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EXPERT REVIEW

Click Chemistry for Drug Delivery Nanosystems

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ABSTRACT The purpose of this Expert Review is to discuss the impact of click chemistry in nanosized drug delivery systems. Since the introduction of the click concept by Sharpless and coworkers in 2001, numerous examples of click reactions have been reported for the preparation and functionalization of polymeric micelles and nanoparticles, liposomes and polymersomes, capsules, microspheres, metal and silica nanoparticles, carbon nanotubes and fullerenes, or bionanoparticles. Among these click processes, Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has attracted most attention based on its high orthogonality, reliability, and experimental simplicity for non-specialists. A renewed interest in the use of efficient classical transformations has been also observed (*e.g.*, thiol-ene coupling, Michael addition, Diels-Alder). Special emphasis is also devoted to critically discuss the click concept, as well as practical aspects of application of CuAAC to ensure efficient and harmless bioconjugation.

KEY WORDS bioconjugation · click chemistry · CuAAC · drug delivery · nanostructure

ABBREVIATIONS

AAC	azide-alkyne cycloaddition
AgNP	silver nanoparticle
AIBN	azobisisobutyronitrile
Alk	alkyne
ATRP	atom transfer radical polymerization
AuNP	gold nanoparticle
Az	azide
BNP	bionanoparticle
BPDS	bathophenanthroline disulphonated disodium salt
CD	cyclodextrin
CMC	critical micelle concentration
CNT	carbon nanotube
CPMV	cowpea mosaic virus
CTA	chain transfer agent
CuAAC	Cu(I)-catalyzed azide-alkyne cycloaddition
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DDS	drug delivery system
DIPEA	*N,N*-diisopropylethylamine
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-acetic acid
DOX	doxorubicin
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EPL	expressed protein ligation
EPR	enhanced permeability and retention
FA	folic acid
FPLC	fast protein liquid chromatography
FR	folate receptor
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
HABA	2-(4-hydroxyphenylazo)benzoic acid
HEMA	2-hydroxyethyl methacrylate
LA	lactic acid
LbL	layer-by-layer
MAA	methacrylic acid
MBP	maltose binding protein
MNP	magnetic nanoparticle
MRI	magnetic resonance imaging
MWCNT	multi-walled carbon nanotube
NHS	*N*-hydroxysuccinimide
NP	nanoparticle
PAA	poly(acrylic acid)
PACA	poly(alkyl cyanocrylate)
PBD	poly(butadiene)
PCL	poly(ε-caprolactone)
PCN	polymer-caged nanobin
PDMA	poly(*N,N*-dimethylacrylamide)

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PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PEO	poly(ethylene oxide)
PET	position emission tomography
PGA	poly-L-glutamic acid
PIC	polyion complex
PLL	poly-L-lysine
PMA	poly(methacrylate)
PMDETA	*N,N,N′,N′,N″*-pentamethyldiethylenetriamine
PMPC	poly(2-methyl-2-carboxyl-propylene carbonate)
PNIPAM	poly (*N*-isopropylacrylamide)
PrMA	propyl methacrylate
PS	poly(styrene)
PTMCC	poly(2-methyl-2-carboxytrimethylene carbonate)
PtNP	platinum nanoparticle
PTQY	photoluminescence quantum yield
PVP	poly(vinyl pyrrolidone)
QD	quantum dot
RAFT	reversible addition-fragmentation chain transfer
RGD	Arg-Gly-Asp
ROS	reactive oxygen species
SiNP	silica nanoparticle
SPAAC	strain-promoted azide-alkyne cycloaddition
SPION	superparamagnetic iron oxide nanoparticle
STEM	scanning transmission electron microscopy
SWCNT	single-walled carbon nanotube
TBTA	tris(benzyltriazolylmethyl)amine
TCEP	tris(carboxyethyl)phosphine
TEC	thiol-ene coupling
THPTA	tris(hydroxypropyltriazolylmethyl)amine
TMS	trimethylsilyl
VNP	viral nanoparticle

"The reaction must be modular wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation. Purification—if required—must be by nonchromatographic methods, such as crystallization or distillation, and the product must be stable under physiological conditions… Click processes proceed rapidly to completion and also tend to be highly selective for a single product: we think of these reactions as being "spring-loaded" for a single trajectory". H. C. Kolb, M. G. Finn and K. B. Sharpless. Angew. Chem., Int. Ed. 40: 2004–2021 (2001).

INTRODUCTION

Over the past few decades, various generations of drug delivery systems (DDS) have been developed for controlled administration of drugs into the body (1). Among them, nanosized carriers are particularly attractive since they provide protection to the cargo while enhancing cellular uptake and selective delivery to specific cells. Since their appearance in the 1970s, a number of nanosized DDS have been described as carriers for delivery and controlled release of drugs, including drug-polymer conjugates and nanoparticulate carriers, where drug is either physically entrapped or covalently attached to the delivery system (2).

Widespread application of DDS in nanomedicine has been prompted by several remarkable discoveries and breakthroughs, the most relevant being (1) minimizing immunogenicity and preventing early clearance of proteins, drugs, and DDS by conjugation to hydrophilic biocompatible polymers, such as poly(ethylene glycol) (PEG) (3); development of active targeting—the selective delivery of drugs to target tissues and cells facilitated by the privileged interaction of ligands on the periphery of drug carriers with cell surface receptors (4); discovery of the enhanced permeability and retention (EPR) effect, also known as passive targeting, to explain selective accumulation of nanosized DDS within solid tumors thanks to fenestrations in the vasculature and poor lymphatic clearance (5).

Organic synthesis as a discipline has accompanied this evolution of drug delivery into a mature field by providing synthetic tools for efficient bioconjugation of drugs, polymers, or targeting ligands, and preparation of novel building blocks and polymeric materials for construction of DDS with new properties (6). Indeed, chemistry is a central science where phenomena occur and properties are defined at the molecular level. The ability of putting together small building blocks into larger structures has been at the core of evolution and can be used to create novel properties and functions. The level of efficiency achieved by biological systems has inspired chemists in search of more efficient processes with production of minimal waste. In this way, concepts such as atom economy (every atom of a reagent should be included in the product of the reaction), energy efficiency (drive to reduce energy requirements of chemical processes), catalysis (aimed at lowering energy input and avoiding use of stoichiometric reagents), step economy (drive to reduce number of synthetic steps), as well as safety and toxicity issues have been adopted in search of more efficient and environmentally friendly procedures (green chemistry) (7).

In this context, Sharpless and coworkers introduced in 2001 the concept of *click chemistry* (8) in an effort to focus attention on easy production of properties rather than challenging structures. The idea is to confine the whole range of chemical transformations available to a set of processes with a high thermodynamic driving force, allowing efficient and easy transformation of "spring-loaded" starting materials into new substances with useful properties.

Herein, we cover the use of click procedures for preparation and functionalization of nanoparticulate DDS. A selection of,

in our view, the most relevant examples reported in the area is presented following a historical perspective. The review begins with a description of characteristics that define a reaction as click and evolution of those criteria when adapted to fields other than those originally considered by Sharpless and coworkers. A critical assessment on the tendency found in literature to label as click many reactions that do not meet these requirements is also included. As the vast majority of examples on application of the click concept to drug delivery nanosystems rely on the Cu (I)catalyzed azide-alkyne cycloaddition (CuAAC) (9,10), the following section discusses advantages and disadvantages of the use of CuAAC in bioconjugation. The need of a correct selection of catalyst, ligand, solvent, and experimental conditions to avoid degradation of the substrates under CuAAC conditions is also addressed. These features are of application not only to bioconjugation but also any other field. After these introductory sections, a selection of literature examples dealing with the use of click reactions (CuAAC, Michael addition, Diels-Alder, thiol-ene coupling (TEC), strain-promoted azide-alkyne cycloaddition (SPAAC)) for preparation and functionalization of nanosized DDS is included. To facilitate reading, these examples have been organized according to the nature of the nanosystem.

- Polymeric Nano- and Microparticulate Delivery Systems (Polymeric Micelles, Polymeric Nanoparticles (NP), Polymersomes and Liposomes, Polymeric Capsules and Microspheres)
- Metal and Silica Nanoparticles (Gold (AuNP) and Noble-Metal Nanoparticles, Magnetic Nanoparticles (MNP), Quantum Dots (QD), Silica Nanoparticles (SiNP))
- Carbon Nanotubes (CNT) and Fullerenes
- Bionanoparticles (BNP)

Keeping in mind the multidisciplinarity of the drug delivery audience, we focus not only on chemical aspects of the click procedures, but also on the merits of the resulting DDS, with emphasis on their usefulness in therapy and diagnosis. Brief comments on the particular advantages of the conjugates produced, drugs encapsulated, efficiency of targeting processes, or existence of *in vivo* or *in vitro* assays are also included. In a complementary follow-up review, we will discuss click chemistry for drug delivery applications with synthetic polymers and dendrimers.

THE CONCEPT OF CLICK CHEMISTRY

In 2001, Sharpless and coworkers introduced the concept of click chemistry in the field of drug discovery (8). In an effort to focus attention on the easy production of properties rather than challenging structures, these authors suggested that: "all searches [of drug candidates] must be restricted to molecules that are easy to make." This strategy was designed to enable the straightforward and economic synthesis of large libraries of new compounds with a minimum synthetic cost. With this aim, it was proposed to confine the whole range of possible chemical transformations to a set of processes with a high thermodynamic driving force (usually higher than 20 $Kcal \cdot mol^{-1}$), which could allow the efficient transformation of "spring-loaded" starting materials into new substances with useful biological properties. In addition, among the highly energetic starting materials available, "those provided by Nature, petroleum chemistry, or easily manufactured from any of those" were selected, preferred candidates being olefins and acetylenes.

Besides being highly exothermic processes, click reactions should be also characterized by high orthogonality, meaning that coupling partners in click processes should have a wide functional group tolerance but selectively react to each other under a broad range of experimental conditions. In this way, the need of protecting groups is avoided. Thanks to these intrinsic characteristics, reactivity and orthogonality, click processes are reliable and clean transformations of broad scope that proceed in quantitative (or near-quantitative) yields, where simple or no purifications are required.

In order to provide an adequate toolbox of chemical transformations, a set of stringent criteria was established for a process to be considered as click (8): "[to] be modular, wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective)…include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed, and simple product isolation. Purification, if required, must be by nonchromatographic methods, such as crystallization or distillation, and the product must be stable under physiological conditions." These very stringent criteria are actually fulfilled only by a narrow set of chemical transformations, mainly comprising carbon-heteroatom bond-forming reactions. Four main groups were originally defined on these basis (8): additions to carbon-carbon multiple bonds (such as epoxidation, dihydroxylation, aziridination, sulfenyl halide addition, and Michael addition chemistry); nucleophilic substitution chemistry (such as ring-opening reactions of strained cycles, *e.g.*, epoxides, aziridines, aziridinium ions, and episulfonium ions); cycloadditions of unsaturated species (such as 1,3-dipolar cycloadditions and Diels-Alder reactions); carbonyl chemistry of the "non-aldol" type (such as formation of ureas, thioureas, aromatic heterocycles, oxime ethers, hydrazones, and amides). The potential of these four groups of click reactions for efficient preparation of complex structures has been highlighted by Sharpless

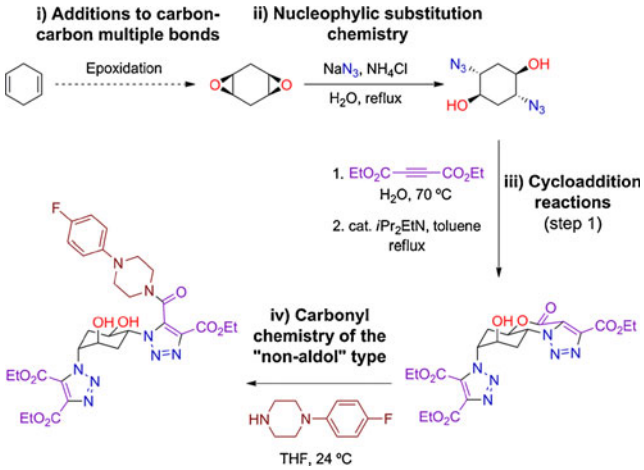

Fig. 1 Synthetic sequence reported by Sharpless and coworkers to highlight the four groups of click reactions originally proposed (ref (8)).

and coworkers in short synthetic sequences as seen in Fig. 1. Interestingly, it is worth pointing out that although the concept of click chemistry has emerged recently, it essentially relies on classical chemical transformations; therefore, publications appearing before the click era (and hence not labeled as click) should be taken into account in this context. An illustrative example is the century-old photochemically/thermally induced radical addition of thiols to alkenes (*i.e.*, thiol-ene coupling, TEC), which has now reappeared as the thio-click reaction.

Among the click reactions proposed so far, the Cu(I)-catalyzed cycloaddition of azides and terminal alkynes (CuAAC) has attracted special interest (Fig. 2). Independently reported by the groups of Meldal (9) and Fokin and Sharpless (10), this reaction displays one of the greatest orthogonaties ever seen. As opposed to classical thermal Huisgen 1,3-dipolar azide-alkyne cycloaddition (AAC), where high temperatures (*ca.* 100°C) and long reaction times are typically required, Cu (I) catalysis significantly reduces the activation barrier, allowing the reaction to proceed with very good rates at room temperature, both in aqueous media and organic solvents (Fig. 2). Moreover, CuAAC is regiospecific, leading exclusively to 1,4-disusbstituited 1,2,3-triazoles, whereas mixtures of regioisomers are obtained under non-catalyzed conditions. In addition, the ease with which azide and alkyne moieties are introduced both chemically (nucleophilic substitution, diazo transfer, incorporated into low molecular weight tags) and biologically (incorporating acetylene and azido amino acids

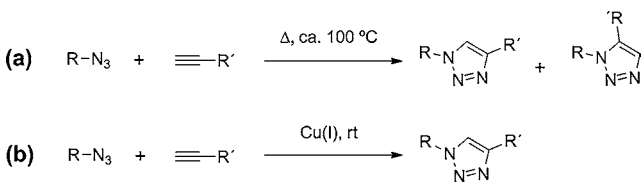

Fig. 2 Thermal (**a**) and Cu(I)-catalyzed (**b**) versions of the Huisgen 1,3-dipolar AAC.

into proteins in either a residue-specific or site-specific fashion) (11), along with their small size and exceptional stability in complex environments, have allowed their incorporation into a vast range of substrates as handled with hidden reactivity. All these features justify CuAAC at the leading edge within click reports.

Since click chemistry was originally defined, an ever increasing number of publications dealing with its application to different areas of research have quickly appeared in the literature. Fields such as polymer and materials science, medicinal chemistry, chemical biology, pharmaceutical sciences, etc. have clearly profited from the click concept (12–15). However, in spite of this favorable reception, a survey of literature reveals that in many publications, even entitled as "click," some fundamental concepts of click chemistry are misinterpreted. For instance, the use of heating to accelerate the reaction rate has been branded by different authors as against the "simple reaction conditions" requirement. Actually, it should be emphasized that click reactions are generally characterized by having high activation energies; therefore, they are often performed by heating (8) (Fig. 1). Use of organic solvents has also been controversial, as sometimes it has been stated that click reactions are only allowed to be performed in water, aqueous mixtures, or neat. In this sense, while water is often the preferred reaction medium, organic solvents can be also used as nicely illustrated by Sharpless and coworkers in their seminal report (Fig. 1) (8).

On the other hand, given the original application of click chemistry to drug discovery, it is clear that the stringent criteria for a click process might be revised when adapted to other fields. This adjustment, however, must be in agreement with the click philosophy. For instance, while stereospecificity is demanded for drug discovery, it may not be important for delivery purposes or in polymer and materials science. Similarly, substrates such as many DDS cannot be considered as "readily available starting materials," as several steps of "non-click" type chemistry are frequently required for their preparation.

However, besides these particular adjustments, some key requirements of click chemistry are sometimes severely distorted in papers claimed as click. A good example is the use of chromatographic purifications that clearly violates the click philosophy and could be only accepted when dealing with macromolecular structures that are difficult to purify by typical techniques demanded of low-molecular-weight compounds (*e.g.*, crystallization or distillation).

To sum up, in spite CuAAC being considered as "the cream of the crop" among click reactions, not every process relying on CuAAC necessarily fulfills the click requisites. In other words, mixing an azide and an alkyne in presence of Cu(I) does not necessarily imply a click process if accompanied, for instance, by low yields or tedious chromatographic purifications. Moreover, experimental conditions

such as catalyst loading, ratio of reagents, and formation of byproducts should also be considered in evaluating overall efficiency and convenience of the process.

Finally, the high impact of the click concept in chemistry and other disciplines has sometimes derived in abusive use of the term click. For instance, a number of reactions that strongly defy the click philosophy have been proposed as new examples of click reactions. On the other hand, well-known and established reactions are now being "relabeled" with the fashionable click tag (*e.g.*, thio-click coupling, thio-bromo click, or Diels-Alder click reactions). In addition, the relevance of CuAAC among the click reactions has sometimes led to labelling this as "the click reaction," obviating all other processes fulfilling the click criteria. Readers must be aware of the above considerations, and, in order to avoid misinterpretations, critical reading is advised.

BIOCONJUGATION *VIA* HUISGEN AZIDE-ALKYNE CYCLOADDITIONS

Bioconjugation techniques play an important role in drug delivery. The conjugation of peptides, antibodies, or aptamers on the surface of DDS allows their active targeting to specific cells and organs. In addition, coupling of synthetic polymers to biomolecules has been described as a method to improve pharmacokinetics and increase stability. Other interesting applications of bioconjugation deal with labeling biomacromolecular therapeutics, such as proteins and nucleic acids, with tags for *in vivo* imaging purposes. With the aim of preserving structural and functional integrity of biomolecules, bioconjugation processes should be ideally performed with fast kinetics and under mild conditions in aqueous media.

Thanks to its high orthogonality, reliability, and experimental simplicity for non-specialists, it is not surprising that CuAAC quickly found application in the bioconjugation arena soon after being described in literature. Unfortunately, however, use of CuAAC in bioconjugation has not been straightforward. Thus, the required Cu(I) catalyst has been demonstrated to induce severe structural damage to biomolecules. Also, under the original conditions reported by Fokin, Sharpless, and coworkers ($CuSO_4$/sodium ascorbate) (10), CuAAC often lacks adequate kinetics at the micromolar range typically employed in bioconjugation. Taking into account the relevance of CuAAC within click chemistry, it is the intention of this section to analyze the encountered problems and proposed solutions for efficient and harmless conjugation of biomolecules *via* CuAAC.

Some limitations of CuAAC in bioconjugation were soon pointed out by Fokin, Sharpless, Finn, and coworkers, who described that cowpea mosaic virus (CPMV) resulted in either degraded or aggregated in presence of Cu and reducing agents used for *in situ* generation of Cu(I) (16). Indeed, the role of Cu in oxidative stress of biomolecules is well known. Cu ions can easily promote generation of reactive oxygen species (ROS), such as H_2O_2, and hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) radicals, which are responsible for biological damage (17). Production of $^{\bullet}OH$, the most powerful among ROS, is mediated by a Fenton reaction involving a transition metal, Cu(I) in this case, and H_2O_2 (Eq. 1) (18). The required H_2O_2 is formed *in situ via* two possible mechanisms: in presence of ascorbate (AH_2), O_2 can be reduced to H_2O_2 in a process catalyzed by traces of Cu(II) or other transition metal ions (Eq. 2) (18); in absence of ascorbate or other reducing agents, Cu(I) can reduce O_2 to H_2O_2 through a two-step process involving mediation of $O_2^{\bullet-}$ (Eqs. 3,4) (17). Presence of ROS during functionalization of biomolecules, such as proteins, nucleic acids, polysaccharides, and lipids, is therefore a major concern, as structural and functional integrity of these substrates might be severely compromised.

$$Cu(I)+H_2O_2 \rightarrow Cu(II)+{}^{\bullet}OH + OH^- \quad (1)$$

$$AH_2+O_2 \rightarrow A+H_2O_2 \quad (2)$$

$$Cu(I)+O_2 \rightarrow O_2{}^{\bullet-}+Cu(II) \quad (3)$$

$$Cu(I)+O_2{}^{\bullet-} + 2H^+ \rightarrow Cu(II)+H_2O_2 \quad (4)$$

Other undesired reactions, such as the homocoupling of terminal alkynes through Cu(I)-catalyzed Glaser reaction, have also been observed under CuAAC conditions (19). Altogether, these secondary processes diminish conjugation efficiency and are particularly undesired in functionalization of macromolecular platforms, as purifications are often eventually impossible.

In a general sense, the aforementioned drawbacks of CuAAC can be efficiently overcome with proper selection of the catalytic system, which usually requires use of a Cu(I)-chelating ligand (20,21). These ligands are designed to stabilize Cu(I) oxidation state, accelerate cycloaddition reaction, prevent formation of undesired byproducts, and sequester Cu ions to reduce structural damage to biomolecules and facilitate purification process. In this regard, tris(benzyltriazolylmethyl)amine (TBTA) (20), tris(hydroxypropyltriazolylmethyl)amine (THPTA) (20), and bathophenanthroline disulphonated disodium salt (BPDS) (21) have been the ligands most usually employed in bioconjugation (Fig. 3).

The tetradentate binding ability of tris(triazolylmethyl) amine ligands allows formation of stable Cu(I) chelates that account for an increase of ~300 mV in redox potential of

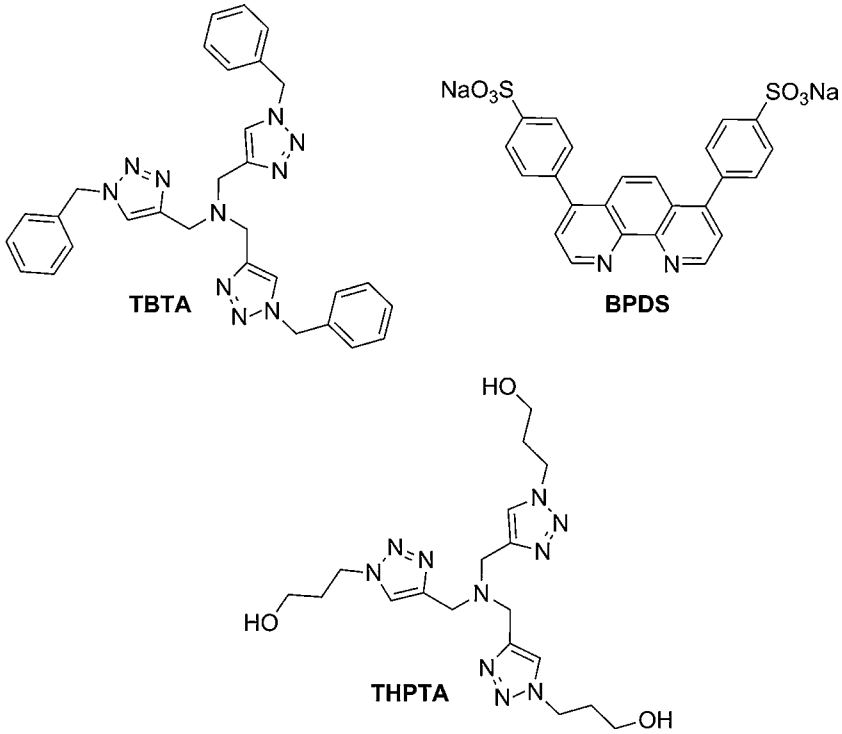

Fig. 3 Cu(I)-chelating ligands commonly employed in CuAAC bioconjugation.

