymer complexes.[222,223] Methylated pyridyl disulfide groups among the polymeric scaffold were the key of this synthetic strategy. On the one hand, the cationic nature of the functional groups initiated the complexation of the RNA, while on the other hand these moieties could undergo thiol–disulfide exchange reaction for crosslinking.[222] In a follow up study, remaining pyridyl disulfide groups after crosslinking were used to PEGylate the complex using a thiol-terminated PEG.[223]

Besides introducing chemical functionalities, thiol–disulfide exchange reaction proved to be favorable for the conjugation of biomacromolecules to colloids, due to the presence of reactive cysteines on their surface. One example is the conjugation of the capsase-3 protein to the interior or the surface of nanogels, prepared of amphiphilic copolymers with pyridyl disulfide moieties. Interior conjugation was achieved by assembly of the copolymer in presence of the protein and the subsequent thiol–disulfide exchange reaction. The assembly was further locked-in with the addition of DTT leading to self-crosslinking of the pyridyl disulfide moieties. In contrast, prenanogel formation and later addition of the protein led to surface functionalization.[224] This represents a perfect example how the accessibility of the reactive groups by the reagent, in this case the protein, dictates the location of the functionalization.

As already mentioned above, an advantage for postfunctionalization strategies that include thiols, is their wide commercial and synthetic availability. Furthermore, the thiol–disulfide exchange reaction allows the conjugation of biomacromolecules through reactive cysteines on their surface. An additional benefit is the compatibility of the pyridyl disulfide moieties, typically used as reactive groups, with radical polymerization processes such as RAFT polymerization. Furthermore, the reversibility of the formed disulfide bond represents an advantage for applications were a release of the conjugated molecules or a disassembly of the colloid triggered by a reductive environment is favorable. If this is not the case, functionalization strategies based on active ester chemistry, thiol–ene or epoxide-ring opening reactions might be good alternatives.

4.3. Schiff Base Reaction

Another postmodification strategy is based on the formation of a Schiff base linkage between an aldehyde and a primary amine. One example is the immobilization of BSA onto micelles formed by self-assembly of aldehyde-functionalized glycopolymers.[225] Furthermore, Jackson and Fulton reported core crosslinked star polymers through covalent imine bond formation between diblock copolymers possessing amino or aldehyde functions within one of their blocks.[226] In a follow up, they also prepared nanogels by crosslinking two preformed methyl methacrylate copolymers containing either aldehydes or amino functions in their backbone.[227]

Another application of this chemistry is the conjugation of hemoglobin into the interior of self-assembled and crosslinked nanostructures. In the presence of an oxidizing agent, pH assembled dextran-*g*-succinic anhydride-*g*-dopamine conjugates were in situ crosslinked and functionalized with aldehyde groups at the same time. The formed aldehyde groups were then used for subsequent hemoglobin conjugation via Schiff base reaction with the lysine amine groups of hemoglobin.[228]

As for other postfunctionalization methods that contain amines, the advantage of commercial and synthetical availability of amines also applies to the Schiff base reaction. Furthermore, this method also allows the conjugation of biomacromolecules such as proteins. An additional advantage of this functionalization strategy is that the many aldehyde containing monomers tolerate radical polymerization processes.[229] Moreover, the introduction of a new functionality does not come along with the introduction of a large passive linker.

4.4. Electrostatic Interaction

Whereas in the previously discussed postfunctionalization reactions a classical covalent bond was formed, there is also the possibility to use electrostatic interactions for postmodification.

This concept has been applied to modify surfaces of colloids. One example for this approach is the surface functionalization of a crosslinked, chlorhexidine loaded acrylate copolymer nanogel with the cationic polyelectrolyte poly(diallyldimethylammonium chloride).[230] The surface modification of polyacrylic acid based nanogels with branched polyethylenimine represents another example.[231]

Besides surface modification, electrostatic interactions have been mainly used for formation and assembly of nanostructures such as micelles. This type of micelles was pioneered by Harada and Kataoka who studied micellar formation through electrostatic interaction between a pair of oppositely charged block copolymers consisting of a PEG block and poly(l-lysine) block or a PEG block and poly(α,β-aspartic acid) block.[232] Since then, these micelles have been subject to many studies.[233–235]

One example for the electrostatic formation and crosslinking of colloidal systems besides micelles was reported by Klinger and co-workers, who presented microgels based on coacervation of ionic copolymers.[236] The assembly of preformed PEO-based triblock copolymers with oppositely charged end-blocks in a microfluidic device, led to well-defined microgels. This approach furthermore enabled the incorporation of functional small molecule payloads via electrostatic interactions.[236]

