Intramolecularly Cross-linked Polymers. From Structure to Function with Applications as Artificial Antibodies and Artificial Enzymes

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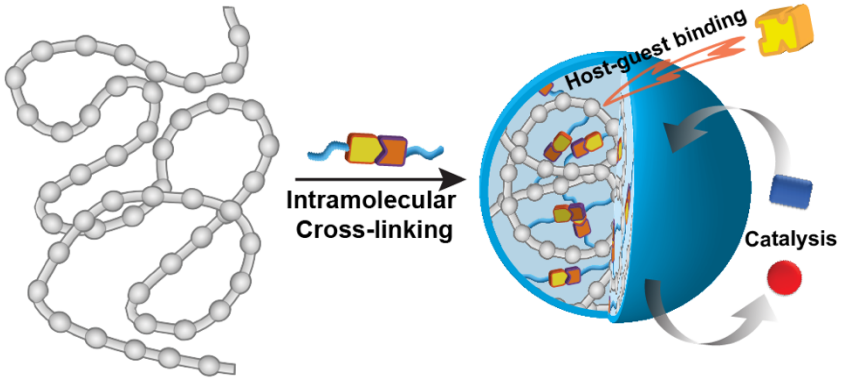
CONSPECTUS: The cross-linking of polymers significantly alters their physical properties, greatly expanding their everyday utility. Indeed, the polymeric networks resulting from linkages between polymer chains are found in everyday materials from soft contact lenses and automobile tires to enamel coatings and high-performance adhesives. In contrast, intramolecularly cross-linked polymers have received far less attention until recent years in large part because they are synthetically more challenging to prepare. In this Account we trace our own efforts to develop the chemistry of intramolecularly cross-linked macromolecules, starting with dendrimers. Dendrimers provided an excellent starting point for investigating intramolecular cross-linking because they are single molecular entities. We showed that the end-groups of dendrimers can be extensively cross-linked using the ring-closing metathesis reaction, and that the discrete structure of the dendrimer provides unique opportunities for characterizing the number and location of the cross-links as well as some physical properties of the macromolecule such as its size and rigidity. Increasing the number of ring-closing metathesis reactions correlated with a reduction in size and an increase in rigidity. The general strategy applied to dendrimers was extended to star polymers and hyperbranched polyglycerols. Each of these macromolecules has a core or an initiating group from which the branches emanate. Linking the end-groups or branches of these polymers presents a unique opportunity to chemically remove the core of the cross-linked macromolecule in a process that is reminiscent of that used to produce covalent molecular imprinted polymers. Recognizing this analogy, we sought a compelling application for cross-linked dendrimers, the first example of unimolecular imprinting, where a single polymer contains a single molecular imprint. The quality of the imprinting was mixed but pointed to an alternative general strategy for molecular imprinting in polymers. The effort also focused attention on synthetic antibodies and the general biomimicry provided by this class of macromolecules. Indeed, cross-linking of polymers either covalently or noncovalently bears a loose resemblance to the folding of proteins into defined three-dimensional shapes. The synthesis and study of cross-linked linear polymers, often called single-chain nanoparticles (SCNP), has emerged as a very active area of research in the past few years. Our experience with the cross-linking of branched polymers combined with an interest in performing organic synthesis within living cells led us to develop copper-containing SCNP as artificial clickases. These polymeric clickases exhibit all the hallmarks of enzymatic catalysis. One clickase containing a polyacrylamide backbone performs low concentration copper-assisted alkyne-azide click reactions with unprecedented rates. Another performs click reactions within living cells. Other organic transformations can be performed intracellularly, some of the most advanced SCNP engaging in concurrent and tandem catalysis with a naturally occurring biocatalyst. By tracing our own efforts, this Account provides a few entry points into the broader literature and also points to both the challenges remaining and overall promising future envisioned for this unique class of functional macromolecules.



1. INTRODUCTION

Chemical cross-linking has been widely used to alter the physical properties of polymeric materials, the vulcanization of rubber being a prototypic example. By linking polymer chains through chemical linkages, materials gain a more rigid structure and potentially a better-defined shape. Numerous functional materials have been developed using this strategy, including thermosets, rubbers, and hydrogels, which have had an incalculable impact on industry and our everyday lives.

The chemical cross-linking of polymers has not been limited to the bulk materials but has also led to create micro- or nano-sized organic particles that encapsulate molecules of interest and deliver them to specific biological targets.[1,2] These cross-linked particles are soluble and have defined sizes, shapes, and chemical composition. However, most such organic nanoparticles contain multiple polymeric chains. In recent years there has been an increased interest in cross-linking single macromolecules with covalent or noncovalent linkages between different polymer segments. Not only are these intramolecularly cross-linked polymers easier to characterize, study, and tune, they are biomimetic, loosely resembling the folding and disulfide cross-linking in proteins.

