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Title[a] Design of Degradable Click Delivery Systems.

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Click chemistry has had a significant impact in the field of materials science over the last ten years, as it has enabled the design of new hybrid building blocks, leading to multifunctional and responsive materials. One key application for such materials is in the biomedical field, such as gene or drug delivery. However, to meet the functional requirements of such applications, tailored degradability of these materials under biological conditions is critical. There has been an increasing interest in combining click chemistry techniques with a range of degradable or responsive building blocks as well as investigating new or milder chemistries to design click delivery systems that are capable of physiologically relevant degradation. This feature article will cover some of the different approaches to synthesize degradable click delivery systems and their investigation for therapeutic release.

Introduction

The development of click chemistry in 2001 has had dramatic impact on the synthesis of advanced materials, due to the ease and versatility of click-based reactions. The concept of a click reaction, as introduced by Sharpless and coworkers,[1] refers to reactions that are highly

quantitative, with minimal or no byproducts, and which are orthogonal to other functionalities present within the materials. Some of the reactions used as examples of click chemistry were existing chemistries, such as Diels-Alder or 1,3 dipolar cycloaddition. In 2002, both Meldal and Sharpless reported that the use of a copper catalyst dramatically enhanced the rate of the existing 1,3 dipolar cycloaddition referred to as copper (I) catalyzed alkyne and azide cycloaddition (CuAAC). This reaction is the most prominent example of click chemistry due to its many advantages such as high selectivity under mild conditions, making it highly attractive for material science applications.[2,3] Later work saw many other existing reactions be classified as click reactions, most significantly thiol reactions with alkenes referred to as thiol-ene. Thiol reactions with alkynes or maleimides have also been referred to as click reactions. However, strict control over reaction conditions is required in these cases to limit side products and thus there is some discussion over their classification as a click reaction.[4,5,6] In addition, various researchers have identified challenges with using CuAAC for biological applications due to the potential toxicity of Cu (I), which has lead to the development of copper free alternatives.[7] The mechanisms of each click reaction, as well as their limitations, have been discussed in detail in comprehensive reviews.[8,9]

Click reactions, while initially being developed for biological applications, have generated significant interest for material scientists, as they allow specific coupling of different building blocks to design new and advanced structures, from two different polymers to form block copolymers through to the combination of polymer and biological hybrids.[10,11] There is a large body of work in the area of polymer conjugates assembled using click reactions; this work is highlighted in several extensive reviews,[10,11] and so will not be covered in this article. However, the high efficiency of these reactions also allows the development of new strategies to assemble engineered polymers into larger structures such as particles or capsules. Such nanoengineered carriers have important applications in various fields, including therapeutic

delivery and imaging due to the precise control over functionality and architecture. For biomedical applications it is particularly important that materials can be designed which have high stability but can be degraded efficiently under physiologically relevant conditions. Most click reactions, such as CuAAC, allow the synthesis of highly stabilized bonds and are not inherently degradable. Therefore, their application to delivery systems or biomedical devices is challenging. The use of versatile and precise chemistry offered by click reactions provides new opportunities for the development of new polymeric carriers with enhanced properties and hence there is a significant need to develop techniques for combining degradable components with these efficient chemistries to design optimized polymeric carriers. Degradable therapeutic carriers often need to be engineered with components which undergo partial or complete fragmentation under physiological conditions, allowing carrier breakdown and subsequent cargo release. There are three main approaches that have investigated to design such systems, including the use of mild Diels-Alder cycloaddition that is an inherently thermoresponsive click reaction, the use of inherently degradable polymeric building blocks, and finally the combination of click chemistry with responsive components, e.g., redox-responsive crosslinkers. This feature article will discuss the degradable click carriers that have been synthesized using each synthetic approach and their potential application in areas such as drug or gene delivery.

1. Thermoresponsive Click Reactions

While the majority of click reactions do not involve an inherently biologically responsive component, Diels-Alder (DA) and related retro-Diels Alder (rDA) cycloadditions are the notable exceptions. Both of these reactions can be controlled by temperature to form cyclized product and uncyclized starting material, respectively. The thermoresponsive nature of these reactions can be tuned by the diene and dienophile chosen.[12] There are many extensively studied Diels-Alder reactions such as between furan/maleimide; however, these reactions