Cu(I)/Cu(II) (20). TBTA was the first member of this family to be identified. The superior protection conferred by TBTA to Cu(I) towards air oxidation renders unnecessary exclusion of O_2 from reaction medium. Nevertheless, a great shortcoming of TBTA is its poor water solubility, which has fuelled development of water-soluble analogs, such as THPTA (22). In addition, partly because of this poor water solubility, the typical rate enhancement of TBTA has been claimed as low, with kinetics comparable to other classical bioconjugation reactions.

BPDS represents an attractive alternative to tris(triazolylmethyl)amine ligands. It exhibits a high solubility in water and an excellent catalytic activity, even under high dilution conditions or when working with small excess of coupling probes (23). Conversely, the BPDS/Cu(I) catalytic system has revealed itself very sensitive to air oxidation, and unless a sacrificial reducing agent is added, the rigorous exclusion of O_2 from reaction medium is required to avoid oxidation of Cu(I).

A survey of literature reveals many examples to illustrate that the correct selection of the catalytic system allows CuAAC to become a safe and efficient tool in bioconjugation. However, despite the use of Cu(I)-chelating ligands, a loss of bioactivity/integrity of proteins and nucleic acids during CuAAC bioconjugation has been reported in several cases (24), which highlight the necessity of more convenient CuAAC protocols. To this end, Finn and coworkers have reported an electrochemically protected version of CuAAC, where Cu(II) is electrochemically reduced to Cu(I) in presence of the desired accelerating ligand (25) (Fig. 4). In this way, the oxidation state of Cu(I) is continuously preserved without addition of any sacrificial reducing agent. In addition, oxidative degradation of substrates by ROS is drastically diminished, as the O_2 present in the reaction medium, which is necessary for generation of ROS (Eqs. 1, 2, 3 and 4), is electrochemically reduced to H_2O (*i.e.*, $O_2+4H^+ + 4e^- \rightarrow 2H_2O$). In a more recent report, this group has proposed a bioconjugation protocol relying on the simplicity of the Cu(II)/ascorbate system and use of THPTA (22) (Fig. 4). Besides the typical ligand-accelerating effect, THPTA was found to mediate decomposition of H_2O_2 produced in reaction medium and to act as a radical scavenger. As previously pointed out by Brown and coworkers (26), a 5-fold excess of THPTA *vs.* Cu(I) was recommended to minimize the oxidative degradation of substrates. Addition of aminoguanidine as carbonyl capturing reagent was also recommended to preserve substrates from reaction with ascorbate and other byproducts derived from ascorbate oxidation (Fig. 4).

Alternatively, more benign Cu-free AAC bioconjugation strategies, not requiring addition of cytotoxic metals and additives, have also appeared. Bertozzi's group has taken advantage of the inherent ring strain of cyclooctynes as an effective way to lower activation barrier of AAC (24) (Fig. 5). This strain-promoted AAC variant named SPAAC has found widespread application in the context of the bioorthogonal chemical reporter strategy for the study of dynamic processes of biomolecules. In addition, SPAAC has recently gained significance in polymer/materials

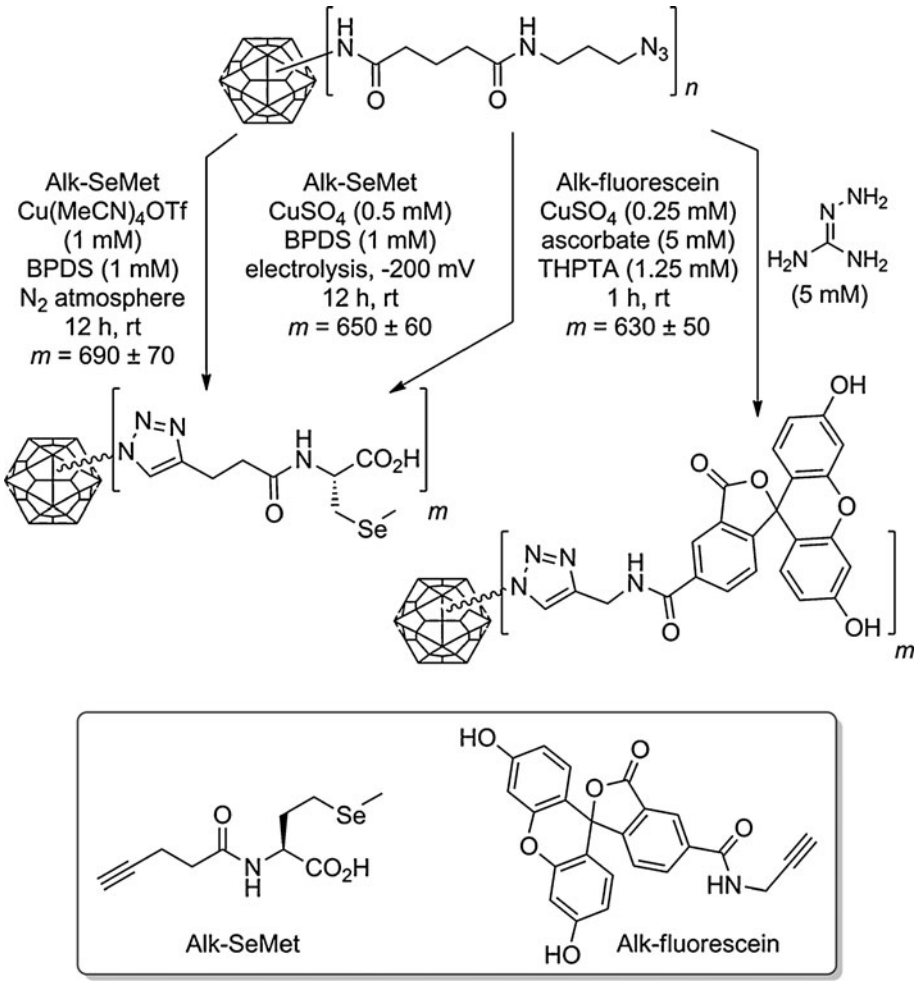

Fig. 4 Functionalization of bacteriophage $Q\beta$ via different CuAAC bioconjugation protocols: Cu(I), BPDS, O_2-free; Cu(II), BPDS, electrolysis; Cu(II), ascorbate, THPTA, aminoguanidine (ref (25) and (22)).

science and drug delivery applications, where presence of Cu prevents the use of CuAAC (11,27). Rate optimization of SPAAC for faster bioconjugation under mild conditions has been reported by the groups of Bertozzi, Boons, and van Hest and van Delft, by means of difluorocyclooctyne (DIFO) (28,29), dibenzocyclooctyne (DIBO) (30), dibenzoazacyclooctyne (DIBAC) (31), biarylazacyclooctynone (BARAC) (32), and bicyclononyne (BCN) (33) derivatives (Fig. 5). The higher reactivity of these reagents compared to unfunctionalized cyclooctyne relates to presence of electron-withdrawing groups and increased ring strain, which have afforded reaction rate values comparable to those of ligand-less CuAAC. Interestingly, a cyclooctyne precursor of reactive DIBO with the triple bond masked as cyclopropenone has been developed by Boons, Popik, and coworkers for photo-triggering of SPAAC under UV irradiation (~350 nm) (34) (Fig. 5).

It must be pointed out that although use of cyclooctynes hampers SPAAC to qualify as a pure click reaction as originally defined (lack of regioselectivity and no readily available starting materials), it essentially matches the click philosophy when dealing with macromolecular structures as those in drug delivery applications.

POLYMERIC NANO- AND MICROPARTICULATE DELIVERY SYSTEMS

Over the past few decades, there has been considerable interest in the use of delivery systems for safe and efficient administration of drugs (1). With this aim, various polymeric nano- and microparticulate systems have been described, including polymeric micelles and NP, polymersomes, and polymeric capsules and microspheres (2). These DDS have been reported to effectively deliver drugs to target sites and hence increase the therapeutic index while reducing undesirable side effects and frequency of administration. In particular, DDS can enhance therapeutic activity by prolonging drug half-life, improving solubility of hydrophobic drugs, reducing immunogenicity, and allowing sustained or stimuli-triggered release of drugs (1,35). In this section, the use of click chemistries for preparation and functionalization

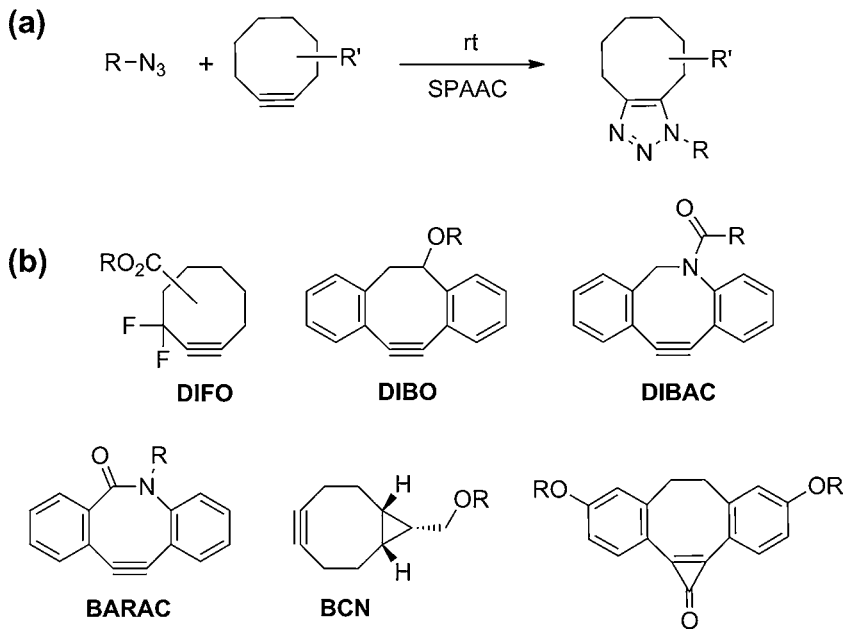

Fig. 5 SPAAC (**a**), and various activated cyclooctyne derivatives used in bioconjugation (**b**).

of different polymeric nano- and microparticulate delivery systems is discussed.

Polymeric Micelles

Polymeric micelles are spherical nanostructures, usually obtained by self-assembly of amphiphilic block copolymers in water, that are considered promising drug carriers (36). They are characterized by a size in the range 20–100 nm (similar to lipoproteins and viruses) and a core-shell structure consisting of a hydrophobic core surrounded by a hydrophilic shell. While the core provides a potential reservoir for hydrophobic drugs, the hydrophilic shell ensures low protein adsorption, improved drug solubility, and long circulation times in the bloodstream. This stealth property and their characteristic small sizes allow selective accumulation of polymeric micelles in tumor tissues due to the known EPR effect (5).

Various click reactions, especially CuAAC, have been used in either the preparation of block copolymers that self-assemble into micelles or the surface functionalization of polymeric micelles. As an example of the former, Sumerlin and coworkers used CuAAC for functionalization of a temperature-responsive block copolymer with folic acid (FA) (37). A thermoresponsive azido-terminated poly(*N*,*N*-dimethylacrylamide)-*b*-poly(*N*-isopropylacrylamide) (N_3-PDMA-*b*-PNIPAM) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization using an azido-functionalized chain transfer agent (CTA), NIPAM and DMA, which was subsequently decorated with a propargyl folate by CuAAC (CuBr, PMDETA, DMF). The resulting block copolymer underwent self-assembly to yield polymeric micelles of ~46 nm at 34°C, as potential drug delivery systems with active targeting for cancer tissues.

Following a similar approach, the groups of Li (38) and Stenzel (39) independently prepared block copolymers incorporating pendant azides/alkenes in one of the blocks that were subsequently functionalized with carbohydrates by means of CuAAC and TEC to give amphiphilic block copolymers. Li and coworkers prepared a series of biodegradable glycopolymers based on poly(ε-caprolactone) (PCL). PCL-*b*-PBrCL was synthesized by ring-opening polymerization, and then bromine groups were transformed into azides and subsequently functionalized with alkynyl saccharides *via* CuAAC (CuBr, PMDETA, DMF) to give amphiphilic block copolymers that self-assembled into micellar aggregates (38).

Taking advantage of TEC, Stenzel and coworkers prepared glycomicelles as potential drug carriers. A block copolymer containing di(ethylene glycol) methyl ether methacrylate and 2-hydroxyethyl methacrylate (HEMA) was prepared by RAFT polymerization and further modified with glucothiose *via* alkene incorporation and TEC (UV light, 2,2-dimethoxy-2-phenyl acetophenone, DMF). The resulting glycosylated block copolymer led to formation of thermoresponsive micelles (39) (Fig. 6).

Several interesting examples on the use of CuAAC for preparation of PEG-dendritic block copolymer-based micelles and other nanostructures have been reported by Dong's group (40–42). For instance, they prepared spherical flower-like micelles of ~50 nm by self-assembly in aqueous solution of dendron-like PCL-*b*-PEG-*b*-dendron-like PCL triblock copolymers prepared by CuAAC (CuBr, PMDETA, DMF) (Fig. 7). These micelles were able to encapsulate the anticancer drug doxorubicin (DOX) with high loading efficiency and displayed a sustained release profile.

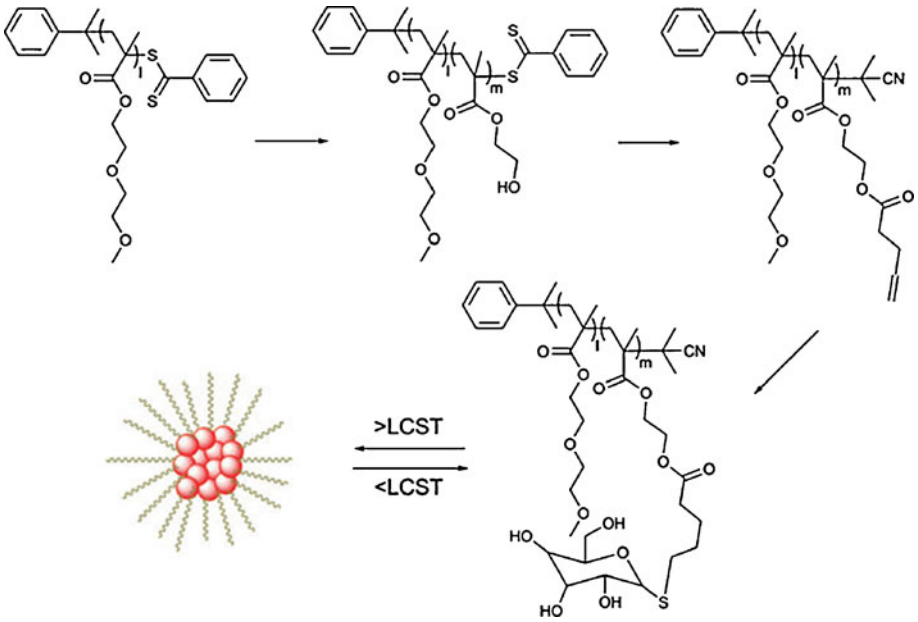

Fig. 6 RAFT/TEC synthetic strategy for the preparation of glucose functionalized copolymers and thermoresponsive micelles. [LCST=lower critical solution temperature]. Reprinted with permission from ref (39).

Recently, Kakkar, Maysinger, and coworkers reported preparation of miktoarm micelles as nanocarriers for nimodipine, a hydrophobic drug employed in the prevention and treatment of delayed ischemic neurological disorders (43). In their approach, miktoarm A_2B polymers (A=PEG, B=PCL) were constructed from a core containing one hydroxyl and two alkynyl groups, which allowed incorporation of two PEG-N_3 chains by CuAAC (CuBr, PMDETA, degassed DMF), followed by ring-opening polymerization of ε-caprolactone. After self-assembly of miktoarm polymers into spherical micelles, nimodipine was loaded with high encapsulation efficiency. *In vitro* studies revealed a slow release with advantageous results in order to reduce frequency of administration and toxicity.

Zhang and coworkers have also employed CuAAC for preparation of core-shell micelles with tumor-triggered targeting properties (44) (Fig. 8). In their approach, an azido-modified α-cyclodextrin (CD), designed to act as a host for the terminal phenyl group at the hydrophilic polymer P(NIPAM-*co*-N-acroyloxysuccinimide), was coupled *via* CuAAC ($CuSO_4$, sodium ascorbate, PMDETA) to an alkynated β-CD, designed to complex hydrophobic PCL bearing a terminal adamantyl group. The CD dimer was used to connect the hydrophilic and hydrophobic segments that subsequently assembled. Resulting micelles were further functionalized with an Arg-Gly-Asp (RGD) peptide as targeting ligand to improve cellular uptake efficacy. PEGylation *via* pH-sensitive imine bonds was employed to protect peptide ligands in normal tissues and body fluids. In addition, DOX was loaded into micelles, and two dyes were conjugated to polymer components to track formation of micelles. The authors demonstrated that the targeting property of micelles was switched on only after removing the PEG segment at the tumor site, which also triggered DOX release by decreasing the lower critical solution temperature of micelles.

CuAAC has also been employed for surface functionalization of polymeric micelles. In one of the first examples, reported by Lutz and coworkers, *in situ* shell functionalization of biocompatible micelles with a hydrophobic core and PEG-based corona was described (45). Surfactants composed of a hydrophobic cholesterol moiety and a hydrophilic polymer segment were synthesized from various oligo(ethylene glycol) methacrylates by atom transfer radical polymerization (ATRP), initiated by cholesteryl-2-bromoisobutyrate. After polymerization, bromine chain-ends were transformed into azides. Some of the resulting polymers formed thermoresponsive micellar aggregates (~100 nm) in aqueous solution that could be decorated with propargyl alcohol as a model ligand by CuAAC ($CuSO_4$, sodium ascorbate).

Poly(alkyl cyanocrylate) (PACA) is a biodegradable polymer well-known in the pharmaceutical field that has been developed for more than two decades by Couvreur's group for preparation of different drug delivery nanostructures (46). Nicolas and coworkers have recently reported preparation of clickable PEGylated PACA micelles from a poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] copolymer, displaying azide functionalities at the end of PEG chains (47) (Fig. 9). CuAAC functionalization was performed directly on the polymer (CuBr, PMDETA, DMF) followed by self-assembly in aqueous solution or onto the surface of preformed micelles in aqueous media ($CuSO_4$, sodium ascorbate). This versatile approach opens the door to ligand-functionalized biodegradable PACA micelles with stealth properties for biomedical applications.

Delivery of ophthalmic drugs using polymeric micelles with targeting RGD peptides is a promising tool for the

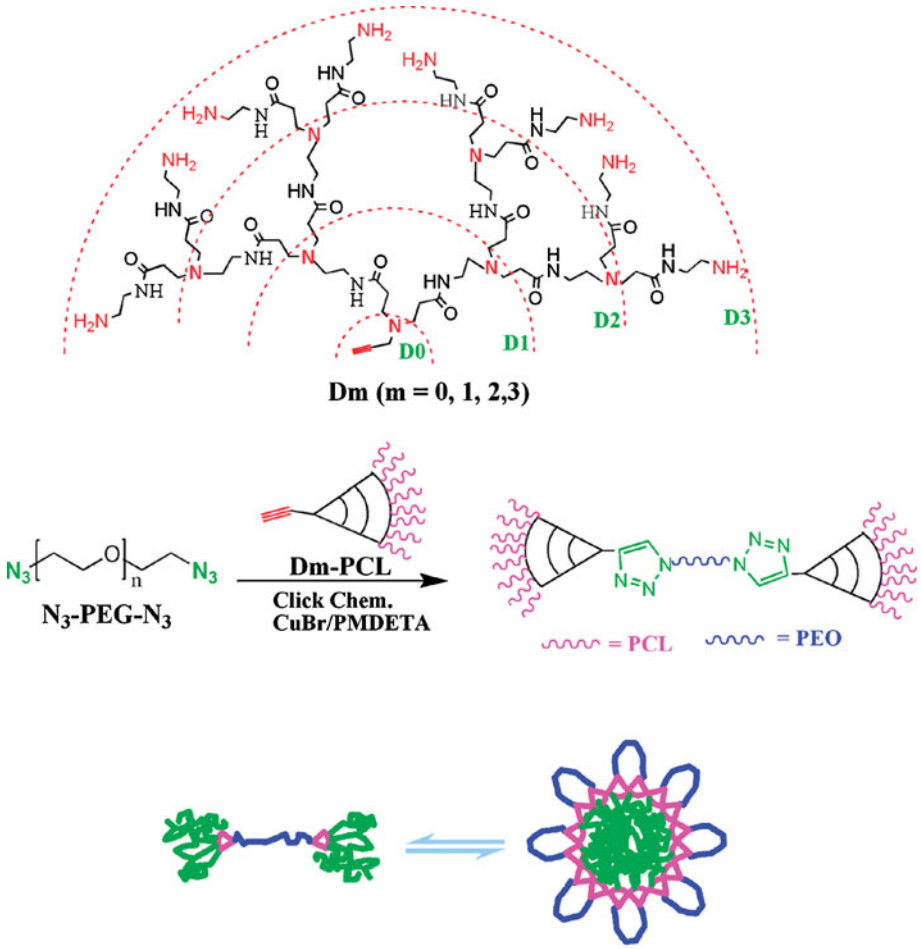

Fig. 7 Synthesis of dendron-like PCL-*b*-PEG-*b*-dendron-like PCL triblock copolymers by CuAAC and self-assembly into flower-like micelles. Reprinted with permission from ref (40).

treatment of a wide range of corneal epithelial defects. With this aim, Shoichet and coworkers have designed peptide-modified micelles by taking advantage of a CuAAC surface decoration (48). An amphiphilic block copolymer was prepared from poly(2-methyl-2-carboxytrimethylene carbonate-*co*-D,L-lactide) [P(TMCC-*co*-LA)] and an amine-terminated PEG-N_3. The resulting P(TMCC-*co*-LA)-*g*-PEG-N_3 copolymer self-assembled in aqueous solution and the azide groups on the surface of micelles were functionalized with alkyne-modified KGRGDS peptides *via* CuAAC in water ($CuSO_4$, sodium ascorbate). Covalently bound RGD peptides maintained bioactivity and binding affinity towards rabbit corneal epithelial cells.

Shoichet's group has also described an elegant route to immuno-polymeric micelles based on Diels-Alder cycloaddition (49) (Fig. 10). An amphiphilic P(TMCC-*co*-LA)-*g*-PEG-furan carrying furan as a terminal diene was prepared and self-assembled. Furan groups on the outer PEG shell were then reacted with a maleimide-modified anti-HER2, a therapeutic antibody used to treat breast cancer. Under these mild conditions, classical low efficiency and loss of activity associated with coupling of sensitive antibodies were overcome. In addition, the procedure takes advantage of increased reaction rate and stereoselectivity of Diels-Alder couplings in aqueous media. The anti-HER2 immuno-micelles specifically bound to HER2-over-expressing cells, demonstrating the strength of this procedure to create bioactive immuno-micelles. As only a few anti-HER2 antibodies on the surface of micelles were necessary for targeting, thousands of PEG-furan chains were available for coupling to a DOX-maleimide conjugate in a subsequent step (50). Resulting DOX-conjugated immuno-micelles represent an entirely new method for localized codelivery of chemotherapeutics and antibodies.