A landmark paper by Kuhn and Majer in 1956 described how intramolecular cross-linking of polymers would produce a significant contraction.[3] Other early examples[4,5] date back to the 1960s and reports of intramolecularly cross-linked polymers continued intermittently over the next half-century. We entered the field with a report describing the complete ring-closing metathesis (RCM) reaction of a third-generation dendrimer containing 24 homoallyl end-groups.[6] As described below, the dendrimer RCM reaction was a major step forward, allowing the cross-linking process to be monitored and characterized in a way not previously available. Since that time, considerable work by others has focused on cross-linking linear polymers. Reviews covering chemical methods to cross-link linear, brush, star, and hyperbranched polymers as well as dendrimers have been published.[7-9]

This Account traces our research into intramolecularly cross-linked polymers starting in 1999.[6] As shown in Figure 1, the focus is on synthesis, cross-linking methodologies, how cross-linking alters the polymer properties, and, finally, applications that include binding and catalysis. Before commencing, it is important to acknowledge in a highly abbreviated and selective fashion other earlier efforts that were influential in our work. Hawker and coworkers reported an intramolecularly cross-linked polystyrene by using a heat induced benzocyclobutene dimerization reaction.[10] Meijer and Palmans developed water-soluble polymers that fold via intramolecular cross-linking created by hydrogen bonding.[11] Lemcoff and coworkers reported single-chain organometallic nanoparticles formed by intramolecular cross-linking

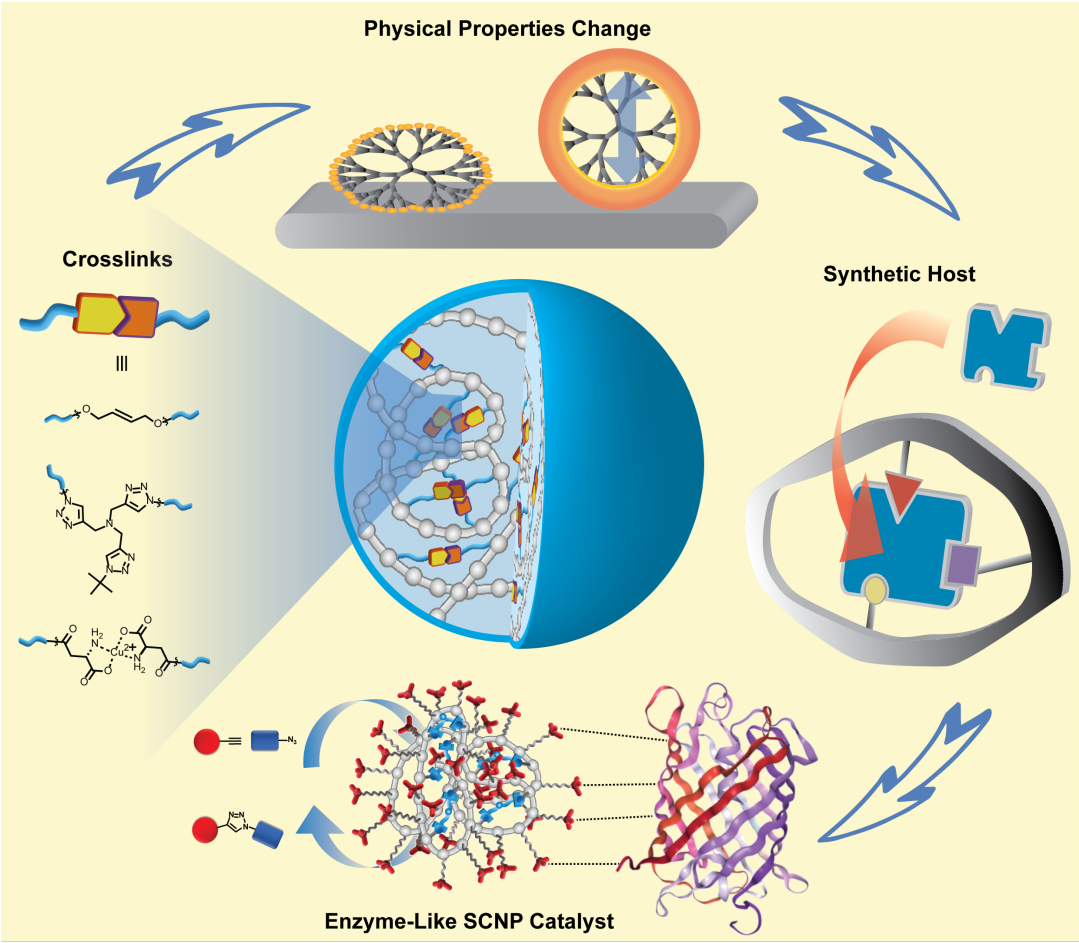


Figure 1. Illustration of the synthesis, properties and applications of intramolecularly crosslinked polymers.

polycyclooctadiene using rhodium(I) ion complexation.[12] The reviews cited above provide a fuller account of the work of others.