Besides colloid formation and stabilization, electrostatic interactions can also be used for the introduction of functional groups into the interior of nanoparticles. A simple route for the design of hydrophilic microgels, containing hydrophobic nanodomains in the interior for the encapsulation of hydrophobic molecules, was established by Möller and co-workers.[237] The hydrophobic domains were formed by hydrophobic wedge shaped sulfonic acid molecules, which were incorporated into the poly(*N*-vinylcaprolactam-*co*-acetoacetylethyl methacrylate-*co*-vinylimidazole) microgels via acid–base interaction between imidazole units and sulfonic acid groups.[237] The authors furthermore showed that changing the alkyl chain length of the amphiphilic wedge-shaped molecules affected the particle size as well as the environmental sensitivity of the microgels (**Figure 25**).[238] In a recent study, an additional azobenzene group was introduced to the microgels. The azobenzene group acted as a spectroscopic and kinetic probe, thus enabling the study of the microenvironment of the microgels with regard to temperature and hydrophobicity.[239] These examples show that noncovalent linkages also allow the preparation of functional nanoparticle libraries, thus offering similar colloidal features for structure-property investigations.

An advantage of this synthetic strategy is its mild reaction conditions. However, a major problem of this functionalization approach is the lack of specificity and, the instability and reversibility of the formed bonds which are dependent, e.g., on pH and ionic strength. This can be considered as an advantage or disadvantage depending on the desired application.

5. Conclusion and Perspective

Postpolymerization modification of reactive precursor particles shows great potential for the design and preparation of functional nanoparticles. Originally, this concept was developed for polymer synthesis, where postpolymerization modification has been used to introduce a variety of functional groups to polymers without changing the degree of polymerization and molecular weight distribution. More recently, this concept was transferred to the preparation of colloids. In a similar manner, postfunctionalization of reactive precursor particles enables the introduction of a broad variety of functional groups into particles while reducing variations in colloidal features, e.g., particle size and size distribution. With this, postpolymerization modification overcomes the two major challenges in the preparation of functional colloids: First, the limited functional group tolerance of some conventional preparation techniques. Second, the lack of comparability between particles with different functionalities.

To demonstrate the potential of particle analogous reactions, this review highlights the milestones in the transfer of postpolymerization modification from linear polymers to colloidal systems. For this, several selected reactions have been reviewed that can be used for the introduction of new functionalities to the surface, the interior or the complete nanoparticles. Discussing these different reactions, it became obvious that there is no universal "one-fits-all" postparticle formation modification

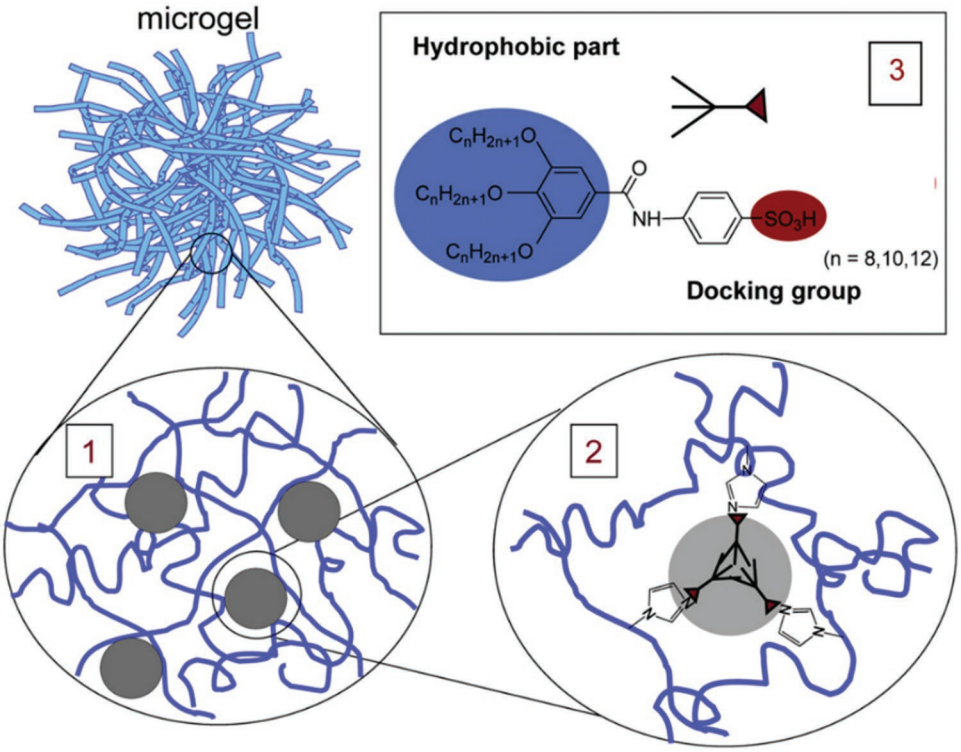

Figure 25. Schematic representation of microgels modified by wedge-shaped amphiphilic sulfonic acid molecules via electrostatic interactions. Reproduced with permission.[238] Copyright 2012, Elsevier Ltd.