Michael addition of thiols to activated olefins has been successfully applied to functionalization of micelles. In an interesting example, the groups of An and Stucky have reported a combination of Michael addition and CuAAC for orthogonal functionalization of the core and shell of polymeric micelles (51) (Fig. 11). Monodisperse core-shell micelles (65–95 nm) were prepared by a RAFT-mediated precipitation polymerization of NIPAM, using a preformed PDMA RAFT polymer as CTA. PNIPAM generated during the process constituted the core in which trithiocarbonate groups were located, while PDMA blocks carrying azido groups formed the shell on the surface. The micelle

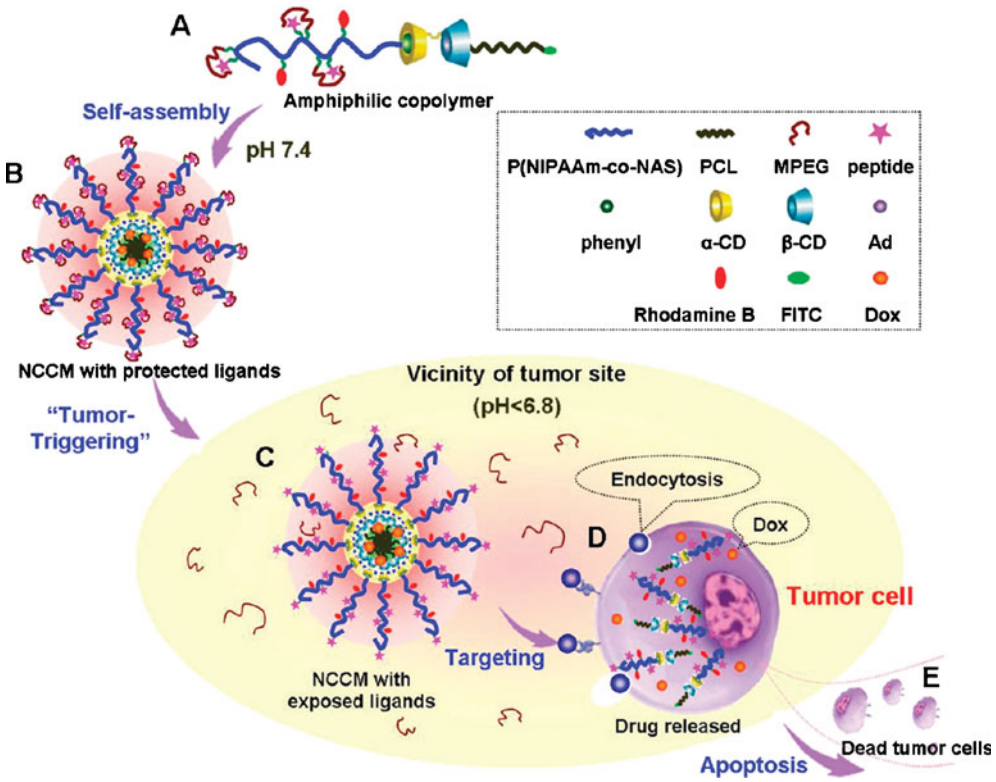

Fig. 8 Formation and cellular behavior of core-shell micelles with switchable tumor-triggered targeting properties. [NAS (*N*-acroyloxysuccinimide); MPEG (methoxy-PEG); Ad (adamantyl); NCCM (non-covalently connected micelle); LCST (lower critical solution temperature)]. (**A**) Diblock copolymer connected by α-β cyclodextrin dimer; (**B**) NCCM with protected RGD peptides at pH 7.4; (**C**) Tumor-triggered de-shielding to switch on the targeting property through removal of PEG segments at pH < 6.8; (**D**) Endocytosis of NCCM and drug release after destruction of the core-shell structure of NCCM at T > LCST; (**E**) Apoptosis of tumor cells. Reprinted with permission from ref (44).

core could be functionalized with a fluorescein *o*-acrylate through a one-pot aminolisis/Michael addition, while the shell was decorated with a dansyl probe *via* CuAAC ($CuSO_4$, sodium ascorbate, DMF-H_2O).

Nguyen's group has shown that drug-containing (indomethacin or DOX) amphiphilic polynorbornenes can self-assemble into therapeutically active core-shell polymer micelles with uniform, tunable diameters (52,53). More recently, the same

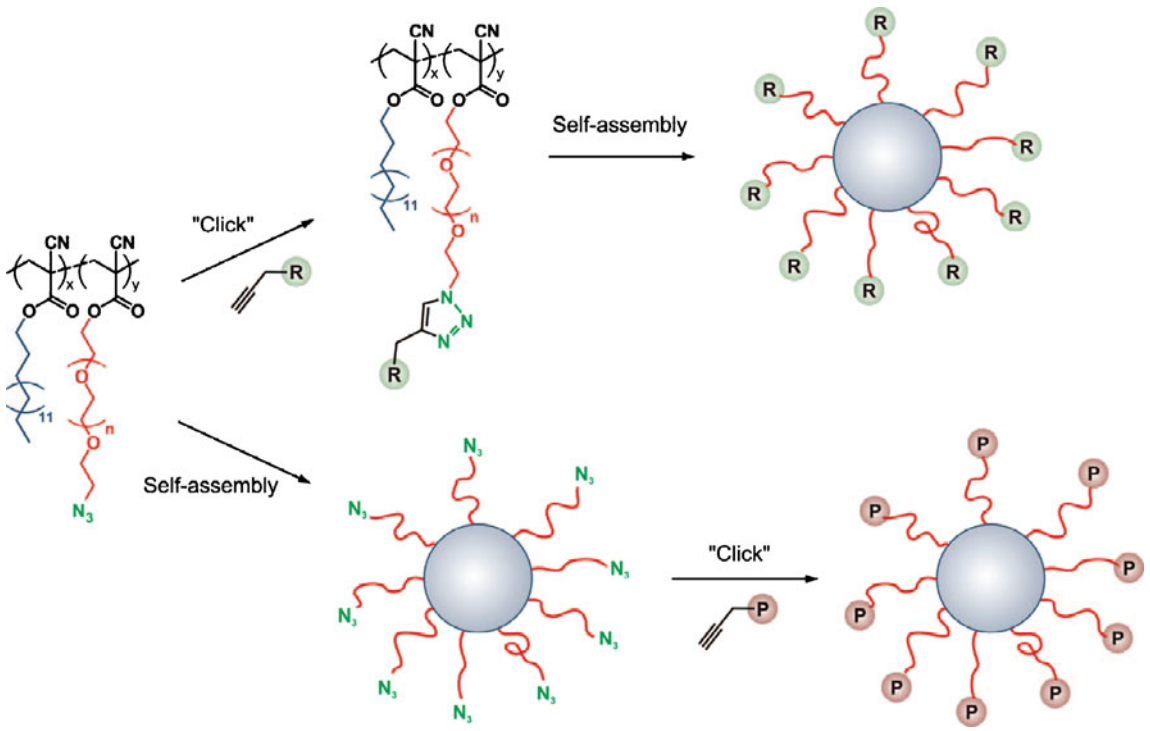

Fig. 9 General approaches to the preparation of functionalized PACA micelles. Reprinted with permission from ref (47).

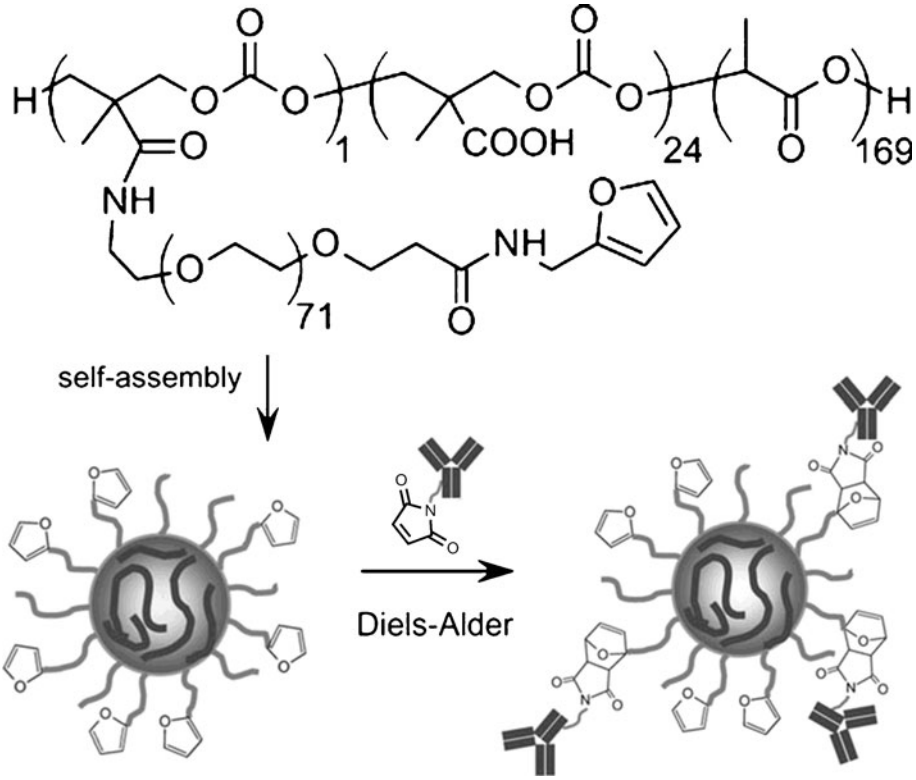

Fig. 10 Self-assembly of P(TMCC-*co*-LA)-*g*-PEG-furan and preparation of immuno-micelles *via* Diels-Alder functionalization with maleimide-modified antibodies. Reprinted with permission from ref (49).

group reported a CuAAC strategy for functionalization of these micelles with various bioactive ligands/particles (54). An indomethacin-containing norbornene monomer and trimethylsilyl (TMS) protected acetylene-terminated hydrophilic hexa(ethylene) glycol norbornene monomer were sequentially polymerized in different ratios by ring-opening metathesis polymerization. After deprotection of the TMS groups, block copolymers self-assembled in water into alkyne-decorated NP that were decorated by CuAAC ($CuSO_4$, sodium ascorbate) with azido-functionalized folate, biotin, or AuNP.

Jing, Chen, and coworkers have reported on surface functionalization of microspheres (55) and biodegradable polymer fibers (56) with proteins and antibodies by means of CuAAC. In a recent paper, an artificial oxygen carrier was constructed by conjugating hemoglobin to biodegradable micelles (57) (Fig. 12). The authors prepared a series of triblock copolymers from PLA, poly(2-methyl-2-carboxyl-propylene carbonate) (PMPC), and PEG, carrying pendant propargyl groups at the PMPC middle block. These amphiphilic copolymers self-assembled in aqueous solution into core-shell micelles. Hemoglobin containing azide groups was conjugated *via* CuAAC ($CuSO_4$, sodium ascorbate, histidine) to propargyl groups on the core surface, a privileged location to facilitate coupling while ensuring

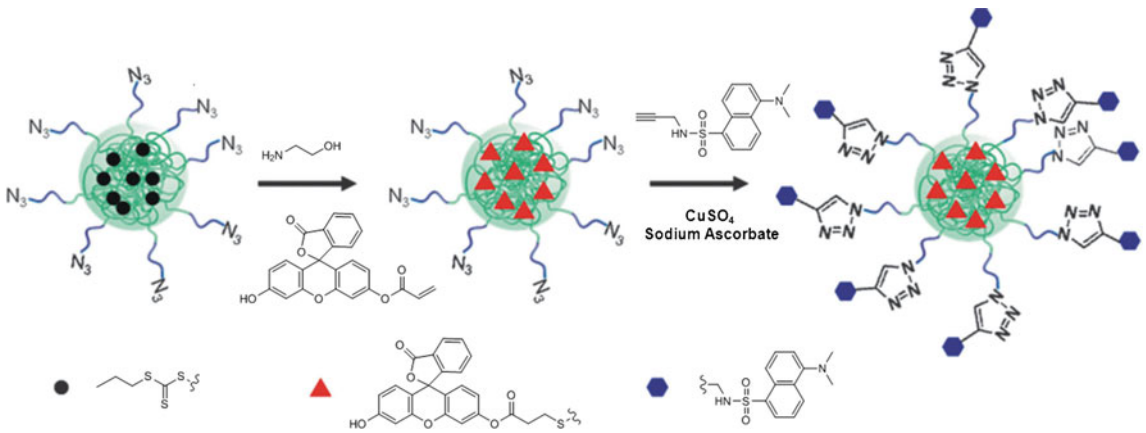

Fig. 11 Preparation of heterodifunctionalized core-shell micelles *via* sequential aminolysis/Michael addition and CuAAC functionalizations. Reprinted with permission from ref (51).

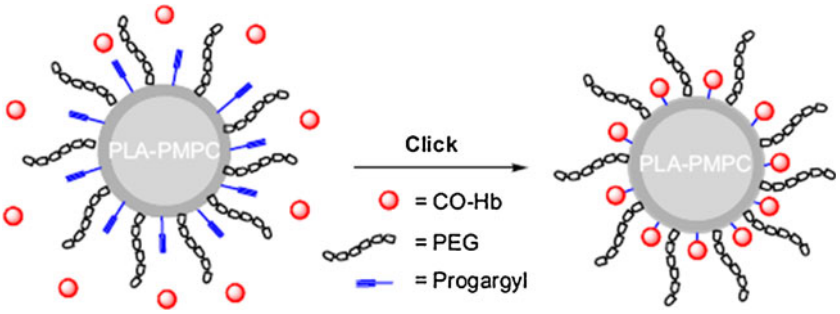

Fig. 12 Conjugation of hemoglobin to the core surface of PLA-*b*-PMPC-*b*-PEG micelles *via* CuAAC. [CO-Hb (carbonylhemoglobin)]. Reprinted with permission from ref (57).

protection of the protein against the immunological system by the PEG corona. Hemoglobin content and oxygen binding ability of micelles were studied, revealing a maximum hemoglobin loading of 70 wt.% and retention of its oxygen-binding ability.

Quite similarly, Liu, Zhao, and coworkers reported preparation of an azido-containing amphiphilic triblock copolymer PEG-*b*-poly(azidoethyl methacrylate)-*b*-poly(methyl methacrylate) that in aqueous media self-assembled into spherical micelles carrying azide groups at the hydrophobic/hydrophilic interface. Conjugation of biotin *via* CuAAC ($CuSO_4$, sodium ascorbate, *t*-BuOH-H_2O) resulted in formation of a functional interface between the hydrophilic shell and the hydrophobic core with demonstrated bioavailability (58).

An interesting family of polymeric micelles is the so-called Polyion Complex (PIC). Originally described by the groups of Kataoka (59) and Kabanov (60), PIC micelles are formed by electrostatic interaction between oppositely charged polymers. Their electrical neutrality, small size, and fairly narrow size distribution make them very appealing for drug delivery purposes. The group of Fernandez-Megia and Riguera recently described CuAAC ($CuSO_4$, sodium ascorbate, *t*-BuOH-H_2O) functionalization of PEG-dendritic block copolymers of the GATG (gallic acid-triethylene glycol) family with peripheral anionic groups. One of the copolymers carrying 27 peripheral sulphates led to PIC micelles of ~25 nm by incubation with poly-L-lysine (PLL) (Fig. 13). These micelles were remarkably stable under physiological conditions (150 mM NaCl, 37°C), high ionic strengths, and even freeze drying-resuspension, as a result of an increased stability granted by the dendritic scaffold (61).

Liu and coworkers recently reported another example of CuAAC in preparation of PIC micelles (62). A combination of ATRP and CuAAC (CuBr, DMF) was used to obtain two oppositely charged graft ionomers: PNIPAM-grafted poly [methacrylic acid-*co*-(3-azidopropyl methacrylate)] [P(MAA-*co*-N_3PrMA)] and PNIPAM-grafted poly{[2-(trimethylammonium)ethyl methacrylate]-*co*-N_3PrMA}. Incubation of both ionomers in aqueous solution led to formation of PIC micelles with thermoresponsive PNIPAM coronas. In a further step, CuAAC ($CuSO_4$, sodium ascorbate) was used again to cross-link the core of micelles, following a strategy previously reported by the groups of Wooley and Hawker (*vide infra*), to ensure their stability against ionic strength and pH changes.

Polymeric Nanoparticles

In spite of favorable features of polymeric micelles for drug delivery applications, these assemblies may suffer from spontaneous dissociation at concentrations below their critical micelle concentration (CMC) and from low stability at high temperatures (and ionic strengths in the case of PIC micelles). A common strategy to confer greater stability to these self-assembled structures is cross-linking of individual core/shell components to give core cross-linked or shell cross-linked NP (63,64). Wooley, Hawker, and coworkers described the first applications of CuAAC in this field in a series of very exciting contributions (65,66). In their first

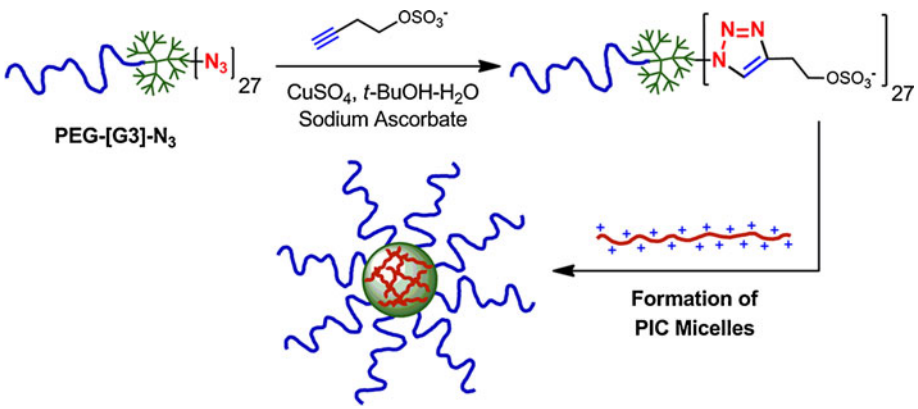

Fig. 13 CuAAC functionalization of PEG-dendritic block copolymers and formation of PIC micelles with PLL. Reprinted with permission from ref (61).

report, an amphiphilic diblock copolymer, poly(acrylic acid)-*b*-poly(styrene) (PAA-*b*-PS), that had been assembled into micelles and partially functionalized throughout the corona with alkynyl groups, was shell cross-linked with a first generation azido-terminated dendrimer by CuAAC ($CuSO_4$, sodium ascorbate) (65) (Fig. 14). The resulting NP benefited from increased stability and presence of imbedded azides into the shell for further functionalization. The complementary approach for preparation of well-defined core cross-linked polymeric NP was realized from an amphiphilic diblock copolymer PAA-*b*-PS, containing alkynyl groups partially incorporated throughout the hydrophobic PS block (66).

Stabilization of block copolymer micelles following similar CuAAC cross-linking approaches has been extensively investigated by different groups in the last few years. For instance, Liu and coworkers described preparation of core cross-linked NP with thermoresponsive cores from PDMA-*b*-P(NIPAM-*co*-azido-propylacrylamide) (67). The same authors in collaboration with Narain's group reported fabrication of two cross-linked micelles with inverted structures from a single water-soluble triblock copolymer composed by poly[2-(2-methoxyethoxy)ethyl methacrylate], PDMA, and poly[2-(diethylamino)ethyl methacrylate] blocks (68).

An interesting application of this cross-linking strategy has been developed by Stenzel's group for preparation of carriers for delivery of cobalt pharmaceuticals (69). A core-shell micelle was prepared by self-assembly of a poly(trimethylsilyl propargyl methacrylate)-*b*-poly[poly(ethylene glycol) methyl ether methacrylate] obtained by RAFT polymerization, after removal of TMS protecting groups. Then, the pendant alkyne groups at the core were used as reactive functional groups for cross-linking *via* CuAAC (Cu wire, $Cu(PPh_3)_3Br$, DIPEA, THF-H_2O), and as ligands for $Co_2(CO)_8$ to generate a macromolecular carrier of the antitumor agents based on (alkyne)$Co_2(CO)_6$. It is worth noting that while Co-loaded micelles were highly toxic to L929 fibroblast cells, the permanent encapsulation of Co complex in the cross-linked NP was shown to reduce toxicity.

Click procedures have been applied not only to covalent cross-linking of micelles, but also to surface decoration of NP. In this regard, the group of Fernandez-Megia and Riguera described the use of SPAAC as an efficient alternative to CuAAC for orthogonal functionalization of polymeric NP that avoids the detrimental effects associated with Cu(I) catalyst (70) (Fig. 15). In their work towards CuAAC functionalization of chitosan-*g*-PEG-N_3 NP as carriers across blood–brain barrier, a severe oxidative depolymerization of the chitosan backbone mediated by $^{\bullet}OH$ was observed, along with high contents of cytotoxic Cu severely compromising their biomedical applications. These detrimental effects, seen also in other polysaccharides (*e.g.*, dextran and hyaluronic acid), led the authors to explore the use of SPAAC as a safer Cu-free coupling technology. In this way, surface functionalization of cross-linked chitosan-*g*-PEG-N_3 NP with a cyclooctyne-derived

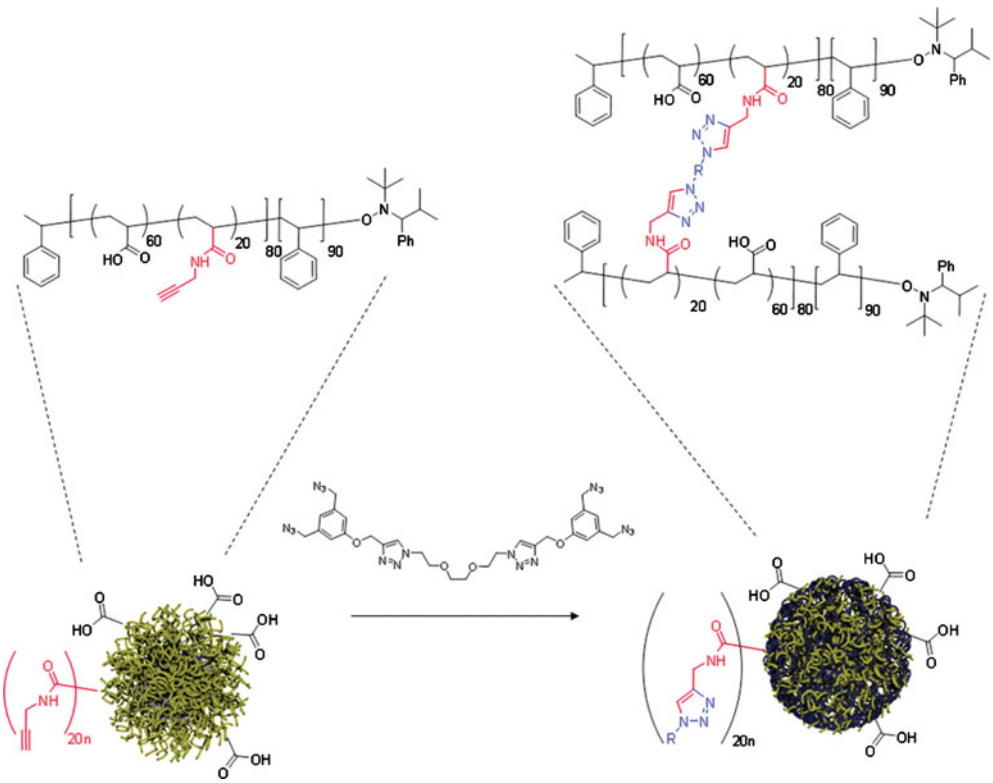

Fig. 14 CuAAC stabilization of alkyne-modified micelles with azido-dendrimer for the preparation of shell cross-linked NP. Reprinted with permission from ref (65).