occur well above physiological temperatures and thus can have limitations for the design of therapeutic carriers. However, recently there has been significant research on new DA combinations, which have quicker reaction rates and more accessible temperature ranges. Barner-Kowollik and coworkers have reported a number of interesting studies on activated dithioesters, which in addition to controlling a RAFT polymerization, can also act as dienophiles. Depending on the structure, it was found that quantitative conversions could be achieved at 50 °C within 24 h.[13] Later work investigated the cycloaddition with cyclopentadiene, which could be obtained within a few minutes at ambient conditions.[13] To date there has been limited use of these milder DA reactions to synthesize engineered structures. There have been a few studies on the synthesis of thermoresponsive hydrogels. One study involved formation of a hydrogel under ambient temperature and its degradation within approximately 10 min at 80 °C (Figure 1A).[14,15] Reutenauer et al. reported the synthesis of thin films that are capable of self-healing at room temperature (Figure 1B).[16] It was demonstrated that films could be cut into pieces and then self-healed when pressed into contact for only 10 s. While the majority of DA reactions occur at temperature ranges above what is relevant for biomedical application, it is evident from these studies that significant progress has been made in developing new milder chemistries. Thus it is anticipated that continued progress will be made in this area.

Recently, another reaction was introduced which encompasses many of the click chemistry requirements; namely it is highly specific, efficient, proceeds under mild conditions and has no byproducts. Termed a bio-click reaction, it involves the *Staphylococcus aureus* enzyme Sortase A, which recognizes protein substrates containing a LPETG peptide motif and cleaves between the threonine and glycine residues. The carboxyl group of threonine subsequently undergoes a nucleophilic attack by the N-terminal of a polyglycine sequence, hence creating a new peptide bond. Recently, this reaction was used to modify a poly(*N*-vinyl pyrollidone)

capsule with a targeting single-chain variable fragment (ScFv).[17] The applicability of this technique to the assembly of polymer carriers would be of interest as the technique relies on peptide functionality, which is inherently degradable.

2. Degradable Delivery Systems Using Biological Components

An alternative approach for the synthesis of degradable click carriers is to use native degradable components such as DNA, polysaccharides or polypeptides.[18] One strategy to design such carriers was developed by Such et al. based on CuAAC facilitated layer-by-layer (LbL) assembly. This approach was based on the alternate deposition of polymers functionalized with either an alkyne or azide moiety in the presence of a copper catalyst.[19,20] Click-LbL assembly is highly versatile and can be performed on planar surfaces or on a sacrificial colloidal template, in which case the template can then be removed to form polymer capsules. A wide range of polymers can be used as long as they are amenable to functionalization with the appropriate click functionalities. Capsules based on poly(L-glutamic acid) and poly(L-Lysine) have been successfully assembled using this approach. Additionally, the capsule properties can be tuned by the assembly conditions (e.g., pH).[21] This approach was also used to assemble polysaccharide-based systems, which are inherently degradable.[22]

Another highly versatile approach was developed by Harth and coworkers based on polymer precipitation of click-functionalized polyesters. It was demonstrated that highly uniform particles can be synthesized by modifying the polyester backbone with either alkyne or allyl functionality, which is then cross-linked with an azide or thiol-functionalized crosslinkers, respectively. The degree of functionality or the ratio of polyester to crosslinker used allows control over the particle size formed (from 20 nm to almost 1 µm in the case of the thiol-ene based system) (Figure 2).[23] Such precise control over particle properties is challenging and

thus this system provides exciting potential to offer fundamental insights into polymer carriers and their interactions in biological systems. Related work by Silvers et al. demonstrated the synthesis of polyesters with both alkyne and alkene functionality, allowing thiol-ene and CuAAC to be used in combination for cross-linking and functionalization.[24] The polyester backbone could be potentially used to tune the degradation by using different combinations of polyester and crosslinker. Later work by Harth and coworkers investigated the formation of similar particles using poly(carbonate); these were of interest as they had slower degradation rates in biological conditions and easier side group modification.[25] Harth et al. also demonstrated that polyester particles could successfully be loaded with a cancer therapeutic and then targeted toward a specific tumor with a response to a radiation trigger, achieved by functionalization with a GIRLRG peptide.[26]

A related technique was recently reported based on alkyne-modified polylactides. In this system, a miniemulsion was formed based on the modified polylactide, and then the droplet was cross-linked by a bifunctional azide crosslinker.[27] An interesting facet of this work was the use of paclitaxel (a well-known cancer drug) as the crosslinker, thus allowing cross-linking and drug loading to be achieved in one simple step. Glyconanocapsules were also synthesized using a step-growth polymerization between 6,6'-diazido-6,6'-dideoxysucrose and bis(propargyloxy)butane in an oil-in-water miniemulsion. Precise control over size is quite challenging using this approach, but could potentially be improved by the efficiency of cross-linking with microwave irradiation (98% conversion after 30 min).[28] It has also been demonstrated polylactide particles could be formed by synthesizing amphiphilic block copolymers using CuAAC and then self-assembly into polymer micelles.[29]