2. INTRA-MACROMOLECULAR REACTIONS

2.1. Covalently Cross-linking Dendrimers and Related Polymers.

To stably cross-link a polymer, it is best to use either irreversible covalent bonds or dynamic (reversible) covalent bonds. Irreversible covalent linkages allow the polymeric nanoparticle to maintain their cross-linked structure under different stresses and in a wider range of environments. This type of cross-linking has been achieved using multiple methods, including alkene metathesis, the click reaction, amide coupling, and photodimerization.[7] Dynamic covalent bonds that can be reversibly broken and reformed provide the polymers with the flexibility to change its three-dimensional structure under certain physical and chemical conditions.[13,14] By taking advantage of this reversible structural transformation, stimuli responsive materials have been developed.[15-17]

Our group began work in this area by studying the RCM reaction of Fréchet-type dendrimers containing homoallyl groups.[6] Each RCM reaction connects two terminal alkene groups with release of a molecule of ethene. A cross-link is defined as a small region in a macromolecule from which at least four chains emanate, and formed by reactions involving groups on existing macromolecules.[18] This definition applies well to linear polymers, but does not address analogous reactions in other polymer architectures such as dendrimers. Nonetheless, if one considers the full AB_2 monomer structure of the dendrimer, the term is appropriate, and functionally the RCM product acts as a cross-link.

The advantage of studying dendrimers arises from their unique structure. Dendrimers are monodisperse, tree-like macromolecules with a discrete chemical structure containing a core unit from which the repeating branches emanate.[19-24] In dendrimer **1** we selected a core with three hydrolyzable ester bonds (Figure 2a) to which Fréchet-type dendrons[25] were attached, each containing homoallylether end-groups.[6] The RCM of **1** was performed with Grubbs first-generation catalyst,[26] and followed by proton nuclear magnetic resonance (^{1}H NMR) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Despite the broadened peaks, the alkene methine proton at δ 5.86 ppm diminished. Convincingly, the MALDI-MS spectra, which could be followed over time shows progressive loss of ethylene units, ultimately reaching nearly full cross-linking with loss of 11 to 12 molecules of ethylene (Figure 2c). After the cross-linking, the core unit in **2** was removed through ester hydrolysis by using KOH, and the dendritic structure was found to remain intact demonstrating that at least one cross-link is formed between the three dendrons of **1**.

The representation of the cross-linked dendrimer in Figure 2a as having the alkene groups on the outside is for convenience. There has been debate about whether dendrimer end-groups are densely packed on the periphery or extensively folded back and distributed throughout the polymer.[27-29] Although the results in Figure 2 do not indicate which model is more appropriate, the discrete and highly branched structure of dendrimers do provide a unique opportunity to examine whether short or long-range cross-links are preferred. This is important because the properties of cross-linked polymer materials are defined not only by their chemical composition but also by the structural topology/connectivity including loop formation and structural imperfections.[30,31] Therefore, dendrimer **3** was synthesized as a simple model to study the preference for short