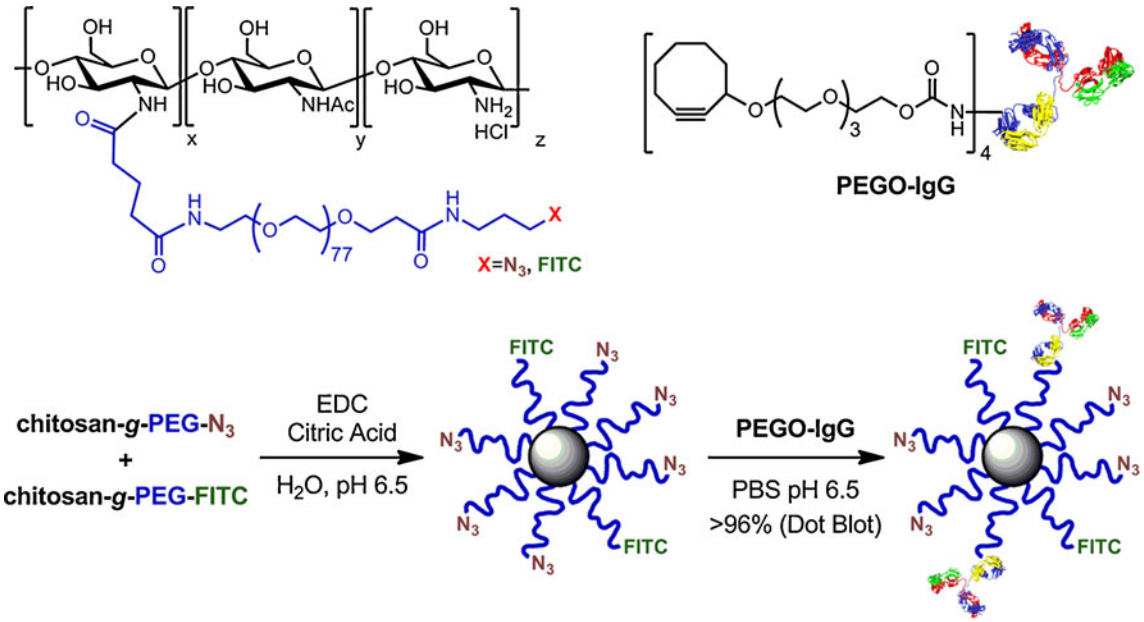

Fig. 15 Preparation of immuno-NP *via* SPAAC decoration. [PEGO (poly(ethylene glycol)octyne); EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)].

anti-BSA antibody proceeded safely and quantitatively under physiological conditions in what represents a step forward in development of environmentally friendly bioconjugation technologies for preparation of immuno-NP.

Polymersomes and Liposomes

Liposomes, self-closed vesicular structures composed of (phospho)lipid bilayers, have attracted considerable attention in drug/gene delivery and diagnosis and reached clinical application, especially for the treatment of cancer and opportunistic diseases (71). Although liposomes are not polymeric DDS, they are included in this section due to their relevance in drug delivery and similarity to polymersomes.

Functionalization of the surface of liposomes with different ligands to target specific cells has been traditionally performed by active esters, Michael additions, or hydrazones. The first example on the use of CuAAC with this purpose was reported by Schuber's group ($CuSO_4$, sodium ascorbate, BPDS), who prepared mannosylated vesicles from liposomes incorporating a synthetic lipid carrying a terminal alkyne (72). Interestingly, use of BPDS as Cu(I)-chelating ligand (2-fold excess over Cu) allowed a large increase in the yield of mannosylation and decrease in reaction time. Similarly, Kros *et al.* described surface functionalization of liposomes with a fluorescent probe (73). By means of fluorescence resonance energy transfer (FRET) studies, they revealed that CuAAC functionalization occurred on the liposome surface. More recently, Cai and coworkers optimized CuAAC conditions for functionalization of liposomes (74). They prepared physically robust clickable polymerized liposomes based on polydiacetylene lipids that were efficiently decorated with multiple functionalities (FITC, coumarin, and a GRGD peptide) without decomposition of liposomes, a feature usually observed for unsaturated liposomes under CuAAC conditions. The improved reaction conditions are based on the use of a Cu(I)-chelating ligand [a tris(triazolylmethyl)amine derivative bearing tetra(ethylenglycol) side chains] that is highly soluble in water and insensitive to air, and has shown to shorten reaction times with reduced amounts of $CuSO_4$ and sodium ascorbate.

Similarly to liposomes, polymersomes have attracted a great deal of attention in the field of drug delivery. They are hollow vesicles characterized by a bilayer membrane built from amphiphilic block copolymers and have many similarities to liposomes but greater stability (lower CMC values) and decreased permeability. Since they were reported by Discher and Hammer's group (75), polymersomes have been the subject of intensive research with applications ranging from imaging agents and delivery vehicles to nanoreactors (76).

The first application of CuAAC to functionalization of polymersomes was reported by van Hest and coworkers (77) (Fig. 16), who reported preparation of azido-decorated polymersomes and their conjugation to bioactive ligands, such as a dansyl probe, biotin, and enhanced green fluorescent protein (EGFP). With this aim, an amphiphilic PS-*b*-PAA was prepared by ATRP, and terminal bromide end groups were substituted for azides. Resulting copolymers were allowed to self-assemble into vesicular aggregates that were incubated with aqueous solutions of alkyne-functionalized ligands under standard CuAAC conditions ($CuSO_4$, sodium ascorbate, TBTA or BPDS). Average functionalization of peripheral azides in the range 40–50%

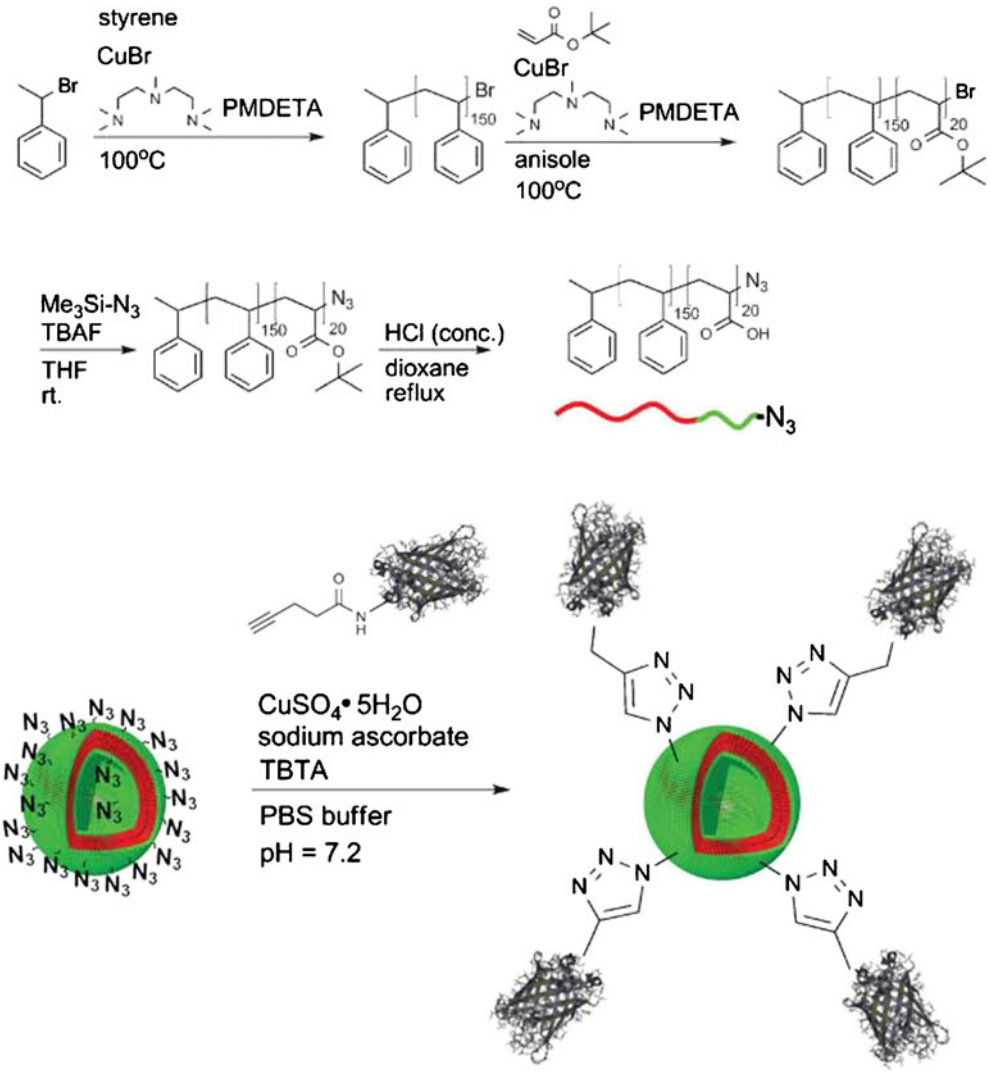

Fig. 16 Preparation of PS-*b*-PAA polymersomes with peripheral azide groups and their functionalization with Alk-EGFP. [TBAF (tetra-*n*-butylammonium fluoride)]. Reprinted with permission from ref (77).

was estimated by fluorescence measurements. Interestingly, no change in vesicle morphology was observed during CuAAC functionalization process.

In an interesting contribution, Gillies and coworkers reported a similar method for attachment of dendrons onto polymer vesicles directed to enhance availability of peripheral ligands for multivalent interaction with biological targets (78) (Fig. 17). Vesicles were prepared by self-assembly of amphiphilic poly(butadiene)-*b*-poly(ethylene oxide) (PBD-*b*-PEO) and PBD-*b*-PEO-N_3, mixed in different ratios. Functionalization of these vesicles with a polyester dendritic scaffold incorporating an alkyne focal point was studied by CuAAC ($CuSO_4$, sodium ascorbate, BPDS). Well-dispersed vesicles were observed after CuAAC functionalization when containing up to 20% of PBD-*b*-PEO-N_3. Under these conditions, full surface decoration resulted. In a more recent report, the same group demonstrated that vesicles functionalized with dendritic mannose ($CuSO_4$, sodium ascorbate, BPDS) show an enhanced affinity (of 1–2 orders of magnitude) towards lectin Concanavalin A (hemagglutination assay), when compared to vesicles containing an equal density of non-dendritic mannose (similar results with dextran-coated iron oxide NP) (79). This binding enhancement has been attributed to the

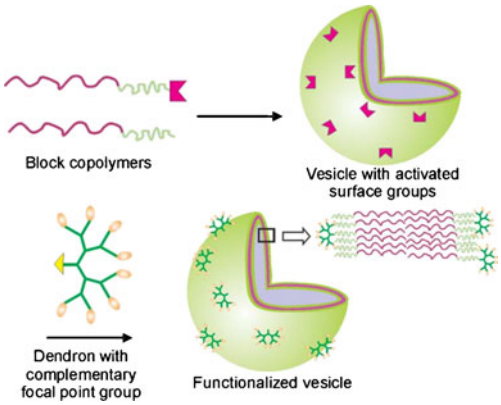

Fig. 17 General approach for the surface functionalization of vesicles with dendritic wedges. Reprinted with permission from ref (78).

ability of dendrons to effectively display ligands and overcome steric inhibition by surrounding polymer chains, which represents an interesting example of the relevance of the ligand presentation on binding to biological targets.

O'Halloran and Nguyen described a drop-in procedure for preparation of polymer-caged nanobins (PCN) from preformed liposomes and a cholesterol-terminated PAA (synthesized by nitroxide-mediated controlled radical polymerization), followed by shell cross-linking (80). PCN surpass some of the problems associated with classical liposomes composed of pH-sensitive, amine-modified lipids, and represent a more robust delivery platform with pH-responsiveness under acidic conditions. In a recent report, the same group described preparation of clickable PCN from DOX-loaded liposomes and a cholesterol-terminated PAA by covalent cross-linking with an alkyne-modified diamine (81) (Fig. 18). Resulting alkyne-functionalized PCN (~125 nm) were decorated with folic acid *via* CuAAC ($CuSO_4$, sodium ascorbate) to give folate-targeted DOX-loaded PCN enhanced potency towards folate receptor (FR)-positive tumor cells (KB and OvCa432), when compared to the untargeted agent. In addition, folate-targeted PCN could discriminate FR-positive tumor cells as a function of the level of cellular FR expression, showing different degrees of potentiation.

Polymeric Capsules and Microspheres

Polymer capsules are hollow spherical systems envisioned as promising materials with application for controlled release of functional molecules, such as drugs or enzymes. They are usually prepared by layer-by-layer (LbL) assembly techniques comprising deposition of alternating polymer layers over sacrificial cores that are removed at a later stage (82). Although LbL has been traditionally driven by electrostatic and hydrogen-bonding interactions, covalently bound films offer the advantage of higher stability due to formation of cross-linked polymer networks (82). For example, Caruso's group relied on a CuAAC-based LbL for preparation of pH-responsive polymer microcapsules by assembly of ultrathin PAA films onto poly(ethylene imine) (PEI)-coated silica particles (83) (Fig. 19). PAA copolymers incorporating ~10% of either alkyne or azide functionalities were synthesized by living radical polymerization. LbL assembly was performed by sequentially exposing PEI-coated particles to PAA-N_3 and PAA-Alk solutions in presence of $CuSO_4$ and sodium ascorbate. After centrifugation, sacrificial silica cores were removed with fluoride to afford single-component capsules with wall thickness of 5 nm. One advantage of this approach relates to the presence of remaining azide or alkyne groups on the shell of capsules amenable to further selective functionalization. Following the same methodology, biodegradable capsules were prepared from homopoly(aminoacids), PLL or poly-L-glutamic acid (PGA), modified with azides and alkynes ($CuSO_4$, sodium ascorbate) (84). These capsules showed a reversible pH-responsive swelling/shrinking behavior, with potential applications for pH-triggered loading/release of bioactive species. In addition, PLL capsules were further PEGylated with NHS-PEG-OMe and NHS-PEG-biotin and their interaction with fluorescently labeled BSA and streptavidin investigated by flow cytometry. Non-specific BSA adsorp-

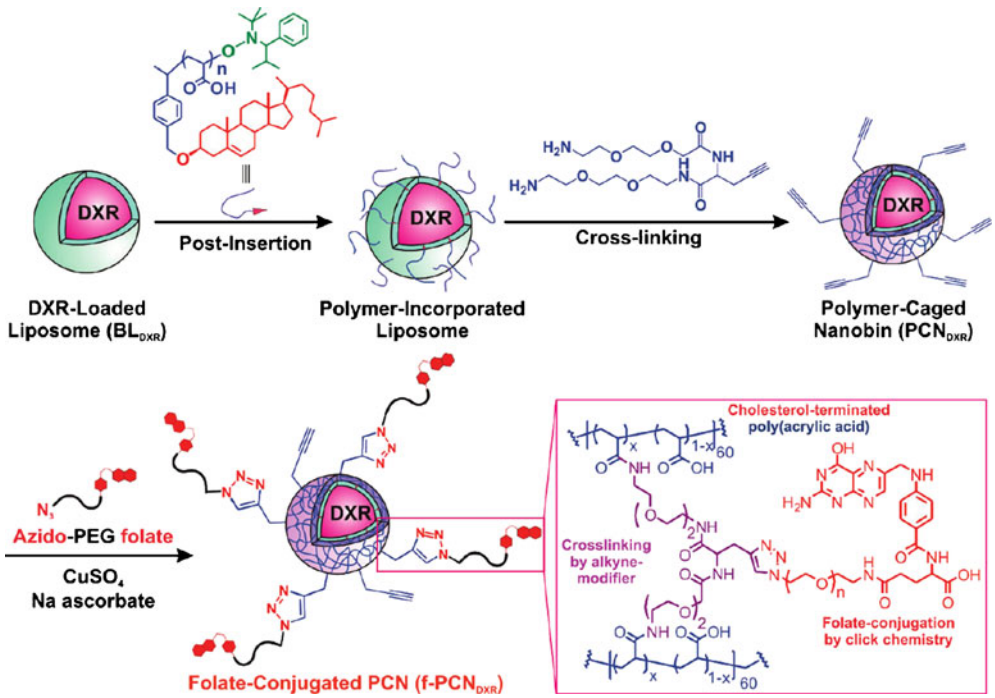

Fig. 18 CuAAC strategy for the preparation of DOX-loaded, folate-conjugated PCN [DXR=doxorrubicin (DOX)]. Reprinted with permission from ref (81).

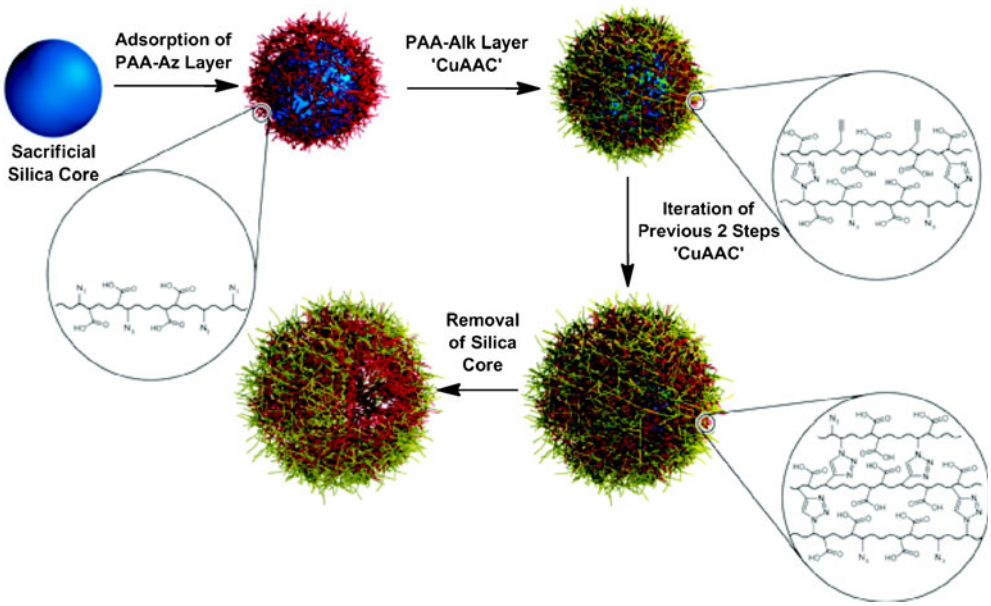

Fig. 19 Schematic representation of the LbL/CuAAC preparation of triazole cross-linked pH-responsive polymer microcapsules from a silica template. Reprinted with permission from ref (83).

tion typical of PLL surfaces was considerably reduced in the PEGylated system, whereas streptavidin specifically bound to biotin. Biodegradability, low-fouling properties, and targeting ability of these capsules make them promising carries for targeted drug delivery.

In a similar way, De Geest and coworkers prepared biodegradable multilayer films and hollow capsules based on dextran (85). $CaCO_3$ microparticles precoated with a layer of PAA-Alk were assembled by LbL with consecutive layers of azido- and alkyne-dextran under CuAAC conditions ($CuSO_4$, sodium ascorbate). Then, hollow capsules were obtained by dissolving the $CaCO_3$ core with EDTA at pH 5.2. Use of carbonate linkages for incorporation of azide and alkyne functionalities along the dextran chain ensured biodegradability of resulting capsules. The same authors also prepared biodegradable microcapsules from azido- and alkyne-dextran by microemulsion using an external aqueous PEG phase ($CuSO_4$, sodium ascorbate) (86). When a FITC-labeled dextran was employed, degradability of capsules was studied under physiological conditions. It was demonstrated that release of entrapped FITC-dextran occurred in a controlled fashion, and rate of release could be modulated by density of cross-linking.

Caruso and coworkers also developed a TEC-based LbL strategy that avoids use of coupling reagents or catalysts (87) (Fig. 20). A LbL assembly based on hydrogen-bonding is preformed on silica particles by depositing consecutive layers of PMAA (carrying thiol or alkene functionalities) and poly (*N*-vinylpyrrolidone) (PVP), that are subsequently cross-linked by irradiation with UV light (256 nm, acetate buffer). Efficiency of this TEC/LbL strategy was demonstrated by lack of disassembly at pH 7 under conditions leading to hydrogen-bonding disruption. The authors took advantage of the presence of remaining thiol or alkene functional groups on the surface of capsules for incorporation of PEG chains. A related CuAAC strategy was more recently employed by the same group to prepare low-fouling polymer capsules decorated with antibodies showing specific binding to colorectal cancer cells (88). In this case, single component PVP capsules were prepared from the consecutive assembly of PVP-Alk and PMAA layers *via* hydrogen-bonding interactions and further CuAAC ($CuSO_4$, sodium ascorbate) cross-linking of the PVP layers with a bifunctional azide linker. After removal of the core template and PMAA layers, remaining alkyne groups on the surface of PVP capsules were conjugated to an azido-PEGylated antibody *via* CuAAC ($CuSO_4$, sodium ascorbate) in presence of a Cu(I)-chelating ligand [tris(4-carboxyl-benzyltriazolylmethyl) amine]. Taking advantage of the same hydrogen-bonding assembly/CuAAC cross-linking sequential approach, Caruso and coworkers recently prepared biodegradable capsules with drug-loaded multilayers as promising carrier systems for biomedical applications (89). For preparation of these capsules, layers of PGA-Alk with/without covalently conjugated DOX molecules were assembled *via* hydrogen-bonding with PVP on silica templates, and then covalently stabilized using a diazide linker ($CuSO_4$, sodium ascorbate). After removal of PVP and the sacrificial template, DOX-loaded PGA capsules were obtained with control over drug dose and position in the multilayer system. These PGA capsules could be enzymatically degraded to release active drug, which was localized in cell nuclei.

Although several methods for preparation of polymeric capsules have been reported so far, they all have in common the necessity of a pre-organized structure or template. A rare example of self-assembly into nanosized capsules not requiring the use of templates was recently reported by Sung, Kim, and coworkers (90), who made use of TEC (UV light, MeOH)

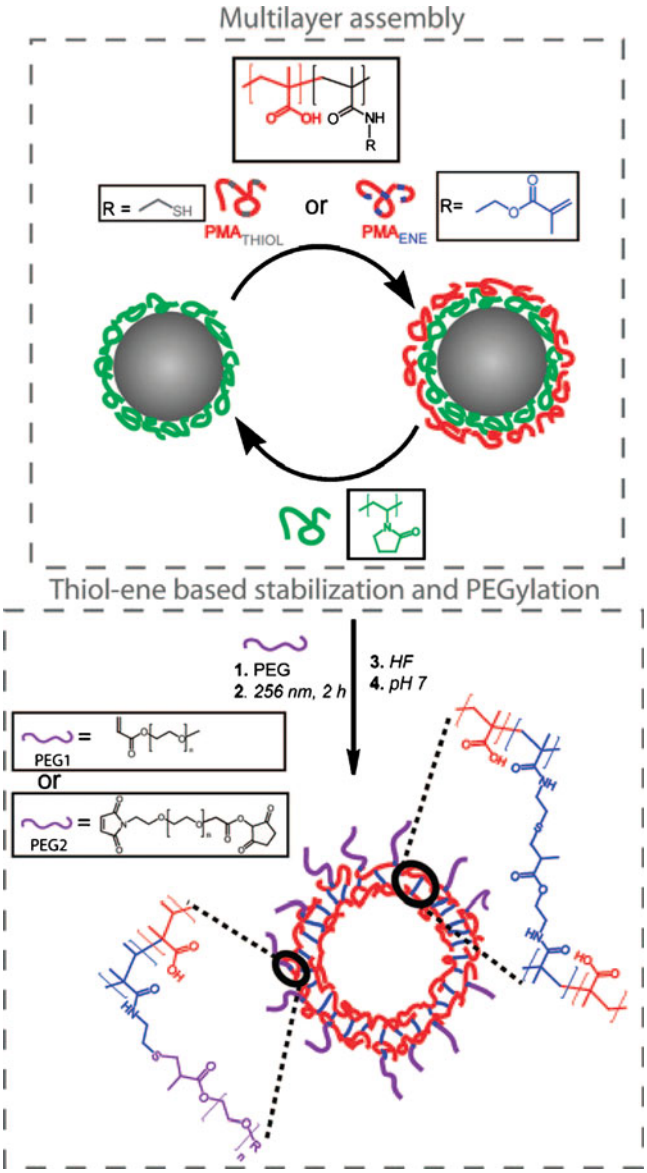

Fig. 20 Preparation of (PVP/PMA-thiol/PVP/PMA-Alk)-coated microparticles by LbL assembly, followed by PEGylation, cross-linking stabilization *via* TEC, and removal of the PVP layers and silica template. Reprinted with permission from ref (87).

between dithiols and a cucurbit[6]uril carrying 12 allyloxy groups at the periphery for preparation of polymer nanocapsules with high stability and relatively narrow size distribution (Fig. 21). Different dyes (carboxyfluorescein, rhodamine) were entrapped inside these capsules. A unique property of these capsules is that they can form highly stable host-guest complexes with polyamines by virtue of unique recognition properties of the accessible molecular cavities exposed on the capsule periphery.