Another type of degradable delivery system that holds promise for therapeutic applications is based on biodegradable poly(alkyl cyanoacrylate) (PACA). Currently in phase III clinical

trials, doxorubicin-loaded PACA nanoparticles have shown improved survival and safety compared to the standard treatment for patients with multidrug resistance hepatocarcinoma.[30] In such delivery systems, it is important that the surface chemistry can be readily functionalized to allow both stealth and targeting capabilities. In recent work, this was achieved by modifying azide functional monomers with specific targeting or stealth components using CuAAC and then combining them within the nanoparticle formulation.[31]

Other researchers have also utilized click chemistry to synthesize bio-hybrids. There are many excellent reviews on these new and emerging polymers[32,33] and thus this will not be focused on in this article. However, the ability of click techniques to combine varied building blocks has opened up the possibility for many innovative and highly functional materials, ranging from micelles and polymersomes through to polymeric or virus particles. An interesting example was recently reported on the assembly of responsive polypeptide polymersomes, obtained by grafting a pH-responsive poly(2-aminoethyl methacrylate hydrochloride) onto a hydrophobic poly(L-glutamate) backbone.[34] It was shown that the polymersome diameter could be varied with pH due to the responsive nature of the polymer side chain, from 150 nm once formed at pH 7.4 to 500 nm at pH 5.5. The polymersomes could be successfully loaded with both doxorubicin (DOX) and model DNA. However, in the case of DOX, release was most efficient at pH 7.4 rather than the acidic pH, which is the reverse of what would be required in most delivery applications. Potentially, cross-linking could be used as an approach to control this release. In another system, Schatz et al.[35] synthesized a simple glycoprotein analogue by coupling an alkyne-modified dextran to an azide functional polypeptide, poly(γ-benzyl-L-glutamate) (PBLG), using CuAAC. This copolymer was found to self-assemble into small polymersomes (45 nm). In a related system, Schatz and coworkers also synthesized a block copolymer of poly(γ-benzyl-L-glutamate) (PBLG) and hyaluronan (HYA) using CuAAC, which formed uniform polymersomes.[36] These materials were also successfully

loaded with doxorubicin and this cargo was stably retained within the polymersomes for several months (10% loss at 4 ºC). Later work demonstrated galactose-containing block copolymers could be used to form self-assembled structure ranging from worm-like to spherical micelles by tuning either composition or assembly conditions.[37] Another approach involved CuAAC coupling of two maltoheptaosyl oligosaccharides where one component was hydrophobic due to the acetylation of the hydroxyl moieties.[38] Highly uniform micelles were formed (30 nm in diameter) which were readily degraded by model enzymes.

The use of biologically derived particles has also generated significant interest for the synthesis of delivery systems due to their controlled size and inherent functionality. There has been a number of elegant studies demonstrating how virus particles can be used as templates for tunable polymer scaffolds. In one such study, virus particles formed from 180 copies of the coat protein bacteriophage QB were modified with azide functionality and then stabilized by alkyne modified poly(2-oxazoline).[39] The coating was tuned by using polymer with single or multiple alkyne functionalities and also by the ratio of polymer to virus particles, allowing films of up to 5 nm to be added to the structure. Another interesting strategy is to combine proteins with synthetic polymers through the use of click chemistry. One study investigated coupling bovine serum albumin (BSA) with poly(styrene) (PS) using CuAAC. It was found the addition of the PS caused the structure to aggregate into micelles where the protein formed the outer shell.[40,41] One of the attractive features of this approach is the potential to incorporate catalytically active proteins on the surface of the assembled structure.

Recently, there has been increasing interest in controlling the shape of delivery systems to influence their biological interactions and biodistribution.[42,43] Early work by Discher and coworkers demonstrated that wormlike micelles of poly(ethylene oxide) amphiphilic copolymers exhibited ten times longer plasma circulation than the corresponding spherical

micelles.[44] One approach to design wormlike structures is to graft multiple end functional polymers onto a comb-like polymer backbone. One such system used CuAAC to graft poly(e-caprolactone) (PCL) -b-poly(ethylene oxide) onto an azide functional poly(methacrylate).[45]