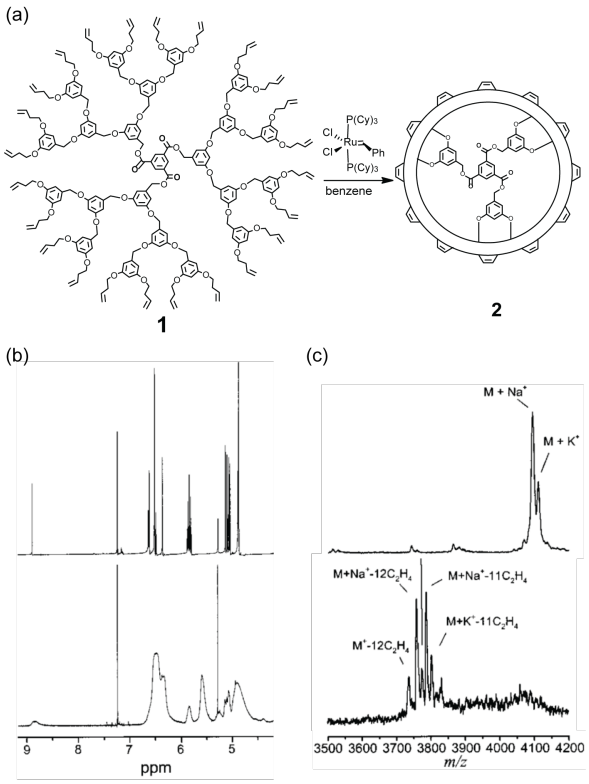


Figure 2. Synthesis of intramolecularly crosslinked dendrimers. (a) Dendrimer crosslinking through the Grubbs catalyst mediated RCM reactions. (b) and (c) NMR and MALDI spectra of dendrimer **1** (above) and crosslinked dendrimer **2** (below). Adapted with permission from ref. 6. Copyright 1999 American Chemical Society.

and long-range RCM reactions and how forming one cross-link might impact the preference for the next.[32]

As shown in Figure 3a, four types of cross-links with increasing loop size are possible and labeled type **a**-**d**. From a purely statistical perspective, the ratio of type **a** : **b** : **c** : **d** would be 1:2:4:8. However, the actual outcome depends on a combination of statistics and the kinetics and, potentially thermodynamics of each type of RCM reaction. Before studying **3**, we investigated these preferences using three small dendrons (**4a**-**c**). It was found that dendron **4a** did not undergo the RCM reaction, indicating type **a** cross-linking to be highly unfavorable (Figure 3b).

The RCM reaction performed on **4b** resulted in three products with about 48% of the product showing partial cross-linking (see Figure 3b). Considering that larger dendrimers underwent full cross-linking, this observation suggests difficulty in building two type **b** cross-links within one **4b** dendron. To compare type **b** and **c** cross-links, fully cross-linked dendrons **5a**, **5b** and **5c** were synthesized through different methods (Figure 3c). Thus, **5a** was obtained from the standard RCM of dendron **4c**. Dendron **4d** with two type **b** cross-links was prepared and underwent RCM to give **5b** and dendron **5c** containing four type **b** cross-links was prepared from **6c**. All three cross-linked dendrons, **5a**, **5b**, and **5c** have the same chemical components but comparison of their ^{1}H NMR spectra is revealing (Figure 3c). Thus, it is clearly seen that **5a** contains mostly type **c** cross-links given the negligible peak intensity between δ 5.9-6.2 ppm. Therefore, the longer-range type **c** connection is more favorable than type **b**.

Finally, the preference for type **d** cross-links was examined by partially cross-linking dendrimer **3** using a short RCM reaction time, followed by hydrolysis, and analysis of the resulting fragments. The relative percentage of di- and mono-dendron product was measured