Several strategies taking advantage of click reactions have been also used for surface functionalization of polymeric microspheres. For example, Chen and coworkers used CuAAC for grafting of proteins onto biodegradable microspheres prepared from a P(LA-*co*-carbonate) carrying pendant alkyne groups (55). With this purpose, azido-decorated BSA was selected as a model protein. Effectiveness of CuAAC conjugation ($CuSO_4$, Cu wire) was demonstrated by confocal microscopy and X-ray photoelectron spectroscopy. In addition, CuAAC and TEC chemistries were employed for surface functionalization of microspheres with polymers as a strategy for enhancing their stability and modifying their properties (91,92). For instance, the groups of Barner-Kowollik, Barner, and Müller recently reported surface modification of poly(divinylbenzene) microspheres with PNIPAM and PHEMA using either TEC (AIBN, acetonitrile) or CuAAC ($CuSO_4$, sodium ascorbate, DMF-H_2O) strategies (92).

METAL AND SILICA NANOPARTICLES

During the last few decades, hard-matter nanosystems, *e.g.*, AuNP and other noble metal NP, MNP, QD, and SiNP, have been extensively investigated because of their unique physical and chemical properties, which strongly depend on their size and shape. Although these nanosystems are of great interest for researchers in different disciplines, they have been the focus of special attention in the biomedical field, where they have found application as drug carriers, labeling agents, vectors for gene therapy, hyperthermia treatments, and magnetic resonance imaging (MRI) (93,94). In addition, some of these nanostructures offer the possibility of simultaneously imaging and treating tumors (theranostic carriers), which may be advantageous over conventional chemotherapies.

Gold and Noble-Metal Nanoparticles

AuNP and other noble-metal NP have emerged as novel nanostructures with exciting applications in therapy and diagnosis (*e.g.*, drug and gene delivery, imaging, phototherapy, and radiotherapy enhancement treatments). Their low inherent toxicity, high surface area, and tunable stability have revealed noble-metal NP as promising platforms for biomedical applications (94,95). Indeed, metallic NP have demonstrated their capability to deliver small drugs and large biomolecules such as peptides, proteins, or nucleic acids. Although monodisperse metal NP can be easily prepared with extremely small sizes (<30 nm), they usually suffer from reduced chemical stability in solution and high tendency to aggregate, which may lead to loss of their unique properties. To overcome this shortcoming, the surface of particles is usually functionalized with various types of ligands (*e.g.*, surfactants, polymers, dendrimers, biomolecules), which in the case of AuNP typically involves thiolated species. In addition to enhanced stability, surface functionalization of the

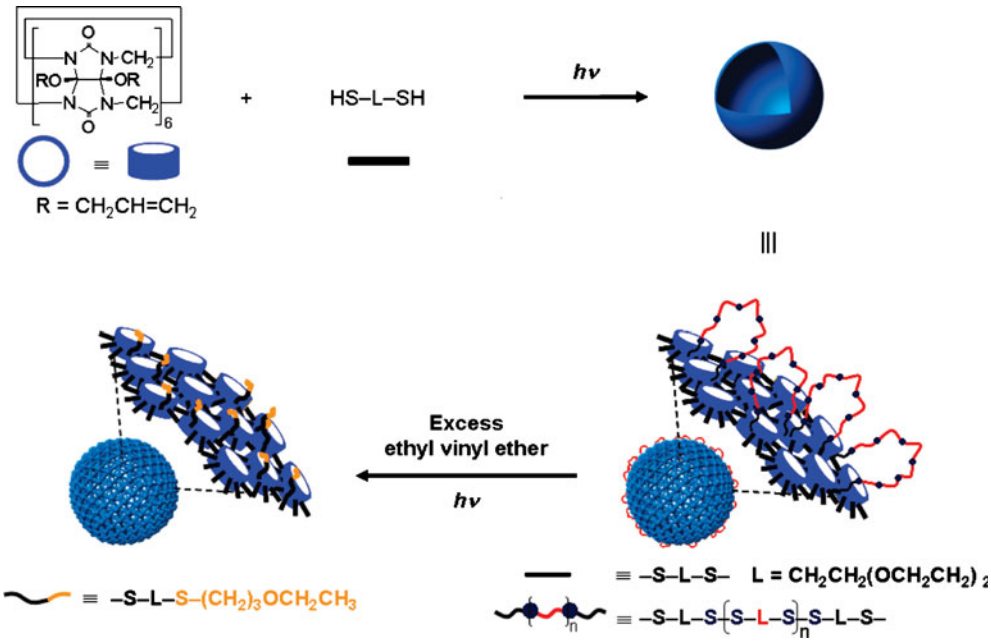

Fig. 21 Synthesis of (allyloxy)$_{12}$cucurbit[6]uril polymer nanocapsules *via* TEC cross-linking. Reprinted with permission from ref (90).

particles has been shown to be a key parameter for optimizing bioavailability and non-immunogenicity. As a result, a great amount of work has been devoted to effective functionalization of metal NP. Among different strategies used for this goal, those based on click chemistries are extremely efficient.

The first example on the use of an AAC coupling for surface decoration of AuNP was reported by Williams and coworkers (96). Azido-functionalized AuNP were decorated with various low-molecular-weight compounds bearing carbonyl groups adjacent to a terminal alkyne for improved reactivity. Although functionalization proceeded in absence of catalyst, use of organic solvents (dioxane or 1:1 hexane/dioxane) and inability to achieve complete functionalization of terminal azides render this protocol of low applicability within the biomedical field. Subsequent attempts to improve reactivity by use of organosoluble Cu catalysts led to particle aggregation or decomposition (96). These shortcomings have been partially solved by Astruc and coworkers, who described experimental conditions for quantitative CuAAC functionalization of azide-decorated AuNP [stoichiometric amounts of $CuSO_4$ and sodium ascorbate, room temperature, THF-H_2O (1:1), N_2 atmosphere] (97). This optimized protocol has proven successful for incorporation of organic, organometallic, dendritic, and short PEG chains on AuNP, but the requirement of large amounts of THF makes it unsuitable for conjugation of proteins or nucleic acids.

Rowan, Brust, and coworkers used CuAAC to efficiently prepare functional enzyme-AuNP conjugates from an acetylene-functionalized *Thermomyces lanuginosus* lipase and azido-decorated water-soluble AuNP ($CuSO_4$, ascorbic acid) (98) (Fig. 22). Successful formation of protein-particle conjugate was confirmed by gel electrophoresis, while a fluorometric lipase activity assay showed that approximately seven fully active lipase molecules per particle had been coupled. Following a similar approach, Rao's group recently reported site-specific conjugation of bioluminescent *Renilla* luciferase proteins to AuNP for sensing protease activity (99). In addition, conjugation of azido-functionalized AuNP to an alkyne-modified DNA duplex was reported by Simon's group (CuBr, TBTA), leading to a chain-like assembly of NP on a DNA template (100). More recently, Pang and coworkers described a new method for preparing azido-bearing AuNP functionalized with Alk-HRP *via* CuAAC ($CuSO_4$, ascorbic acid) rendering AuNP-HRP conjugates with retained catalytic activity (101).

CuAAC has also been effectively employed for surface functionalization of silver (AgNP) and platinum (PtNP) nanoparticles. In various recent examples, Li and

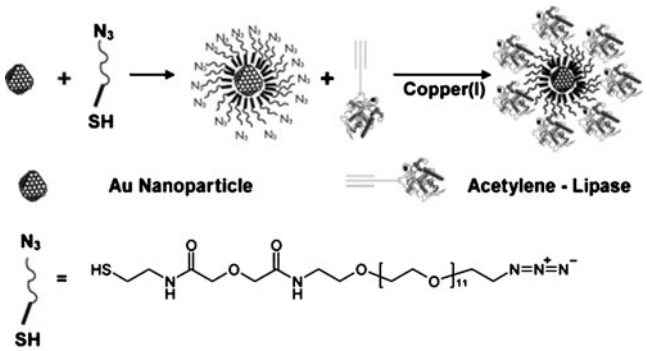

Fig. 22 Functionalization of AuNP with a lipase *via* CuAAC. Reprinted with permission from ref (98).

coworkers reported preparation of highly selective colorimetric sensors for Cd(II) and Co(II) based on triazole-ester (102) and triazole-carboxyl (103) modified AgNP prepared by CuAAC ($CuSO_4$, sodium ascorbate). Similarly, Carrot and coworkers have taken advantage of CuAAC (CuBr, PMDETA, dimethylacetamide) to graft biocompatible coronas of PEG and PCL onto azido-functionalized PtNP as hybrid structures with potential applications in biodetection (104).

Other click reactions that have found application for surface functionalization of noble-metal NP include Michael addition and Diels-Alder. For instance, Chechik's group prepared shell cross-linked AuNP with improved stability *via* Michael addition of various amino-dendrimers [poly(amido amine) and diaminobutane] to acrylate-terminated particles (105). Also, reversible surface functionalization of AuNP has been accomplished by Beyer's group by means of a Diels-Alder coupling strategy (106). AuNP were first functionalized with a thiol-terminated PS-*b*-PEG prepared by Diels-Alder of PS and PEG blocks. Subsequent thermal treatment of these particles caused retro Diels-Alder leading to AuNP functionalized by PS chains.

Magnetic Nanoparticles

In the last decade, MNP based on maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4) have consolidated as promising materials in biomedicine due to applications mainly in drug delivery (93), MRI, and hyperthermia therapy (107). For these applications, particles must display high magnetic saturation, biocompatibility, and a functionalized surface. Although iron oxide NP with low dispersity are easily produced, they present an intrinsic instability, which is normally overcome by installing protecting shells. This coverage not only protects MNP from degradation, but also provides anchoring sites for functionalization with active molecules or reactive groups.

Turro's group reported CuAAC conditions for surface functionalization of γ-Fe_2O_3 NP with low-molecular-weight organic species and polymers (*e.g.*, α-acetylene-poly(*tert*-butyl acrylate)) (108). With this aim, short ligands containing carboxylic or phosphonic acid groups were designed as strong anchors to bind the NP surface. Presence of a terminal azide or alkyne group in these ligands provided orthogonal functionality on the surface for further chemical modification by CuAAC ($CuSO_4$, sodium ascorbate, DMSO-H_2O).

A more biocompatible CuAAC protocol avoiding the need of organic solvents for effective functionalization of MNP (silica oxide-coated Fe_3O_4) with biologically relevant ligands was described by Lin and coworkers (109). Under reported conditions ($CuSO_4$, tris(carboxyethyl)phosphine (TCEP), TBTA, pH 8), mannose, biotin, the FLAG peptide, Tn antigen, and various proteins (EGFP and maltose binding protein (MBP)) were incorporated on the MNP surface and detected using fluorescently labeled antibodies/proteins. When MNP containing azides or alkynes at the surface were reacted with complementary alkyne- or azido-terminated mannose, the alkynated mannose was more efficiently immobilized. This faster kinetic of the alkyne counterpart in solution is consistent with the required Cu(I) coordination of the alkyne. A similar preference has been reported when labeling CPMV (16,23) and microarray slides (110). Interestingly, the activity of a MBP covalently immobilized on the MNP at its C terminus by site-specific CuAAC was higher than random amide bond formation, in agreement with the C terminus being distant from the MBP binding site.

Prosperi and coworkers reported an interesting one-pot biofunctionalization of MNP (γ-Fe_2O_3) *via* diazo transfer followed by *in situ* CuAAC (111). Since a common way to modify iron oxide NP involves treatment with γ-aminopropyl triethoxysilane to give amino-functionalized MNP, these authors explored the possibility of generating azido functionalities from corresponding amines *via* Cu(II)-catalyzed diazo transfer (112) and their subsequent funtionalization *via* CuAAC. In this one-pot protocol, the Cu(I) species required for CuAAC are easily generated *in situ* by addition of sodium ascorbate to Cu(II) salts necessary for diazo transfer (biphasic CH_2Cl_2-H_2O mixture). By application of this procedure, biofunctionalization of MNP with lactose and HSA has proceeded successfully and with retained biological activity.

In a recent report, Mirkin and coworkers employed CuAAC ($CuSO_4$, sodium ascorbate, THPTA, DMSO-H_2O) to conjugate alkynated oligonucleotides to superparamagnetic iron oxide nanoparticles (SPION) functionalized with azides (113). The resulting densly functionalized SPION exhibited good cellular uptake by HeLa cells without the need of transfection agents. Another example of SPION bioconjugation was reported by Tsourkas and coworkers (114), who combined expressed protein ligation (EPL) with CuAAC to produce a highly efficient and site-specific conjugation strategy that was employed in preparation of SPION labeled with HER2/neu-targeted affibodies. Affibodies expressed in bacteria were first conjugated to an alkynated fluorescent peptide *via* EPL. CuAAC was then used for conjugation to azido-labeled SPION ($CuSO_4$, sodium ascorbate, bathocuproinedisulfonic acid). The resulting HER2-SPION proved to be highly potent and receptor-specific in both *in vitro* cell studies and murine tumor models. In addition, the authors demonstrated application of this EPL-CuAAC strategy to other nanostructures, such as liposomes and dendrimers.

MNP are also useful in magnetic hyperthermia, a promising cancer thermotherapy based on the exothermic properties of magnetic materials under the influence of an alternating current magnetic field (107). In order to

specifically target certain tumors, MNP can be conjugated with tumor-specific targeting ligands, thereby minimizing undesired side effects. In a recent report, Yogo and coworkers prepared FA-functionalized Fe_3O_4 NP by CuAAC as a cancer targeting system with application in hyperthermia treatment (115). Taking into account the low chemoselectivity in the derivatization of FA through carboxylic acids and the relevance of the α-carboxyl group for high-affinity binding to the FR, these authors developed a novel alkynation method at the heterocycle moiety by treatment with 2,3-dibromopropionyl chloride followed by HBr elimination. The resulting Alk-FA was subsequently reacted with azido-terminated MNP under standard CuAAC conditions (CuI, DMSO-H_2O). Then, the authors prepared SPION functionalized with FA and CD as novel devices for simultaneous drug delivery and hyperthermia treatment (116). With this aim, *β*-CD was used as nanocontainer for the anticancer drug tamoxifen and as anchoring point for covalent CuAAC (CuI, 70°C) functionalization of SPION with FA. Controlled release of tamoxifen was achieved by applying a high frequency magnetic field at an optimum temperature for hyperthermia, demonstrating that induction heat can be useful not only for hyperthermia treatment, but also as a driving force for drug release.

Perez and coworkers also relied on CuAAC for incorporation of a FA-N_3 derivative at the PAA-Alk coating of iron oxide NP (CuI, DMF-H_2O) (117) (Fig. 23). In addition, taxol and a near-infrared dialkylcarbocyanine dye were encapsulated within hydrophobic pockets at the polymeric matrix, resulting in a multifunctional (imaging and therapy) and multimodal (magnetic and fluorescent) folate-derivatized NP. This system provided targeted drug delivery to lung carcinoma A549 cells that over-express FR, without affecting normal cells not over-expressing this receptor. Similarly, Weissleder and coworkers prepared dextran-coated SPION functionalized with radionuclide ^{18}F in high yield *via* CuAAC ($CuSO_4$, sodium ascorbate, BPDS, DMSO-H_2O) for *in vivo* PET (position emission tomography) imaging (118,119).

Bhatia and coworkers investigated *in vivo* targeting of iron oxide NP to tumors (120). CuAAC ($CuSO_4$, sodium ascorbate) was selected to specifically conjugate the highly functionalized cyclic tumor targeting peptide LyP-1 to azido-functionalized PEGylated magneto-fluorescent NP. LyP-1 has been reported to bind p32, a mitochondrial protein that is over-expressed and aberrantly localized at the surface of tumor cells, macrophages, and lymphatic endothelial cells in certain experimental tumors and in human cancers. The resulting NP were able to direct their binding to p32-expressing tumor cells *in vitro*. More importantly, these NP were stable for >5 h in bloodstream after intravenous administration, allowing them to extravasate into tumors,

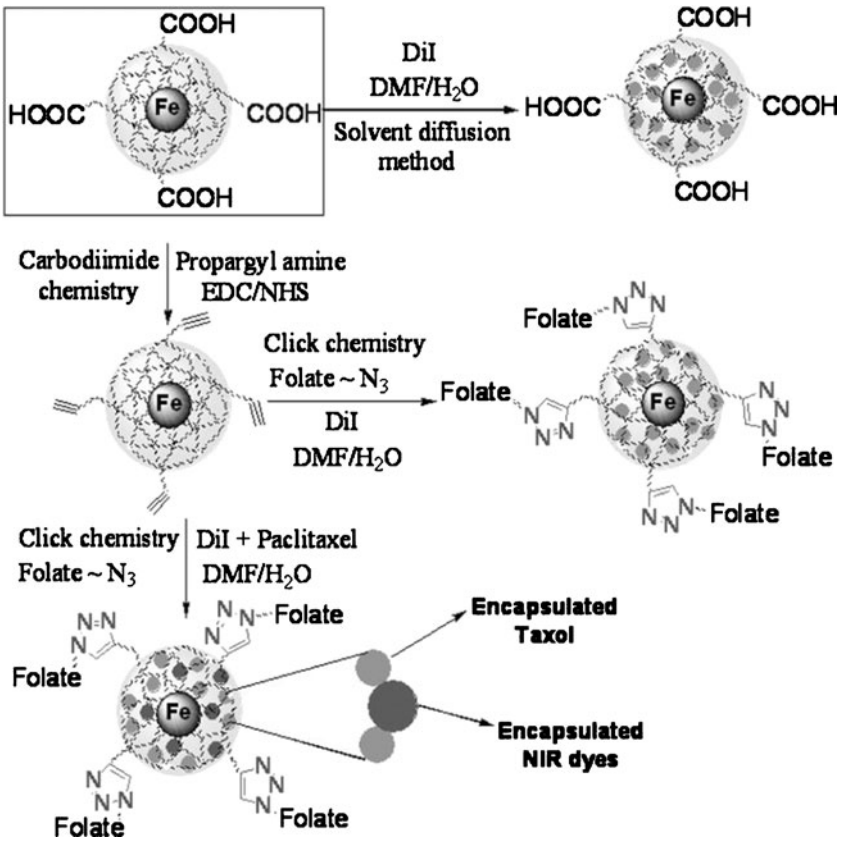

Fig. 23 Schematic representation of the preparation of theranostic and multimodal iron oxide NP. [NIR (near-infrared); paclitaxel=taxol. DiI (dialkylcarbocyanine fluorophores)]. Reprinted with permission from ref (117).

penetrate tumor interstitium, and specifically bind to p32-expressing cells. Altogether, these findings not only support the excellent features of CuAAC in bioconjugation (orthogonality, efficiency, aqueous compatibility), but confirm stability of clicked complexes *in vivo* in blood and tumor environments.

Although thiol-ene chemistry has been widely employed in the field of polymeric colloidal systems, its use for modification of MNP is still limited, with only a few recent examples being reported. One of the main shortcomings in the application of this chemistry to MNP is difficulty synthesizing alkene-immobilized particles because C-C double bonds undergo polymerization at the high temperature required in this process. This limitation has been recently overcome by Yogo and coworkers by employing allyl groups, which do not undergo polymerization (121). The authors reported modification of allyl-functionalized Fe_3O_4 NP with cysteine *via* a one-pot approach hydrolysis-condensation of Fe(III) allylacetylacetonate and TEC (AIBN, EtOH-H_2O, 60°C). A different strategy was employed by Warner and coworkers, who employed Fe_3O_4-mercaptopropionic acid NP further decorated with allyl diphosphonic acid or ester ligands by TEC (benzophenone, UV light, H_2O or MeOH) (122).

Quantum Dots

In a similar fashion to noble-metal NP, inorganic semiconductor nanocrystals known as QD have drawn attention in the fields of diagnosis and imaging due to their unique fluorescence properties, such as broad absorption, narrow and symmetric emission spectra, long luminescence lifetime, and good photostability (123). These optical properties can be tuned as a function of size and surface functionality. In spite of these favorable properties, development of efficient and mild bioconjugation strategies for surface functionalization of QD with biologically relevant molecules remains an important challenge to be addressed. Unfortunately, some of the most popular click reactions have severe limitations in their application for this goal. For instance, as thiols are commonly employed as stabilizing ligands in preparation of QD, their use for surface functionalization *via* TEC or Michael additions is severely restricted since ligand exchange at QD surface may occur. On the other hand, application of CuAAC suffers from two main drawbacks: harsh conditions usually employed for preparation of QD are incompatible with presence of azide or alkyne groups in stabilizing ligands, so ligand exchange strategies have to be employed for incorporation of these functional groups, which usually result in reduced photoluminescence quantum yields (PTQY); and Cu catalysts promote strong inhibition of QD luminescence. These shortcomings were early illustrated by Binder's group in functionalization of CdSe QD by CuAAC (124). A ligand exchange method was employed to decorate trioctylphosphine oxide QD with azide or alkyne functional groups. This process resulted in a reduction of PTQY of up to 63%. In addition, CuAAC derivatization of the resulting alkyne-functionalized conjugate (CuBr, DIPEA, TBTA, THF) led to a dramatic decrease of PTQY, so thermal AAC conditions had to be implemented.

An effective way to overcome the above-mentioned drawbacks in QD functionalization is application of convenient Cu-free cycloadditions. Texier and coworkers recently described application of SPAAC for functionalization of PEG-decorated CdSe/ZnS QD and their application to *in vitro* imaging of cell membrane glycoproteins (125). A cyclooctyne-functionalized QD was prepared from a commercially available CdSe/ZnS/PEG-NH_2 QD and then functionalized with an azido-tagged mannosamine in presence and absence of Cu (Fig. 24). While SPAAC decoration had a minor effect on PTQY (7% as compared to cyclooctyne-functionalized QD), addition of Cu led to a marked decrease of >65%.

In another approach, Bewendi and coworkers relied on the inverse-electron-demand Diels-Alder cycloaddition between tetrazine and strained alkenes (norbornene) to functionalize CdSe QD with Alexa 594 and an epidermal growth factor (EGF) (126). A ligand exchange protocol was employed for surface doping of QD with multiple norbornene units, which resulted in a reduction of 40% in PTQY. No additional quenching of fluorescence occurred during Diels-Alder cycloaddition. Interestingly, EGF-QD proved efficient for *in vitro* visualization of A431 human carcinoma cells *via in situ* labeling or with preformed conjugates (Fig. 25).