3. Degradable Delivery Systems Using Responsive Components

Another important strategy to synthesize degradable polymeric carriers is to use building blocks where at least one is cleavable with specific stimuli. This is an attractive approach for the design of therapeutic delivery systems as it allows degradation to be triggered at a specific location (e.g., inside a cell). Recently, this technique has been utilized by Caruso and coworkers to synthesize a range of polymer capsules with low-fouling[46] or responsive capabilities.[47,48] The capsules were designed by assembling an alkyne functional polymer through LbL assembly and then stabilized by using a bisazide crosslinker. This crosslinker could be designed with a responsive component, including disulfide functionality, which is cleavable under reducing conditions inside the cell cytoplasm. In one study, alkyne-modified poly(2-diisopropylaminoethyl methacrylate) (PDPA) was used to assemble polymer capsules. PDPA is of interest for delivery systems as it is a pH-responsive polymer that is sensitive to the variations in cellular pH. At pH < pK_a (6.4) PDPA undergoes a charge-shifting transition from hydrophobic (water insoluble) to hydrophilic (water soluble) due to protonation of tertiary amine groups. The PDPA capsules were stabilized by a second bioresponsive component, a disulfide cleavable linker. Disulfides moieties are commonly used in delivery systems as they are relatively stable in the bloodstream but degrade once within a cell. The PDPA capsules were found to respond synergistically to pH and redox triggers, allowing precise control over therapeutic release.[47] The combination of two responsive mechanisms within the system was fundamental as it allowed the capsules to have high stability in conditions which mimic the bloodstream, but degrade very rapidly once in endosomal conditions, even in the presence of small amounts of glutathione (GSH) (0.1 mM). This work

was later extended to show degradation rate could be further tuned by varying the amount of crosslinker used and thus the stability of the capsule wall (Figure 3).[48] Micelles with the capability for dual response have also been designed by incorporation of disulfide and o-nitrobenzyl methyl ester groups within the polymer building block.[49] These micelles responded rapidly to UV light but more slowly to disulfide cleavage agents.

The LbL approach outlined above was also used to design a low-fouling polymer capsule based on poly(*N*-vinyl pyrrolidone).[50] It was demonstrated these capsules could be loaded with model DNA and their degradation tuned by the choice of crosslinker.[51] Later work involved the functionalization of these capsules using excess click functionality with a model antibody for targeting colorectal cancer (HuA33 Ab).[46] These capsules showed specific binding to cells even when the target cell was present as less than 0.1% of the total cell population. In related work, capsules based on poly(ethylene glycol) were functionalized with a targeting ScFv, to selectively bind to blood clots.[52] This approach was also used to design poly(L-glutamic acid) PGA capsules with tunable degradation.[53,54] It was demonstrated a PGA-DOX conjugate could be incorporated within the capsule structure and this conjugate was active upon release within the cell.[53] Later work showed PGA capsules loaded with either DOX and paclitaxel could partially overcome multidrug resistance in a model cell line.[55]

A related technique was used by Zhao and coworkers to form stabilized surfactant micelles.[56,57] They demonstrated the formation of cross-linked micelles using a range of responsive crosslinkers, with good retention of hydrophobic cargo over a period of six months.[57] It was found that the cargo was released extremely rapidly when the crosslinker was cleaved (<1 min) due to the high stress present within the micelle structure below the critical micelle concentration. An alternative delivery system with responsive properties was developed recently by Dam and Caruso[58] based on cross-linked poly(rotaxane).

Poly(rotaxanes) are formed by the self-assembly of poly(ethylene glycol) within the core of a cyclodextrin. In this system, the structure was stabilized by a click functional capping group. This cap was connected through a responsive disulfide linkage allowing release of the structure in the presence of disulfide cleavage agents. The poly(rotaxanes) were stabilized on a silica surface using CuAAC and then cross-linked through further disulfide cross-linking. Capsules formed from these films could be loaded with DOX and degraded in 90 min at 37 °C in the presence of 5 mM GSH.

Katayama and coworkers developed an elegant approach to gene delivery recently based on peptide-modified linear poly(ethylene imine) (PEI) (Figure 4).[59] The peptide sidechain was modified with the substrate for protein kinase Cα (PKCα) which underwent phosphorylation when in the cytosol. This allowed triggered release of DNA, which was complexed to the polymer-peptide conjugate and subsequent expression. This study demonstrated expression could be switched on or off by the choice of peptide sequence within the PEI structure. This control over expression was demonstrated both in vitro and in vivo. This approach relies on using in-built biological intelligence in the form of the peptide to tune material properties. The use of specific biological molecules within a larger synthetic material is an exciting area of research as it enhances the controlled response to biological conditions. It is expected we will see many emerging delivery systems based on this idea in the future.