strategy. In order to enable an evaluation of the discussed functionalization methods, various criteria such as availability of the reagents, the "functionality-to-spacer-ratio," the compatibility with the preparation method as well as the field of application were examined.

When the focus lies on the accessibility and availability of the monomers and reagents, it becomes obvious that functionalization strategies sensitive toward thiols or amines, such as epoxide ring-opening, thiol–ene and thiol-Michael reaction as well as reactions including active esters, benefit from the wide commercial, natural and synthetical availability of amines and thiols. Additionally, the natural occurrence of amines and thiols in biomacromolecules, attributed to amino acids such as lysin or cysteine, facilitates the conjugation of proteins and enzymes via these functionalization strategies, since no modification is required prior to the conjugation step. In this regard, the above-mentioned functionalization strategies stand out against CuAAC, SPAAC and DA.

A similar picture emerges with respect to the ratio between the introduced functionality and the formed connecting points. Especially, postparticle formation modifications using CuAAC, SPAAC, DA, or thiol–maleimide Michael addition result in the formation of large nonfunctional spacers between the colloids and the newly installed group that might have an impact on the actual desired properties of the introduced functionality. Thus, these synthetic strategies are better suitable for the introduction of high molecular weight functionalities, since the ratio between spacer and functionality is shifted toward the functional moiety, e.g., proteins. For the introduction of small groups such as carboxylic acids, functionalization methods such as radical thiol–ene, epoxide ring-opening or reactions based on active esters are better suited.

Another point to consider is the compatibility of the functionalization strategy with the preparation method of the reactive precursor colloids. For example, alkene bearing monomers applied for thiol–ene reaction show incompatibilities with radical polymerization techniques, thereby complicating the preparation of the respective precursor colloids. However, most functionalization methods tolerate radical polymerization techniques and thereby enable standard particle preparation methods such as emulsion polymerization. Especially, PFP active ester reactions proofed as versatile functionalization strategy that is compatible with a large variety of different particle formation techniques including emulsion, miniemulsion, as well as crosslinking of micelles. However, the functionalization via PFP active esters has the drawback that PFP homopolymers are poorly soluble in water, which limits their utility in aqueous systems.

In the end, the field of application and the associate requirements for the particle functionalization reaction have a huge influence on the decision on the postparticle functionalization method. For example, reaction in a complex biological environment requires bioorthogonal functionalization strategies to prevent cross-reactions. Especially, CuAAC and SPAAC are known to proceed bioorthogonal, thereby fulfilling this requirement. However, the toxicity of copper limits the application of CuAAC in a biological environment. Therefore, SPAAC might be the best suitable functionalization strategies even though it comes with some disadvantages as an unfavorable "functionality-to-spacer-ratio" and additional experimental steps that are required for the introduction of the reactive groups to the protein/enzyme and colloids.

Besides the incorporation of new functionalities into nanoparticles, the current trend in postpolymerization modification of colloids goes toward the preparation of functional nanoparticle libraries. Typically, the preparation of nanoparticle libraries is not performed in complex biological environment, thus bioorthogonality plays a minor role. Therefore, postfunctionalization strategies such as radical thiol–ene, thiol-Michael addition, epoxide-ring opening, and methods based on active esters are beneficial, due to the availability of the reagents, the "functionality-to-spacer-ratio" and the compatibility with the preparation method of the colloids.

The formation of particle libraries enables the determination of important structure–property relationships, leading the way to more effective and efficient colloid synthesis. The development will help to establish universal concepts and criteria for the development of functional colloids. Thus, precise tailoring of nanoparticles to their respective application will be enabled, e.g., personalized nanomedicine. On the long term, this will help to narrow down the tremendous number of different, not comparable nanostructures, thus resulting in more transparency in this research area.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

click chemistry, functional nanoparticle libraries, particle analogous reaction, postfunctionalization, structure–property relations

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