Silica Nanoparticles

SiNP represent another class of nanomaterials with potential applications in drug delivery, biodetection, and diagnosis that possess excellent biocompatibility, virtually no

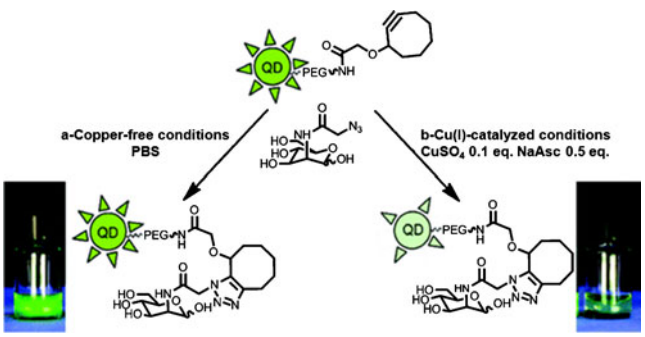

Fig. 24 SPAAC surface functionalization of QD in the presence and absence of Cu(I), and effect of reaction conditions on QD luminescence. Reprinted with permission from ref (125).

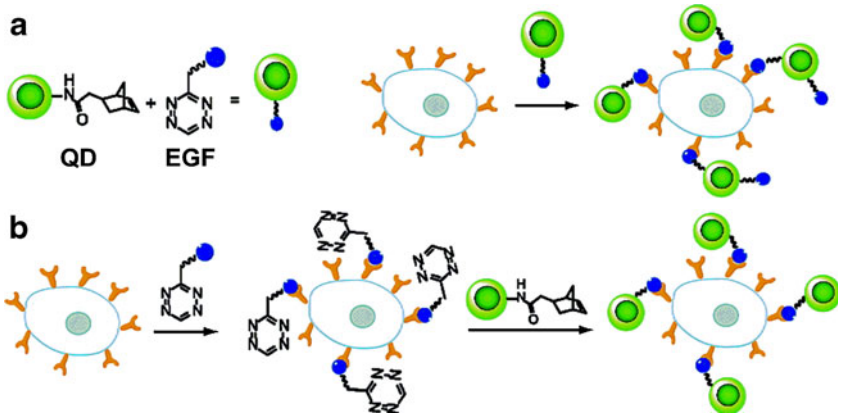

Fig. 25 Labeling of cells with preformed QD-EGF constructs (**a**). *In situ* conjugation of QD to EGF on live cells *via* tetrazine-norbornene Diels-Alder cycloaddition (**b**). Reprinted with permission from ref (126).

toxicity, and reactive functional groups on the surface available for functionalization (127). Moreover, a variety of functional groups can be attached to the silica surface *via* silane linkers. Following this principle, Brittain's group reported a combination of RAFT polymerization and CuAAC to graft polymers onto SiNP (128,129), using an unprotected alkyne-terminated RAFT CTA to prepare alkyne-terminated polymers and block copolymers, *i.e.*, PS, polyacrylamide, PMA, and PS-*b*-PMA, which were subsequently grafted onto azido-functionalized SiNP with high density of grafting ($CuSO_4$, sodium ascorbate, DMSO-H_2O or DMF-H_2O). Using the same CTA, these authors also reported tandem RAFT polymerization/CuAAC procedure to modify the surface of SiNP in a controlled manner.

Janus-type SiNP decorated with biomolecules on one hemisphere and biocompatible polymer brushes on the other have huge potential applications in targeted drug delivery, as well as development of switchable devices, optical probes, and emulsion stabilizers. Liu, Zhao, and coworkers reported the use of CuAAC for sequential functionalization of azide-modified SiNP embedded on the surface of PS particles used as scaffolds (130) (Fig. 26). In this approach, the originally exposed hemisphere of SiNP was coupled to an alkynated biotin derivative by means of CuAAC ($CuSO_4$, sodium ascorbate, *t*-BuOH-H_2O). After removal of excess biotin and catalyst by extensive dialysis, the product was collected by centrifugation. Then, PS was removed by dispersion in THF followed by centrifugation, yielding SiNP with biotin groups on one hemisphere and azide groups on the other. Bioactivity of the conjugated biotin molecules was confirmed by an avidin/HABA competitive binding assay. Remaining azide groups on the surface were coupled to an alkynated PEG through a second CuAAC ($CuSO_4$, sodium ascorbate, H_2O), leading to Janus-type SiNP differently functionalized with biotin and PEG at both hemispheres.

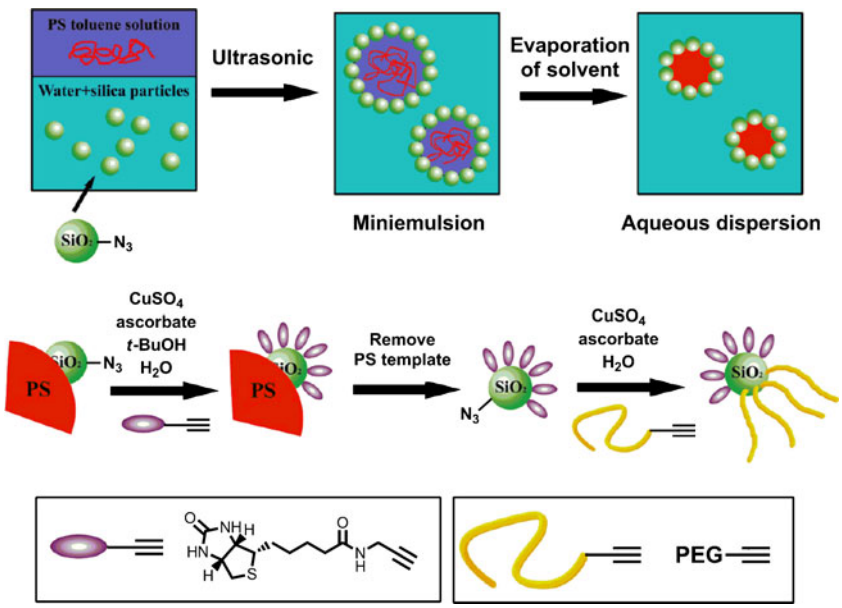

Fig. 26 Schematic representation of the preparation of Janus-type SiNP. Reprinted with permission from ref (130).

Müller and coworkers reported a new type of hybrid NP consisting of a fluorescent inorganic silica-like core and a biocompatible polymer shell containing terminal alkynes amenable to functionalization by CuAAC (131). With this aim, block copolymers composed of a poly[oligo(ethylene glycol)acrylate] hydrophilic block and a hydrophobic block containing trimethoxysilane-functionalized and dye-substituted monomers [(3-acryloxypropyl)-trimethoxysilane and 1-pyrenebutyl acrylate] were prepared by RAFT polymerization. For polymerization, an alkynated CTA was used to introduce a terminal alkyne group at the hydrophilic block. Selection of trimethoxysilane monomer at the hydrophobic block was based on its ability to undergo cross-linking into a stable silsesquioxane network under basic conditions for increased stability. Additionally, pyrene containing monomers were included as fluorescent tags. After self-assembly of block copolymers, the resulting micelles were core cross-linked by mild treatment with ammonia. Resulting alkynated particles were functionalized with Rhodamine B by means of CuAAC under drastic conditions (CuBr, bipy, 80°C, degassed DMF). The potential of these stabilized fluorescent SiNP as biocompatible carriers for intracellular delivery was nicely demonstrated by *in vitro* experiments on lung cancer cells.

An interesting report on the dual, bioorthogonal labeling of SiNP was recently described by Kele, Wolfbeis, and coworkers by sequentially exploiting SPAAC and CuAAC reactions (132,133) (Fig. 27). With this aim, SiNP were doped with several copies of a coumarin dye and azide reporter groups. Resulting fluorescent NP were then conjugated *via* SPAAC with a synthetic dipeptide containing both a cyclooctyne and a terminal alkyne. Subsequent CuAAC labeling of alkynated peptides with a specifically designed azido-coumarin fluorescent dye yielded dually labeled NP as efficient FRET systems. The same authors adapted this strategy for preparation of diagnostic NP carrying a heptapeptide for detection of MMP-2, an enzyme of the collagenase family that plays a key role in several physiological processes, including cancer progression. Successful application of the same FRET pair for labeling of proteins (BSA) paves the way for use of this methodology in construction of dually labeled conjugates for alternative biomedical purposes.

In a recent example, Bein's group reported CuAAC funtionalization of core-shell colloidal mesoporous SiNP for preparation of stimuli-responsive porous materials for controlled release of guest molecules (134). Colloidal mesoposous SiNP with azide groups at the outer shell were prepared and functionalized by CuAAC ($CuSO_4$, sodium ascorbate, THPTA) with double-stranded oligodeoxyribonucleotides incorporating an azide and a biotin label at different strands. As proof of concept, fluorescein selected as model compound was encapsulated and then the pores closed by complexation to avidin. These functionalized SiNP showed a programmable thermoresponsive behavior that allowed controlled release of guest molecules after melting the DNA molecular valves, a characteristic encoded by the length of the oligonucleotide employed.

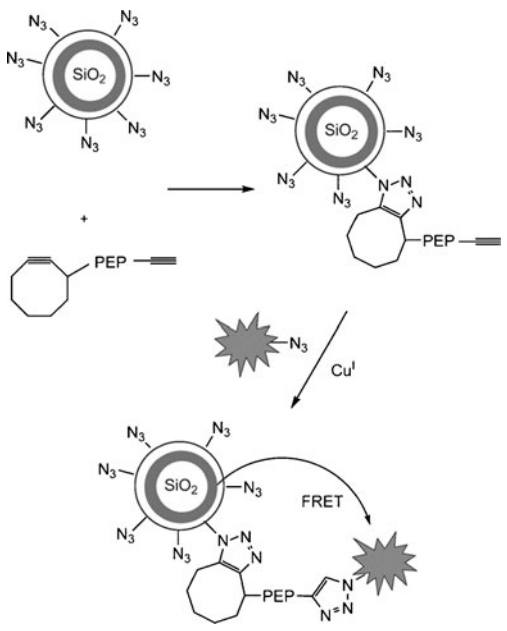

Fig. 27 Schematic representation of the SPAAC conjugation of a cyclooctyne/alkyne peptide to azido-functionalized fluorescent SiNP, and their further CuAAC labeling with an azido-FRET acceptor. [PEP (peptide)]. Reprinted with permission from ref (133).

CARBON NANOTUBES AND FULLERENES

CNT and fullerenes are nanostructures composed solely of carbon atoms that have been thoroughly studied in a wide variety of disciplines due to their unique physical and chemical properties (135). Fullerenes are highly symmetric cage molecules, of which C_{60} (Buckminsterfullerene) is the foremost member: a truncated icosahedron with a diameter of 0.7 nm, containing 60 carbon atoms forming 20 hexagons and 12 pentagons. Similarly, CNT are composed of carbon atoms arranged in benzene rings, forming graphene sheets rolled up to give cylinders with typical diameters in the range 1–100 nm. Two main types of CNT exist, single-walled (SWCNT) and multi-walled carbon nanotubes (MWCNT), the latter formed by several concentric layers of rolled graphene. One of the main shortcomings for development of biomedical applications of fullerenes and CNT is their poor solubility in aqueous media. As a result, great efforts have been dedicated to increase their solubility in water and biocompatibility by different strategies, including chemical modification by click chemistry (136,137).

For instance, Adronov and coworkers reported conditions for surface functionalization of SWCNT by means of CuAAC

(138). With this aim, *p*-aminophenyl propargyl ether was reacted with SWCNT using solvent-free diazotization/coupling procedure leading to alkyne-decorated SWCNT with a high degree of functionalization. Conditions for the conjugation of this SWCNT with an azido-terminated PS (prepared by ATRP and subsequent bromine end-group substitution with NaN_3) involved CuI and DBU in DMF. In an attempt to improve biocompatibility and water solubility of SWCNT, Zheng and coworkers exploited this strategy for covalent linkage of azido-functionalized β-CD (CuI, DBU, DMF, 70°C) (139) (Fig. 28). Inclusion complexation behavior of this artificial receptor with quinine was studied in aqueous solution by fluorescence spectroscopy. The higher biocompatibility and rich guest recognition properties derived from CD make this CD-SWCNT hybrid a promising platform for materials science and drug delivery applications.

An alternative versatile approach to modify surfaces of CNT was described by Gao's group by a combination of CuAAC "grafting onto" and ATRP "grafting from" strategies (140). A clickable macroinitiator $PBrN_3PrMA$, carrying bromides for ATRP as well as azido groups for CuAAC, was synthesized and coupled to alkynated SWCNT/MWCNT by CuAAC (CuBr, PMDETA, DMF). Poly(*n*-butylmethacrylate), PS, and PEG were subsequently grafted onto/from resulting CNT *via* ATRP/CuAAC to afford CNT-supported amphiphilic polymer brushes with controlled degree of grafting.

Because of their unique electronic properties, applications of functionalized CNT are mainly focused on the fields of solar energy conversion, electronics, and sensing. In this regard, several groups have reported application of CuAAC for functionalization of CNT with AuNP (141), MNP (142), and porphyrin dendrons (143) as potential platforms for novel electronic materials. In addition, CuAAC has been applied for preparation of sugar-based amphiphiles capable of binding CNT surfaces through π-π

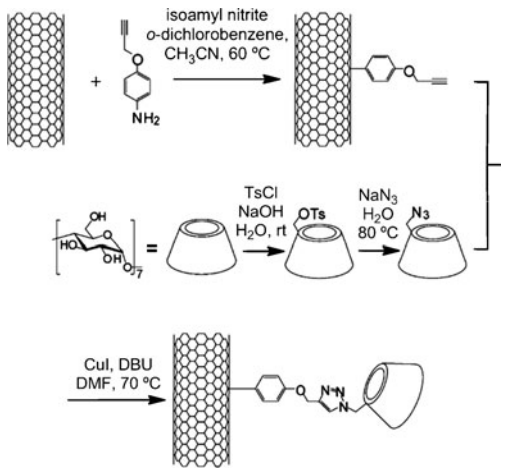

Fig. 28 Synthesis of CD-modified SWCNT. Reprinted with permission from ref (139).

interaction and function as homogeneous bioactive coatings to reduce cytotoxicity and facilitate targeting (144). The combination of these principles (good electronic properties, reduced toxicity, and active targeting) points out potential applications of CNT in drug delivery, where click chemistry will play a critical role as coupling technology.

Functionalized fullerenes, especially those based on the C_{60} platform, have been recently investigated as novel materials in nanomedicine. Thus, C_{60} has been used for development of HIV-1 protease inhibitors, drug and gene delivery systems, or novel photodynamic therapy and MRI contrast agents (137).

Application of CuAAC for construction of functionalized fullerenes has allowed preparation of complex conjugates where previous synthetic approaches failed. In one example by Isobe, Nakamura, and coworkers, C5-symmetric multivalent glycoconjugates with suitable spatial orientation for inhibition of pentameric Shiga-like toxin proteins have been prepared from a pentaalkynyl C_{60} derivative and various unprotected azido-functionalized carbohydrates, including P^k trisaccharide present in the natural ligand globotriaosylceramide (Gb-3) ($CuBr{\cdot}SMe_2$, *i*-Pr_2EtN, DMSO, 40°C) (145) (Fig. 29).

Shortly after, Nierengarten and coworkers prepared symmetrical C_{60} hexakis-dendritic adducts bearing 12 peripheral azide or alkyne groups functionalized with various ligands, including ferrocene, a porphyrin, and G2 Fréchet-type dendrons ($CuSO_4$, sodium ascorbate, biphasic CH_2Cl_2-H_2O mixture) (146,147) (Fig. 29). This methodology also enabled preparation of difunctionalized fullerenes *via* sequential double CuAAC approach starting from a C_{60} hexaadduct bearing 10 azides and 2 TMS-protected alkyne groups (147). Biologically relevant ligands such as carbohydrates have been attached to the periphery of C_{60} using this methodology. Some resulting "sugar balls" have been shown to be efficient inhibitors of glycosidase activity, and in some cases, a positive multivalent effect was observed (148).

CuAAC has also allowed preparation of polymer-C_{60} hybrids. In the first example, reported by Cheng (149), a PS-C_{60} conjugate was obtained by reacting azido-terminated PS of different molecular weights with a monoalkynated fullerene derivative (CuBr, PMDETA, toluene).

Steinmetz, Finn, Manchester, and coworkers explored the advantages of combining fullerenes and viral nanoparticles (VNP) for biomedical applications, such as photodynamic tumor therapy (150). VNP were decorated with C_{60} using classical amide formation conditions and CuAAC. An Alk-PEG-C_{60} conjugate was prepared and reacted with an azide-modified VNP ($CuSO_4$, sodium ascorbate, THPTA, aminoguanidine), resulting in soluble and stable hybrids with significantly higher loading than amides. Covalent incorporation of C_{60} was verified by Western blotting and STEM. Structural integrity of VNP-

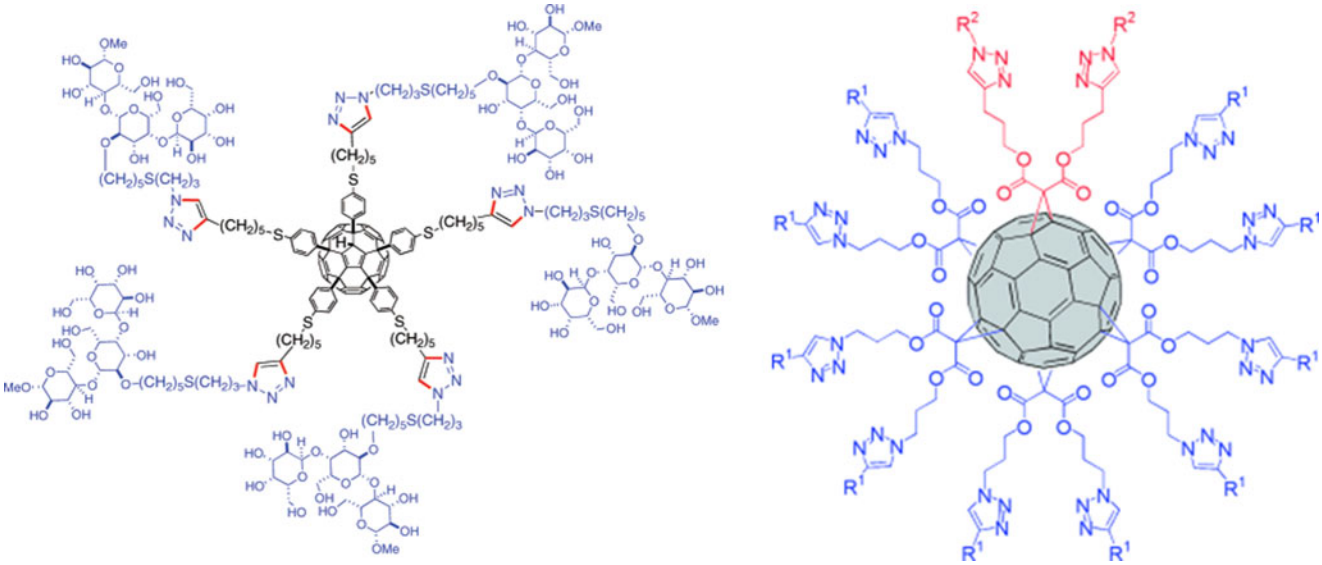

Fig. 29 CuAAC functionalized fullerenes prepared by the groups of Isobe and Nakamura (*left*), and Nierengarten (*right*). Reprinted with permission from ref (145) and (147).

PEG-C_{60} hybrid was confirmed by SEC, TEM, STEM, and native gel electrophoresis. Cellular uptake of a dye-labeled VNP-PEG-C_{60} was studied in HeLa human cancer cells using confocal microscopy, which revealed that internalization was not inhibited by C_{60} units.

Application of TEC to functionalization of fullerenes has been limited by their reactivity towards thiyl radicals (151), which can lead to undesired byproducts. In spite of this, the group of Nierengarten has taken advantage of higher reactivity of the methacrylate group to decorate the surface of C_{60} with model thiols (AIBN, benzene, 80°C) (152). In addition, the authors exploited the orthogonality of TEC (AIBN, degassed THF, reflux) and CuAAC ($CuSO_4$, sodium ascorbate, biphasic CH_2Cl_2-H_2O mixture) to demonstrate the advantages of sequential click reactions to yield differently functionalized fullerenes with potential applications in the biomedical field (Fig. 30).

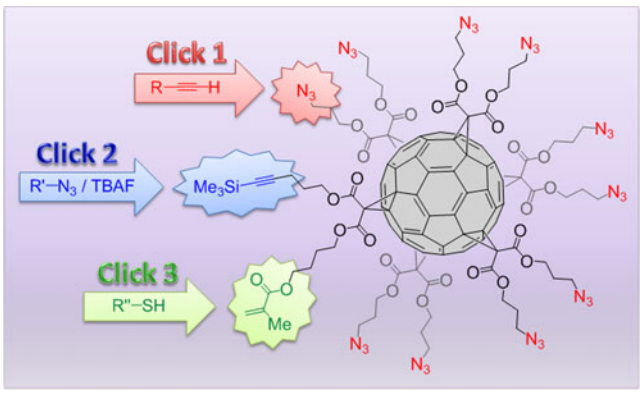

Fig. 30 Functionalization of fullerene hexaadducts by means of sequential CuAAC and TEC. Reprinted with permission from ref (152).

BIONANOPARTICLES

BNP are supramolecular protein assemblies such as viruses, virus-like particles, ferritins, and enzyme complexes, receiving great attention as pre-fabricated, biocompatible, and biodegradable scaffolds for a wide variety of nanotechnology applications, including drug delivery (153). Since azides and alkynes are inert towards biological environment, CuAAC has revealed as an excellent tool for functionalization of BNP. Fokin, Sharpless, Finn, and coworkers reported the first CuAAC functionalization of a BNP, the CPMV, a 31-nm structurally rigid icosahedral assembly of 60 identical copies of a two-protein asymmetric unit around a single-stranded RNA (16). With this purpose, the surface of CPMV was first decorated with 60 azide or alkyne groups at reactive lysine or cysteine residues in a controlled way. Adequate CuAAC experimental conditions involved use of $CuSO_4$, TBTA, and either TCEP or a Cu wire (5% *t*-BuOH in PBS, pH 8). Under these conditions, quantitative functionalization of CPMV with 60 copies of a complementary azide- or alkyne-functionalized fluorescein resulted, without compromising structural integrity of the viral particles.

In spite of the successful bioconjugation, some limitations of CuAAC for functionalization of biomacromolecules were pointed out by the authors. Thus, in absence of TBTA, viral capsides resulted in being degraded or aggregated by Cu ions or reducing agents used to generate Cu(I) *in situ* (16). As a result, great efforts have been devoted to minimize these shortcomings of CuAAC in bioconjugation. As previously described, this goal has been generally achieved by proper selection of a Cu(I)-chelating ligand, intended to stabilize oxidation state of Cu(I), increase reaction rate, and sequester Cu ions to facilitate purification. TBTA is indeed a good

example of Cu(I)-chelating ligand. Its tetradentate binding ability allows formation of Cu(I) chelates so stable that exclusion of O_2 from reaction medium is not required to avoid oxidation of Cu(I) (20). On the other side, its poor solubility in water has encouraged the search of alternative ligands with increased solubility (*i.e.*, BPDS and THPTA).

BPDS in presence of Cu(I) salts (*e.g.*, $Cu(MeCN)_4OTf$, $Cu(MeCN)_4PF_6$, CuBr) represents an interesting water-soluble alternative to TBTA. Finn and coworkers reported successful CuAAC functionalization of CPMV under these conditions with a plethora of biologically relevant substrates, such as complex carbohydrates (sialyl Lewis X, blood group A antigen, tri-LacNAc, globo-H, and a tetrasaccharide galectin-4 ligand), peptides (RGD and a portion of protective antigen of anthrax toxin), polymers (PEG and a glycopolymer produced by ATRP), a DOTA-Gd complex, and the iron carrier protein transferrin (HEPES (usually better results than Tris or PBS buffers), pH 8, rigorous exclusion of O_2) (23,154–156) (Fig. 31). Interestingly, the high catalytic activity of BPDS renders unnecessary the use of a large excess of coupling probes. As previously observed with MNP (109) and microarray slides (110), CuAAC on CPMV containing peripheral azides proceeded more efficiently than with alkynes.