4. Future Perspectives

The development of new materials with enhanced properties depends on harnessing versatile and intelligent chemistries such as click chemistry-based techniques. However, the design of polymer carriers for biomedical applications relies on efficient and controllable degradation of these materials under specific biological conditions. Thus, the synthesis of polymeric carriers using click chemistry relies on combining this powerful technique with a range of degradable

or responsible building blocks. Already, there has been interesting work in this area with the use of tunable crosslinkers and a range of degradable biopolymers, although there are still a number of challenges to be addressed. Targeting to specific cells, trafficking within a cell and control over degradation rate are all areas that still require ongoing research. Recent work has demonstrated that click chemistry can be used to post-functionalize particles/carriers with high efficiency.[8,60] The ability to control the properties of therapeutic delivery systems should lead to a greater understanding regarding the interaction of these materials with biological systems. This understanding is integral to obtaining eventual benefits from these materials in biomedicine. As highlighted in this article, the versatility of these click processes allows components to be designed with an almost limitless array of responsive and degradable components. It is expected that click-based delivery systems will continue to push the boundaries of what is achievable from nanoengineered materials and thus provide potential for the next generation of therapeutic delivery systems.

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Figure 1. (A) Thermoresponsive, reversible polymerization via hetero-Diels-Alder chemistry of a pyridyl dithioester trilinker with a bis(cyclopentadienyl) poly(methyl methacrylate) dilinker (TFA = trifluoroacetic acid).[15] (B) Reversible cross-linking by Diels-Alder chemistry between fulvene-functionalized glycol-based polymers and a cyanfumarate dilinker at room temperature.[16] (A) Reproduced with permission.[15] Copyright 2012, Wiley-VCH. (B) Reproduced with permission.[16] Copyright 2009, Wiley-VCH.

Figure 2. Scheme of nanoparticle formation using thiol-ene (left) and CuAAC (right) click cross-linking. TEM images of nanoparticles assembled using (A) thiol-ene click cross-linking prepared from a 12 h reaction with 5% allyl-functionalized polymer and 3 equivalent thiol to allyl ratio, (B) thiol-ene click cross-linking prepared from a 12 h reaction with 12% allyl functionalized polymer and 1 equivalent thiol to allyl ratio, (C) CuAAC cross-linking prepared at 45 °C with 5% alkyne functionalized polymer and 4 equivalent azide to alkyne ratio, and (D) CuAAC cross-linking prepared at room temperature with 12% alkyne functionalized polymer and 2 equivalent azide to alkyne ratio. Reprinted with permission.[23] Copyright 2012, *Macromolecules*.

Figure 3. pH-responsive PDPA capsules synthesized via CuAAC using a redox-sensitive crosslinker (top). The degradation rate of PDPA capsules was tuned by controlling the degree of cross-linking from 65% to 98% by varying the amount of crosslinker added to the system (bottom). Reproduced with permission.[48] Copyright 2012, *ACS Nano*.

Figure 4. (A) Illustration of LPEI(S)-pDNA polyplex cancer cell-targeted gene delivery system, responsive to intracellular protein kinase Cα (PKCα), which is hyperactivated in various cancer cells and tissues. LPEI(S) and LPEI(S) are linear poly(ethyleneimine)-peptide conjugate synthesized via CuAAC, with LPEI(A) as the control. (B) Phosphorylation reactions of LPEI-peptide conjugates and pristine peptide, implying sufficient reactivity of the grafted peptides on LPEI(S). (C) Light scattering intensity (LSI) changes of polymer-pDNA polyplex after the addition of PKCα. (D) Direct injection of LPEI-pDNA polyplex into HepG2 cancer tissues or normal tissues. PPC(S) and PPC(A) are the previous gene carriers developed that are failed to respond to some cancer cell lines, used for comparison. Reproduced with permission.[59] Copyright 2012, American Chemical Society.

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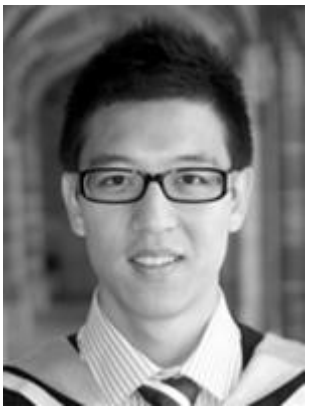

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The development of click chemistry has provided a versatile toolbox of building blocks for materials assembly, **allowing the design of next-generation materials for applications in biomedicine.** However, for the effective design of materials for therapeutic applications it is critical to achieve controlled or triggered degradation. This feature article highlights the development of degradable click carriers and their investigation in biological conditions.

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Design of Degradable Click Delivery Systems.