The BPDS/Cu(I) system has been also exploited by Finn, Manchester, and coworkers for functionalization of CPMV with FA (157). With the aim of overcoming the natural targeting ability of CPMV towards several mammalian cell lines and tissues *in vivo* and redirecting it to alternative targets, FA was selected as a model ligand. FA was attached to CPMV by CuAAC through a short PEG linker and the specific recognition by tumor cells bearing the FR studied. Incorporation of PEG-FA completely eliminated the background binding of CPMV and allowed specific recognition by tumor cells bearing the FR. Analysis of different loadings of FA on CPMV revealed high density loadings may not be necessary for efficient targeting to tumor cells. In a more recent contribution, Finn's group also relied on the BPDS/Cu(I) system for CuAAC functionalization of the capsid of bacteriophage *Qβ* labeled with unnatural amino acids, containing azides or terminal alkynes introduced by site-directed mutagenesis (158).

The combination of BPDS and Cu(I) salts has been adopted by many other research groups as standard CuAAC conditions for bioconjugation of BNP. For example, Fisher and Manchester used these conditions for functionalization of the rod-shaped potato virus X with fluorescent dyes (159). Wang and coworkers chemoselectively functionalized the surface of horse spleen apoferritin, a cage-like protein structure composed of 24 structurally equivalent subunits (160). A total of 4 reactive lysines per subunit (K83, K97, K104, K143) were first functionalized with terminal alkynes and subsequently decorated with an azido-coumarin by CuAAC. Interestingly, while initial CuAAC attempts with $CuSO_4$/ascorbic acid or $CuSO_4$/TCEP led to aggregation or denaturation of the apoferritin, use of CuBr/BPDS afforded functionalized intact protein particles as confirmed by size-exclusion FPLC and TEM. Incorporation of one

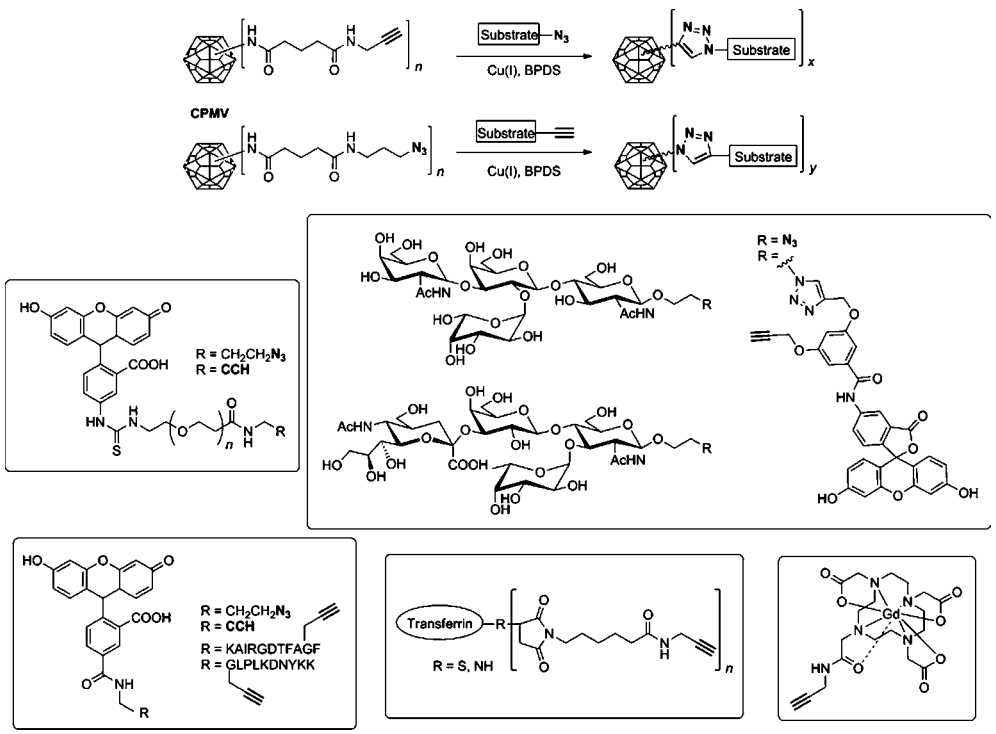

Fig. 31 Functionalization of CPMV with biologically relevant ligands, polymers, and imaging agents *via* CuAAC.

coumarin per subunit was confirmed by MALDI-MS analyses of a V8 protease digests.

More recently, Carrico and coworkers employed these CuAAC conditions (CuBr/BPDS) for selective functionalization of human adenovirus type 5 (161). Effective decoration of the viral surface was accomplished *via* metabolic incorporation of *N*-azidoacetylglucosamine into fiber protein during virus production, and subsequent CuAAC conjugation with alkynated derivatives of the FLAG peptide, tetramethylrhodamine, and FA. Remarkably, a folate-decorated adenovirus encoding a GFP in the viral genome showed a 3-4-fold increase GFP expression in FR-expressing mouse breast carcinoma cells (4 T1) compared to the unmodified adenovirus.

Young, Douglas, and coworkers also reported a CuAAC strategy based on use of BPDS and $Cu(MeCN)_4OTf$ for synthesis of a cross-linked dendritic network in the interior of porous heat shock protein cage (Hsp) (162). With this aim, a mutant HspG41C carrying a genetically introduced cysteine reactive site on the interior surface of each 24 identical subunits was selected. Dendritic structures were grown within the cage by reacting cysteine residue with *N*-propargyl bromoacetamide and subsequent iterative CuAAC couplings with 2-azido-1-azidomethyl ethylamine and tripropargyl amine to generate an internal branched structure that finally cross-linked (Fig. 32). The resulting internally functionalized cages were monodisperse and very similar in size to native protein cage (SEC, TEM, DLS). In addition, cross-linked cages benefited from extended thermal stability to at least

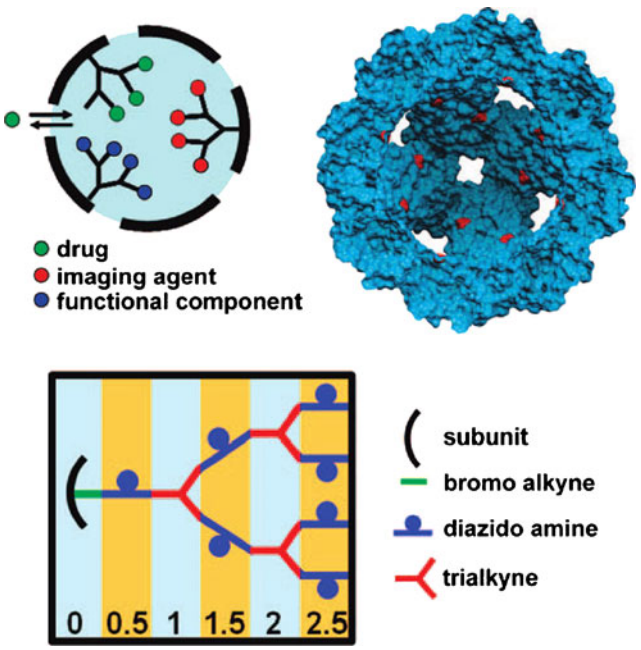

Fig. 32 Rationale and strategy for fabricating a hybrid protein cage/dendritic structure. Reprinted with permission from ref (162).

120°C and an enhanced internal carrying capacity with potential application in drug delivery.

As seen above, use of BPDS in connection with Cu(I) salts has allowed efficient and clean functionalization of various types of BNP under conditions that avoid degradation and aggregation by Cu, ROS, and reducing agents. Nevertheless, high sensitivity of this catalytic system to air oxidation (requiring rigorous exclusion of O_2 or use of a sacrificial reducing agent) demanded more user-friendly reaction conditions for widespread application of CuAAC in bioconjugation. With this aim, Finn's group developed new experimental procedures, including an electrochemically triggered CuAAC (25) and use of the water-soluble ligand THPTA (22). In the first approach, Cu(II) is electrochemically reduced to Cu(I) in presence of coupling substrates and BPDS (Fig. 4). Under these conditions, no reducing agent is required and O_2 present in the reaction medium is reduced to H_2O (*i.e.*, $O_2+4H^+ + 4e^- \rightarrow 2H_2O$). Consequently, degradation processes by ROS or reducing agents are minimized (25). The versatility of this methodology was demonstrated by conjugating up to 650 copies of an alkynated selenomethionine to azido-modified bacteriophage *Qβ* ($CuSO_4$, BPDS, electrolysis, −200 mV) with comparable outcome to that previously reported under O_2-free conditions (25) (Fig. 4). The second approach involves a protocol relying on simplicity and reliability of the Cu(II)/ascorbate system and water solubility of THPTA (22). THPTA, originally reported by Fokin (20), had been previously claimed by Brown and coworkers as an effective ligand for harmless CuAAC functionalization of nucleic acids (26). In addition to the known Cu(I)-chelating properties, Finn and coworkers reported THPTA as radical scavenger, strongly accelerating decomposition of H_2O_2 generated in reaction medium (5-fold excess of THPTA *vs.* Cu(I)). Under these conditions, use of the carbonyl capturing reagent aminoguanidine (stoichiometric aminoguanidine/ascorbate ratio) is also recommended to minimize adverse effects of ascorbate byproducts on amine-containing substrates. Following this protocol, functionalization of bacteriophage *Qβ* with up to 630 copies of a fluorescein dye or 50 BSA molecules has been efficiently accomplished (Fig. 4).

The advantages of this CuAAC bioconjugation protocol [Cu(II)/ascorbate/THPTA] have been also demonstrated by the group of Douglas for internal functionalization of the mutant protein cage HspG41C with a branched iron-phenanthroline-based coordination polymer (163) and also by the group of Finn for covalent functionalization of bacteriophage *Qβ* with C_{60} Buckyballs (150), and the human iron-transfer protein transferrin, a high affinity ligand for receptors upregulated in a variety of cancer cells (164). Transferrin conjugation to *Qβ* particles allowed specific recognition by transferrin receptors and cellular internalization through clathrin-mediated endocytosis, as

determined by fluorescence microscopy on cells expressing GFP-labeled clathrin light chains. More recently, these conditions have been also applied by Finn and coworkers for labeling mammalian cells in culture with no loss in cell viability (5 min incubations) (165), paving the way for safer application of CuAAC in bioorthogonal chemical reporter strategies.

CONCLUSIONS

Over the past few decades, various generations of nanosized drug delivery systems (DDS) have been developed for controlled administration of drugs into the body. Organic synthesis has accompanied this process by providing synthetic tools for preparation of novel materials for construction of DDS with new properties, as well as for efficient bioconjugation of drugs, polymers, and targeting ligands. Indeed, as this ability of putting together small building blocks into larger structures has been at the core of evolution, nature has inspired chemists in the search for more efficient processes with production of minimal waste. In this context, and in an effort to focus attention on the easy production of properties rather than on challenging structures, Sharpless and coworkers introduced in 2001 the concept of click chemistry. The idea was to confine the whole range of chemical transformations to a set of orthogonal processes with high thermodynamic driving force, allowing efficient and easy transformation of "spring-loaded" starting materials into new substances with useful properties. Four main groups of click reactions were defined on this basis: additions to carbon-carbon multiple bonds, nucleophilic substitutions, cycloadditions, and carbonyl chemistry of the "non-aldol" type. Among them, Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has emerged as a paradigm in click chemistry, attracting most attention, and finding application in many areas of research. In addition, as the click concept clearly goes beyond CuAAC, it has also triggered the search for more efficient, orthogonal, and harmless conjugation protocols of wide application, and experimental simplicity for non-specialists. Also, introduction of the click concept has attracted renewed interest on the use of efficient classical transformations. Firmly established reactions before the click era, such as thiol-ene coupling, Michael addition, or Diels-Alder, are clear examples of this type.

As we have shown, drug delivery has greatly benefited from click chemistry. Numerous examples of click procedures have been recently reported for preparation and functionalization of nanosized DDS, including polymeric micelles and nanoparticles, liposomes and polymersomes, capsules, metal and silica nanoparticles, carbon nanotubes and fullerenes, or bionanoparticles. In addition, great efforts have been focused on development of efficient bioconjugation protocols to ensure fast and high yielding functionalizations.

Nevertheless, rapid and favorable acceptance of the click concept in drug delivery and other areas should not screen challenges still ahead. For instance, a survey of literature reveals that in many publications entitled "click," some of the fundamental concepts of click chemistry are misinterpreted. Also, taking CuAAC as a leading example of a recently described click reaction, it is interesting to note that under the original conditions reported by Fokin, Sharpless and cowokers, presence of Cu and reducing agents is detrimental for biomacromolecules (*i.e.*, nucleic acids, proteins, polysaccharides), and hence reaction conditions have been adapted to ensure their harmless functionalization.

As a result, the complexity of nanosized DDS (where polymers, low-molecular-weight molecules, and biomacromolecules coexist into well-organized nanostructures) reveals itself an attractive test ground with rich future for application/development of established/new click reactions. In addition, development of new processes with high reaction kinetics and biocompatibility within the cellular environment, selective labeling of specific targets using wild metabolic routes, or design of easily accessible reactive tags with long shelf-life are undoubtedly roads to ride for biomedical applications of click chemistry, where drug delivery will find an endless source of inspiration.

ACKNOWLEDGMENTS & DISCLOSURES

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REFERENCES

1. Hoffman AS. The origins and evolution of "controlled" drug delivery systems. J Contr Release. 2008;132:153–63.
2. Wang B, Siahaan TJ, Soltero R. Drug delivery: principles and applications. Hoboken: Wiley; 2005.
3. van Vlerken L, Vyas T, Amiji M. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. Pharm Res. 2007;24:1405–14.
4. Byrne JD, Betancourt T, Brannon-Peppas L. Active targeting schemes for nanoparticle systems in cancer therapeutics. Adv Drug Deliv Rev. 2008;60:1615–26.
5. Maeda H. Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. Bioconjugate Chem. 2010;21:797–802.
6. Uchegbu IF, Schätzlein AG. Polymers in drug delivery. Boca Raton: CRC Press; 2006.
7. Anastas P, Eghbali N. Green chemistry: principles and practice. Chem Soc Rev. 2010;39:301–12.
8. Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. Angew Chem, Int Ed. 2001;40:2004–21.
9. Tornøe CW, Christensen C, Meldal M. Peptidotriazoles on solid phase: [1–3]-Triazoles by regiospecific copper(I)-catalyzed 1,3-

dipolar cycloadditions of terminal alkynes to azides. J Org Chem. 2002;67:3057–64.
10. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew Chem, Int Ed. 2002;41:2596–9.
11. Debets MF, van der Doelen CWJ, Rutjes FPJT, van Delft FL. Azide: a unique dipole for metal-free bioorthogonal ligations. Chembiochem. 2010;11:1168–84.
12. Iha RK, Wooley KL, Nyström AM, Burke DJ, Kade MJ, Hawker CJ. Applications of orthogonal "click" chemistries in the synthesis of functional soft materials. Chem Rev. 2009;109:5620–86.
13. Meldal M, Tornøe CW. Cu-Catalyzed azide-alkyne cycloaddition. Chem Rev. 2008;108:2952–3015.
14. Fokin VV, Wu P. Catalytic azide-alkyne cycloaddition: reactivity and applications. Aldrichim Acta. 2007;40:7–17.
15. van Dijk M, Rijkers DTS, Liskamp RMJ, van Nostrum CF, Hennink WE. Synthesis and applications of biomedical and pharmaceutical polymers *via* click chemistry methodologies. Bioconjugate Chem. 2009;20:2001–16.
16. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG. Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition. J Am Chem Soc. 2003;125:3192–3.
17. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. 1990;186:1–85.
18. Fry SC. Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. Biochem J. 1998;332:507–15.
19. Duxbury CJ, Cummins D, Heise A. Glaser coupling of polymers: side-reaction in Huisgens "click" coupling reaction and opportunity for polymers with focal diacetylene units in combination with ATRP. J Polymer Sci, Part A: Polymer Chem. 2009;47:3795–802.
20. Chan TR, Hilgraf R, Sharpless KB, Fokin VV. Polytriazoles as copper(I)-stabilizing ligands in catalysis. Org Lett. 2004;6:2853–5.
21. Lewis WG, Magallon FG, Fokin VV, Finn MG. Discovery and characterization of catalysts for azide-alkyne cycloaddition by fluorescence quenching. J Am Chem Soc. 2004;126:9152–3.
22. Hong V, Presolski SI, Ma C, Finn MG. Analysis and optimization of copper-catalyzed azide-alkyne cycloaddition for bioconjugation. Angew Chem, Int Ed. 2009;48:9879–83.
23. Gupta SS, Kuzelka J, Singh P, Lewis WG, Manchester M, Finn MG. Accelerated bioorthogonal conjugation: a practical method for the ligation of diverse functional molecules to a polyvalent virus scaffold. Bioconjugate Chem. 2005;16:1572–9.
24. Agard NJ, Prescher JA, Bertozzi CR. A strain-promoted [3+2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. J Am Chem Soc. 2004;126:15046–7.
25. Hong V, Udit AK, Evans RA, Finn MG. Electrochemically protected copper(I)-catalyzed azide-alkyne cycloaddition. Chembiochem. 2008;9:1481–6.
26. Kumar R, El-Sagheer AH, Tumpane J, Lincoln P, Wilhelmsson LM, Brown T. Template-directed oligonucleotide strand ligation, covalent intramolecular DNA circularization and catenation using click chemistry. J Am Chem Soc. 2007;129:6859–64.
27. Baskin JM, Bertozzi CR. Copper-free click chemistry: bioorthogonal reagents for tagging azides. Aldrichim Acta. 2010;43:15–23.
28. Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, *et al.* Copper-free click chemistry for dynamic *in vivo* imaging. Proc Natl Acad Sci USA. 2007;104:16793–7.
29. Codelli JA, Baskin JM, Agard NJ, Bertozzi CR. Second-generation difluorinated cyclooctynes for copper-free click chemistry. J Am Chem Soc. 2008;130:11486–93.
30. Ning X, Guo J, Wolfert MA, Boons G-J. Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast Huisgen cycloadditions. Angew Chem, Int Ed. 2008;47:2253–5.
31. Debets MF, van Berkel SS, Schoffelen S, Rutjes FPJT, van Hest JCM, van Delft FL. Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation *via* copper-free (3+2) cycloaddition. Chem Commun. 2010;46:97–9.
32. Jewett JC, Sletten EM, Bertozzi CR. Rapid Cu-free click chemistry with readily synthesized biarylazacyclooctynones. J Am Chem Soc. 2010;132:3688–90.
33. Dommerholt J, Schmidt S, Temming R, Hendriks LJA, Rutjes FPJT, van Hest JCM, *et al.* Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells. Angew Chem, Int Ed. 2010;49:9422–5.
34. Poloukhtine AA, Mbua NE, Wolfert MA, Boons G-J, Popik VV. Selective labeling of living cells by a photo-triggered click reaction. J Am Chem Soc. 2009;131:15769–76.
35. Farokhzad OC, Langer R. Impact of nanotechnology on drug delivery. ACS Nano. 2009;3:16–20.
36. Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev. 2001;47:113–31.
37. De P, Gondi SR, Sumerlin BS. Folate-conjugated thermoresponsive block copolymers: highly efficient conjugation and solution self-assembly. Biomacromolecules. 2008;9:1064–70.
38. Xu N, Wang R, Du F-S, Li Z-C. Synthesis of amphiphilic biodegradable glycocopolymers based on poly(ε-caprolactone) by ring-opening polymerization and click chemistry. J Polymer Sci, Part A: Polymer Chem. 2009;47:3583–94.
39. Chen G, Amajjahe S, Stenzel MH. Synthesis of thiol-linked neoglycopolymers and thermo-responsive glycomicelles as potential drug carrier. Chem Commun. 2009;1198–200.
40. Yang Y, Hua C, Dong C-M. Synthesis, self-assembly, and *in vitro* doxorubicin release behavior of dendron-like/linear/dendron-like poly(ε-caprolactone)-*b*-poly(ethylene glycol)-*b*-poly(ε-caprolactone) triblock copolymers. Biomacromolecules. 2009;10:2310–8.
41. Peng S-M, Chen Y, Hua C, Dong C-M. Dendron-like polypeptide/linear poly(ethylene oxide) biohybrids with both asymmetrical and symmetrical topologies synthesized *via* the combination of click chemistry and ring-opening polymerization. Macromolecules. 2009;42:104–13.
42. Hua C, Peng S-M, Dong C-M. Synthesis and characterization of linear-dendron-like poly(ε-caprolactone)-*b*-poly(ethylene oxide) copolymers *via* the combination of ring-opening polymerization and click chemistry. Macromolecules. 2008;41:6686–95.
43. Soliman GM, Sharma R, Choi AO, Varshney SK, Winnik FM, Kakkar AK, *et al.* Tailoring the efficacy of nimodipine drug delivery using nanocarriers based on A_2B miktoarm star polymers. Biomaterials. 2010;31:8382–92.
44. Quan C-Y, Chen J-X, Wang H-Y, Li C, Chang C, Zhang X-Z, *et al.* Core-shell nanosized assemblies mediated by the α-β cyclodextrin dimer with a tumor-triggered targeting property. ACS Nano. 2010;4:4211–9.
45. Lutz J-F, Pfeifer S, Zarafshani Z. "*In situ*" functionalization of thermoresponsive polymeric micelles using the "click" cycloaddition of azides and alkynes. QSAR Comb Sci. 2007;26:1151–8.
46. Nicolas J, Couvreur P. Synthesis of poly(alkyl cyanoacrylate)-based colloidal nanomedicines. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2009;1:111–27.
47. Nicolas J, Bensaid F, Desmaële D, Grogna M, Detrembleur C, Andrieux K, *et al.* Synthesis of highly functionalized poly(alkyl cyanoacrylate) nanoparticles by means of click chemistry. Macromolecules. 2008;41:8418–28.
48. Lu J, Shi M, Shoichet MS. Click chemistry functionalized polymeric nanoparticles target corneal epithelial cells through RGD-cell surface receptors. Bioconjugate Chem. 2009;20:87–94.
49. Shi M, Wosnick JH, Ho K, Keating A, Shoichet MS. Immunopolymeric nanoparticles by Diels-Alder chemistry. Angew Chem, Int Ed. 2007;46:6126–31.

50. Shi M, Ho K, Keating A, Shoichet MS. Doxorubicin-conjugated immuno-nanoparticles for intracellular anticancer drug delivery. Adv Funct Mater. 2009;19:1689–96.
51. An Z, Tang W, Wu M, Jiao Z and Stucky GD. Heterofunctional polymers and core-shell nanoparticles *via* cascade aminolysis/ Michael addition and alkyne-azide click reaction of RAFT polymers. Chem Commun. 2008;6501–3.
52. Bertin PA, Watson KJ, Nguyen ST. Indomethacin-containing nanoparticles derived from amphiphilic polynorbornene: a model ROMP-based drug encapsulation system. Macromolecules. 2004;37:8364–72.
53. Bertin PA, Smith D, Nguyen ST. High-density doxorubicin-conjugated polymeric nanoparticles *via* ring-opening metathesis polymerization. Chem Commun. 2005;3793–5.
54. Krovi SA, Smith D, Nguyen ST. "Clickable" polymer nanoparticles: a modular scaffold for surface functionalization. Chem Commun. 2010;46:5277–9.
55. Han Y, Shi Q, Hu J, Du Q, Chen X, Jing X. Grafting BSA onto poly[(L-lactide)-*co*-carbonate] microspheres by click chemistry. Macromol Biosci. 2008;8:638–44.
56. Shi Q, Chen X, Lu T, Jing X. The immobilization of proteins on biodegradable polymer fibers *via* click chemistry. Biomaterials. 2008;29:1118–26.
57. Shi Q, Huang Y, Chen X, Wu M, Sun J, Jing X. Hemoglobin conjugated micelles based on triblock biodegradable polymers as artificial oxygen carriers. Biomaterials. 2009;30:5077–85.
58. Wang X, Liu L, Luo Y, Zhao H. Bioconjugation of biotin to the interfaces of polymeric micelles *via in situ* click chemistry. Langmuir. 2009;25:744–50.
59. Harada A, Kataoka K. Formation of polyion complex micelles in an aqueous milieu from a pair of oppositely-charged block copolymers with poly(ethylene glycol) segments. Macromolecules. 1995;28:5294–9.
60. Kabanov AV, Bronich TK, Kabanov VA, Yu K, Eisenberg A. Soluble stoichiometric complexes from poly(N-ethyl-4-vinylpyridinium) cations and poly(ethylene oxide)-block-polymethacrylate anions. Macromolecules. 1996;29:6797–802.
61. Sousa-Herves A, Fernandez-Megia E, Riguera R. Synthesis and supramolecular assembly of clicked anionic dendritic polymers into polyion complex micelles. Chem Commun. 2008;3136–8.
62. Zhang J, Zhou Y, Zhu Z, Ge Z, Liu S. Polyion complex micelles possessing thermoresponsive coronas and their covalent core stabilization *via* "click" chemistry. Macromolecules. 2008;41:1444–54.
63. O'Reilly RK, Hawker CJ, Wooley KL. Cross-linked block copolymer micelles: functional nanostructures of great potential and versatility. Chem Soc Rev. 2006;35:1068–83.
64. Read ES, Armes SP. Recent advances in shell cross-linked micelles. Chem Commun. 2007;3021–35.
65. Joralemon MJ, O'Reilly RK, Hawker CJ, Wooley KL. Shell click-crosslinked (SCC) nanoparticles: a new methodology for synthesis and orthogonal functionalization. J Am Chem Soc. 2005;127:16892–9.
66. O'Reilly RK, Joralemon MJ, Hawker CJ, Wooley KL. Preparation of orthogonally-functionalized core click cross-linked nanoparticles. New J Chem. 2007;31:718–24.
67. Jiang X, Zhang J, Zhou Y, Xu J, Liu S. Facile preparation of core-crosslinked micelles from azide-containing thermoresponsive double hydrophilic diblock copolymer *via* click chemistry. J Polymer Sci, Part A: Polymer Chem. 2008;46:860–71.
68. Jiang X, Zhang G, Narain R, Liu S. Fabrication of two types of shell-cross-linked micelles with "inverted" structures in aqueous solution from schizophrenic water-soluble ABC triblock copolymer *via* click chemistry. Langmuir. 2009;25:2046–54.
69. Withey ABJ, Chen G, Nguyen TLU, Stenzel MH. Macromolecular cobalt carbonyl complexes encapsulated in a click-cross-linked micelle structure as a nanoparticle to deliver cobalt pharmaceuticals. Biomacromolecules. 2009;10:3215–26.
70. Lallana E, Fernandez-Megia E, Riguera R. Surpassing the use of copper in the click functionalization of polymeric nanostructures: a strain-promoted approach. J Am Chem Soc. 2009;131:5748–50.
71. Sawant RR, Torchilin VP. Liposomes as "smart" pharmaceutical nanocarriers. Soft Matter. 2010;6:4026–44.
72. Hassane FS, Frisch B, Schuber F. Targeted liposomes: convenient coupling of ligands to preformed vesicles using "click chemistry". Bioconjugate Chem. 2006;17:849–54.
73. Cavalli S, Tipton AR, Overhand M, Kros A. The chemical modification of liposome surfaces *via* a copper-mediated [3+2] azide-alkyne cycloaddition monitored by a colorimetric assay. Chem Commun. 2006;3193–5.
74. Kumar A, Erasquin UJ, Qin G, Li K, Cai C. "Clickable", polymerized liposomes as a versatile and stable platform for rapid optimization of their peripheral compositions. Chem Commun. 2010;46:5746–8.
75. Discher BM, Won Y-Y, Ege DS, Lee JC-M, Bates FS, Discher DE, *et al.* Polymersomes: tough vesicles made from diblock copolymers. Science. 1999;284:1143–6.
76. LoPresti C, Lomas H, Massignani M, Smart T, Battaglia G. Polymersomes: nature inspired nanometer sized compartments. J Mater Chem. 2009;19:3576–90.
77. Opsteen JA, Brinkhuis RP, Teeuwen RLM, Löwik DWPM, van Hest JCM. "Clickable" polymersomes. Chem Commun. 2007;3136–8.
78. Li B, Martin AL, Gillies ER. Multivalent polymer vesicles *via* surface functionalization. Chem Commun. 2007;5217–9.
79. Martin AL, Li B, Gillies ER. Surface functionalization of nanomaterials with dendritic groups: toward enhanced binding to biological targets. J Am Chem Soc. 2009;131:734–41.
80. Lee S-M, Chen H, Dettmer CM, O'Halloran TV, Nguyen ST. Polymer-caged lipsomes: a pH-responsive delivery system with high stability. J Am Chem Soc. 2007;129:15096–7.
81. Lee S-M, Chen H, O'Halloran TV, Nguyen ST. "Clickable" polymer-caged nanobins as a modular drug delivery platform. J Am Chem Soc. 2009;131:9311–20.
82. Quinn JF, Johnston APR, Such GK, Zelikin AN, Caruso F. Next generation, sequentially assembled ultrathin films: beyond electrostatics. Chem Soc Rev. 2007;36:707–18.
83. Such GK, Tjipto E, Postma A, Johnston APR, Caruso F. Ultrathin, responsive polymer click capsules. Nano Lett. 2007;7:1706–10.
84. Ochs CJ, Such GK, Städler B, Caruso F. Low-fouling, biofunctionalized, and biodegradable click capsules. Biomacromolecules. 2008;9:3389–96.
85. De Geest BG, Van Camp W, Du Prez FE, De Smedt SC, Demeester J, Hennink WE. Degradable multilayer films and hollow capsules *via* a "click" strategy. Macromol Rapid Commun. 2008;29:1111–8.
86. De Geest BG, Van Camp W, Du Prez FE, De Smedt SC, Demeester J, Hennink WE. Biodegradable microcapsules designed via 'click' chemistry. Chem Commun. 2008;190–2.
87. Connal LA, Kinnane CR, Zelikin AN, Caruso F. Stabilization and functionalization of polymer multilayers and capsules *via* thiol-ene click chemistry. Chem Mater. 2009;21:576–8.
88. Kamphuis MMJ, Johnston APR, Such GK, Dam HH, Evans RA, Scott AM, *et al.* Targeting of cancer cells using click-functionalized polymer capsules. J Am Chem Soc. 2010;132:15881–3.
89. Ochs CJ, Such GK, Yan Y, van Koeverden MP, Caruso F. Biodegradable click capsules with engineered drug-loaded multilayers. ACS Nano. 2010;4:1653–63.
90. Kim D, Kim E, Lee J, Hong S, Sung W, Lim N, *et al.* Direct synthesis of polymer nanocapsules: self-assembly of polymer hollow spheres through irreversible covalent bond formation. J Am Chem Soc. 2010;132:9908–19.

91. Breed DR, Thibault R, Xie F, Wang Q, Hawker CJ, Pine DJ. Functionalization of polymer microspheres using click chemistry. Langmuir. 2009;25:4370–6.
92. Goldmann AS, Walther A, Nebhani L, Joso R, Ernst D, Loos K, *et al.* Surface modification of poly(divinylbenzene) microspheres *via* thiol-ene chemistry and alkyne-azide click reactions. Macromolecules. 2009;42:3707–14.
93. Arruebo M, Fernández-Pacheco R, Ibarra MR, Santamaría J. Magnetic nanoparticles for drug delivery. Nano Today. 2007;2:22–32.
94. Sperling RA, Rivera Gil P, Zhang F, Zanella M, Parak WJ. Biological applications of gold nanoparticles. Chem Soc Rev. 2008;37:1896–908.
95. Boisselier E, Astruc D. Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity. Chem Soc Rev. 2009;38:1759–82.
96. Fleming DA, Thode CJ, Williams ME. Triazole cycloaddition as a general route for functionalization of Au nanoparticles. Chem Mater. 2006;18:2327–34.
97. Boisselier E, Salmon L, Ruiz J, Astruc D. How to very efficiently functionalize gold nanoparticles by "click" chemistry. Chem Commun. 2008;5788–90.
98. Brennan JL, Hatzakis NS, Tshikhudo TR, Dirvianskyte N, Razumas V, Patkar S, *et al.* Bionanoconjugation *via* click chemistry: the creation of functional hybrids of lipases and gold nanoparticles. Bioconjugate Chem. 2006;17:1373–5.
99. Kim Y-P, Daniel WL, Xia Z, Xie H, Mirkin CA, Rao J. Bioluminescent nanosensors for protease detection based upon gold nanoparticle-luciferase conjugates. Chem Commun. 2010;46:76–8.
100. Fischler M, Sologubenko A, Mayer J, Clever G, Burley G, Gierlich J, Carell T, Simon U. Chain-like assembly of gold nanoparticles on artificial DNA templates *via* "click chemistry". Chem Commun. 2008;169–71.
101. Zhang M-X, Huang B-H, Sun X-Y, Pang D-W. Clickable gold nanoparticles as the building block of nanobioprobes. Langmuir. 2010;26:10171–6.
102. Li H, Yao Y, Han C, Zhan J. Triazole-ester modified silver nanoparticles: click synthesis and Cd^{2+} colorimetric sensing. Chem Commun. 2009;4812–4.
103. Yao Y, Tian D, Li H. Cooperative binding of bifunctionalized and click-synthesized silver nanoparticles for colorimetric Co^{2+} sensing. ACS Appl Mater Interfaces. 2010;2:684–90.
104. Drockenmuller E, Colinet I, Damiron D, Gal F, Perez H, Carrot G. Efficient approaches for the surface modification of platinum nanoparticles *via* click chemistry. Macromolecules. 2010;43:9371–5.
105. Koenig S, Chechik V. Shell cross-linked Au nanoparticles. Langmuir. 2006;22:5168–73.
106. Costanzo PJ, Beyer FL. Thermally driven assembly of nanoparticles in polymer matrices. Macromolecules. 2007;40:3996–4001.
107. Mornet S, Vasseur S, Grasset F, Duguet E. Magnetic nanoparticle design for medical diagnosis and therapy. J Mater Chem. 2004;14:2161–75.
108. White MA, Johnson JA, Koberstein JT, Turro NJ. Toward the syntheses of universal ligands for metal oxide surfaces: controlling surface functionality through click chemistry. J Am Chem Soc. 2006;128:11356–7.
109. Lin PC, Ueng SH, Yu SC, Jan MD, Adak AK, Yu CC, *et al.* Surface modification of magnetic nanoparticle *via* Cu(I)-catalyzed alkyne-azide [2+3] cycloaddition. Org Lett. 2007;9:2131–4.
110. Lin P-C, Ueng S-H, Tseng M-C, Ko J-L, Huang K-T, Yu S-C, *et al.* Site-specific protein modification through Cu^{I}-catalyzed 1,2,3-triazole formation and its implementation in protein microarray fabrication. Angew Chem, Int Ed. 2006;45:4286–90.
111. Polito L, Monti D, Caneva E, Delnevo E, Russo G, Prosperi D. One-step bioengineering of magnetic nanoparticles *via* a surface diazo transfer/azide-alkyne click reaction sequence. Chem Commun. 2008;621–3.
112. Nyffeler PT, Liang C-H, Koeller KM, Wong C-H. The chemistry of amine-azide interconversion: catalytic diazotransfer and regioselective azide reduction. J Am Chem Soc. 2002;124:10773–8.
113. Cutler JI, Zheng D, Xu X, Giljohann DA, Mirkin CA. Polyvalent oligonucleotide iron oxide nanoparticle "click" conjugates. Nano Lett. 2010;10:1477–80.
114. Elias DR, Cheng Z, Tsourkas A. An intein-mediated site-specific click conjugation strategy for improved tumor targeting of nanoparticle systems. Small. 2010;6:2460–8.
115. Hayashi K, Moriya M, Sakamoto W, Yogo T. Chemoselective synthesis of folic acid-functionalized magnetite nanoparticles *via* click chemistry for magnetic hyperthermia. Chem Mater. 2009;21:1318–25.
116. Hayashi K, Ono K, Suzuki H, Sawada M, Moriya M, Sakamoto W, *et al.* High-frequency, magnetic-field-responsive drug release from magnetic nanoparticle/organic hybrid based on hyperthermic effect. ACS Appl Mater Interfaces. 2010;2:1903–11.
117. Santra S, Kaittanis C, Grimm J, Perez JM. Drug/dye-loaded, multifunctional iron oxide nanoparticles for combined targeted cancer therapy and dual optical/magnetic resonance imaging. Small. 2009;5:1862–8.
118. Devaraj NK, Keliher EJ, Thurber GM, Nahrendorf M, Weissleder R. ^{18}F labeled nanoparticles for *in vivo* PET-CT imaging. Bioconjugate Chem. 2009;20:397–401.
119. Nahrendorf M, Keliher E, Marinelli B, Waterman P, Feruglio PF, Fexon L, *et al.* Hybrid PET-optical imaging using targeted probes. Proc Natl Acad Sci USA. 2010;107:7910–5.
120. von Maltzahn G, Ren Y, Park J-H, Min D-H, Kotamraju VR, Jayakumar J, *et al. In vivo* tumor cell targeting with "click" nanoparticles. Bioconjugate Chem. 2008;19:1570–8.
121. Hayashi K, Ono K, Suzuki H, Sawada M, Moriya M, Sakamoto W, *et al.* One-pot biofunctionalization of magnetic nanoparticles via thiol−ene click reaction for magnetic hyperthermia and magnetic resonance imaging. Chem Mater. 2010;22:3768–72.
122. Rutledge RD, Warner CL, Pittman JW, Addleman RS, Engelhard M, Chouyyok W, *et al.* Thiol−ene induced diphosphonic acid functionalization of superparamagnetic iron oxide nanoparticles. Langmuir. 2010;26:12285–92.
123. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, *et al.* Quantum dots for live cells, *in vivo* imaging, and diagnostics. Science. 2005;307:538–44.
124. Binder WH, Sachsenhofer R, Straif CJ, Zirbs R. Surface-modified nanoparticles *via* thermal and Cu(I)-mediated "click" chemistry: generation of luminescent CdSe nanoparticles with polar ligands guiding supramolecular recognition. J Mater Chem. 2007;17:2125–32.
125. Bernardin A, Cazet A, Guyon L, Delannoy P, Vinet F, Texier I, *et al.* Copper-free click chemistry for highly luminescent quantum dot conjugates: application to *in vivo* metabolic imaging. Bioconjugate Chem. 2010;21:583–8.
126. Han H-S, Devaraj NK, Lee J, Hilderbrand SA, Weissleder R, Bawendi MG. Development of a bioorthogonal and highly efficient conjugation method for quantum dots using tetrazine-norbornene cycloaddition. J Am Chem Soc. 2010;132:7838–9.
127. Slowing II, Trewyn BG, Giri S, Lin VSY. Mesoporous silica nanoparticles for drug delivery and biosensing applications. Adv Funct Mater. 2007;17:1225–36.
128. Ranjan R, Brittain WJ. Combination of living radical polymerization and click chemistry for surface modification. Macromolecules. 2007;40:6217–23.
129. Ranjan R, Brittain WJ. Tandem RAFT polymerization and click chemistry: an efficient approach to surface modification. Macromol Rapid Commun. 2007;28:2084–9.

130. Zhang J, Wang X, Wu D, Liu L, Zhao H. Bioconjugated Janus particles prepared by *in situ* click chemistry. Chem Mater. 2009;21:4012–8.
131. Müllner M, Schallon A, Walther A, Freitag R, Müller AHE. Clickable, biocompatible, and fluorescent hybrid nanoparticles for intracellular delivery and optical imaging. Biomacromolecules. 2010;11:390–6.
132. Kele P, Mezö G, Achatz D, Wolfbeis OS. Dual labeling of biomolecules by using click chemistry: a sequential approach. Angew Chem, Int Ed. 2009;48:344–7.
133. Achatz DE, Mezö G, Kele P, Wolfbeis OS. Probing the activity of matrix metalloproteinase II with a sequentially click-labeled silica nanoparticle FRET probe. Chembiochem. 2009;10:2316–20.
134. Schlossbauer A, Warncke S, Gramlich PME, Kecht J, Manetto A, Carell T, *et al.* A programmable DNA-based molecular valve for colloidal mesoporous silica. Angew Chem, Int Ed. 2010;49:4734–7.
135. Guldi DM, Rahman GMA, Sgobba V, Ehli C. Multifunctional molecular carbon materials-from fullerenes to carbon nanotubes. Chem Soc Rev. 2006;35:471–87.
136. Bianco A, Kostarelos K, Partidos CD, Prato M. Biomedical applications of functionalised carbon nanotubes. Chem Commun. 2005;571–7.
137. Partha R, Conyers JL. Biomedical applications of functionalized fullerene-based nanomaterials. Int J Nanomed. 2009;4:261–75.
138. Li H, Cheng F, Duft AM, Adronov A. Functionalization of single-walled carbon nanotubes with well-defined polystyrene by "click" coupling. J Am Chem Soc. 2005;127:14518–24.
139. Guo Z, Liang L, Liang J-J, Ma Y-F, Yang X-Y, Ren D-M, *et al.* Covalently β-cyclodextrin modified single-walled carbon nanotubes: a novel artificial receptor synthesized by "click" chemistry. J Nanopart Res. 2008;10:1077–83.
140. Zhang Y, He H, Gao C. Clickable macroinitiator strategy to build amphiphilic polymer brushes on carbon nanotubes. Macromolecules. 2008;41:9581–94.
141. Voggu R, Pal S, Pati SK, Rao CNR. Semiconductor to metal transition in SWNTs caused by interaction with gold and platinum nanoparticles. J Phys Condens Matter. 2008;20:215211.
142. He H, Zhang Y, Gao C, Wu JY. 'Clicked' magnetic nanohybrids with a soft polymer interlayer. Chem Commun. 2009;1655–7.
143. Palacin T, Khanh HL, Jousselme B, Jegou P, Filoramo A, Ehli C, *et al.* Efficient functionalization of carbon nanotubes with porphyrin dendrons *via* click chemistry. J Am Chem Soc. 2009;131:15394–402.
144. Wu P, Chen X, Hu N, Tam UC, Blixt O, Zettl A, *et al.* Biocompatible carbon nanotubes generated by functionalization with glycodendrimers. Angew Chem Inter Ed. 2008;47:5022–5.
145. Isobe H, Cho K, Solin N, Werz DB, Seeberger PH, Nakamura E. Synthesis of fullerene glycoconjugates *via* a copper-catalyzed Huisgen cycloaddition reaction. Org Lett. 2007;9:4611–4.
146. Iehl J, Pereira de Freitas R, Delavaux-Nicot B, Nierengarten J-F. Click chemistry for the efficient preparation of functionalized [60] fullerene hexakis-adducts. Chem Commun. 2008;2450–2452.
147. Iehl J, Nierengarten J-F. A click-click approach for the preparation of functionalized [5:1]-hexaadducts of C_{60}. Chem Eur J. 2009;15:7306–9.
148. Compain P, Decroocq C, Iehl J, Holler M, Hazelard D, Mena Barragán T, *et al.* Glycosidase inhibition with fullerene iminosugar balls: a dramatic multivalent effect. Angew Chem, Int Ed. 2010;49:5753–6.
149. Zhang W-B, Tu Y, Ranjan R, van Horn RM, Leng S, Wang J, *et al.* "Clicking" fullerene with polymers: synthesis of [60]fullerene end-capped polystyrene. Macromolecules. 2008;41:515–7.
150. Steinmetz NF, Hong V, Spoerke ED, Lu P, Breitenkamp K, Finn MG, *et al.* Buckyballs meet viral nanoparticles: candidates for biomedicine. J Am Chem Soc. 2009;131:17093–5.
151. Cremonini MA, Lunazzi L, Placucci G, Krusic PJ. Addition of alkylthiyl and alkoxy radicals to C60 studied by ESR. J Org Chem. 1993;58:4735–8.
152. Iehl J, Nierengarten J-F. Sequential copper catalyzed alkyne-azide and thiol-ene click reactions for the multiple functionalization of fullerene hexaadducts. Chem Commun. 2010;46:4160–2.
153. Lee LA, Wang Q. Adaptations of nanoscale viruses and other protein cages for medical applications. Nanomedicine: NBM. 2006;2:137–49.
154. Gupta SS, Raja KS, Kaltgrad E, Strable E, Finn MG. Virus-glycopolymer conjugates by copper(I) catalysis of atom transfer radical polymerization and azide-alkyne cycloaddition. Chem Commun. 2005;4315–7.
155. Prasuhn JDE, Yeh RM, Obenaus A, Manchester M, Finn MG. Viral MRI contrast agents: coordination of Gd by native virions and attachment of Gd complexes by azide-alkyne cycloaddition. Chem Commun. 2007;1269–71.
156. Kaltgrad E, Sen Gupta S, Punna S, Huang C-Y, Chang A, Wong C-H, *et al.* Anti-carbohydrate antibodies elicited by polyvalent display on a viral scaffold. Chembiochem. 2007;8:1455–62.
157. Destito G, Yeh R, Rae CS, Finn MG, Manchester M. Folic acid-mediated targeting of Cowpea mosaic virus particles to tumor cells. Chem Biol. 2007;14:1152–62.
158. Strable E, Prasuhn DE, Udit AK, Brown S, Link AJ, Ngo JT, *et al.* Unnatural amino acid incorporation into virus-like particles. Bioconjugate Chem. 2008;19:866–75.
159. Steinmetz NF, Mertens ME, Taurog RE, Johnson JE, Commandeur U, Fischer R, *et al.* Potato virus X as a novel platform for potential biomedical applications. Nano Lett. 2010;10:305–12.
160. Zeng Q, Li T, Cash B, Li S, Xie F, Wang Q. Chemoselective derivatization of a bionanoparticle by click reaction and ATRP reaction. Chem Commun. 2007;1453–5.
161. Banerjee PS, Ostapchuk P, Hearing P, Carrico I. Chemoselective attachment of small molecule effector functionality to human adenoviruses facilitates gene delivery to cancer cells. J Am Chem Soc. 2010;132:13615–7.
162. Abedin MJ, Liepold L, Suci P, Young M, Douglas T. Synthesis of a cross-linked branched polymer network in the interior of a protein cage. J Am Chem Soc. 2009;131:4346–54.
163. Lucon J, Abedin MJ, Uchida M, Liepold L, Jolley CC, Young M, *et al.* A click chemistry based coordination polymer inside small heat shock protein. Chem Commun. 2010;46:264–6.
164. Banerjee D, Liu AP, Voss NR, Schmid SL, Finn MG. Multivalent display and receptor-mediated endocytosis of transferrin on virus-like particles. Chembiochem. 2010;11:1273–9.
165. Hong V, Steinmetz NF, Manchester M, Finn MG. Labeling live cells by copper-catalyzed alkyne-azide click chemistry. Bioconjugate Chem. 2010;21:1912–6.