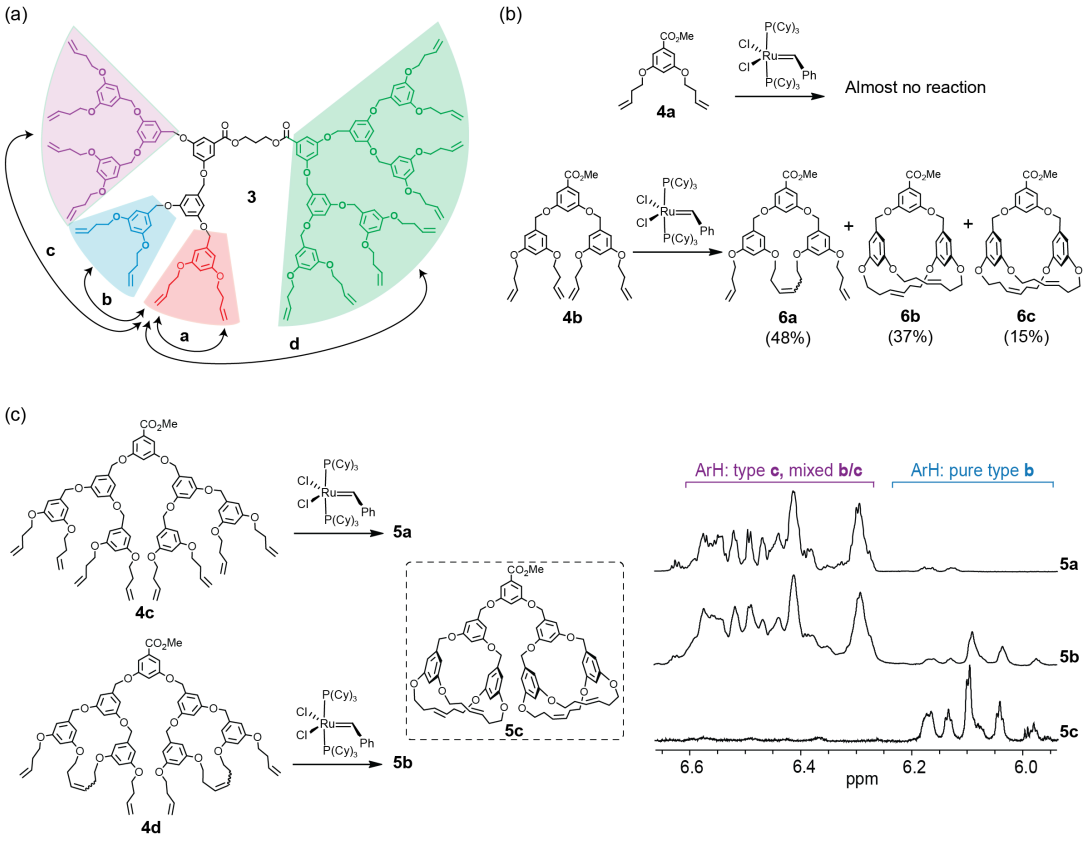


Figure 3. (a) Four possible RCM connections in **3**. (b) Model RCM reactions in smaller dendrons. (c) Synthesis and ^{1}H NMR spectra of crosslinked dendrons **5a**, **5b** and **5c**. Adapted with permission from ref. 32. Copyright 2004 American Chemical Society.

by SEC, which provides an estimate of the preference for inter- vs. intra-dendron RCM. It was found that type **d** cross-links were slightly more favorable than type **c**. Putting all of the data together led to the conclusion that the relative preference of cross-links observed is type **d** ≥ type **c** > type **b** >> type **a**, indicating that larger loops are the favored cross-links. These preferred connections may reflect kinetic or thermodynamic factors or a mixture of both because the RCM cross-linking may undergo dynamic exchange of cross-links.[33,34]

In collaboration with Rainer Haag, we extended this chemistry to polyglycerol dendrimers and hyperbranched polymers.[35-37] The former are an ideal choice for RCM cross-linking because in their iterative synthesis each generation proceeds through an intermediate with allyl ether end-groups. The cross-linked polyglycerol shell was shown to surround a perylenediimide fluorophore and be made water-soluble by dihydroxylation.[38] Finally, we also showed 4- and 8-armed star polymers with porphyrin cores and alkene-containing polystyrene arms could undergo RCM cross-linking.[39]

2.2. Covalently Cross-linking Linear Polymers.

In the past few years, there has been a broad and growing interest in cross-linked linear polymers, which have become known as single-chain nanoparticles (SCNPs).[7-9,40] Linear polymers are less compact than dendrimers and generally easier to prepare, while at the same time offering greater versatility in structure and function. For these reasons, we applied the RCM reaction to cross-link single-chain polymers with the goal of developing a practical synthesis of fluorescent water-soluble organic nanoparticles with a single reactive group for conjugation.[41] The parent polymers used were prepared through ring-opening metathesis polymerization (ROMP) using Grubbs 3rd generation catalyst.[42,43] Polymer **7** was post-functionalized with terminal alkenes by treating with tri-*O*-allyl-TRIS (**8**).

The intramolecular cross-linking was subsequently performed on **9** by using the RCM reaction mediated with Grubbs 1st generation

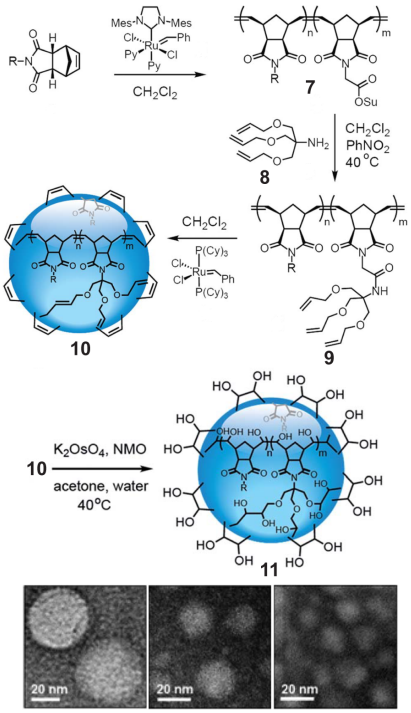


Figure 4. Synthesis and TEM images of SCNP **11** using a sequential ROMP and RCM process. Adapted with permission from ref. 41. Copyright 2014 Royal Society of Chemistry.

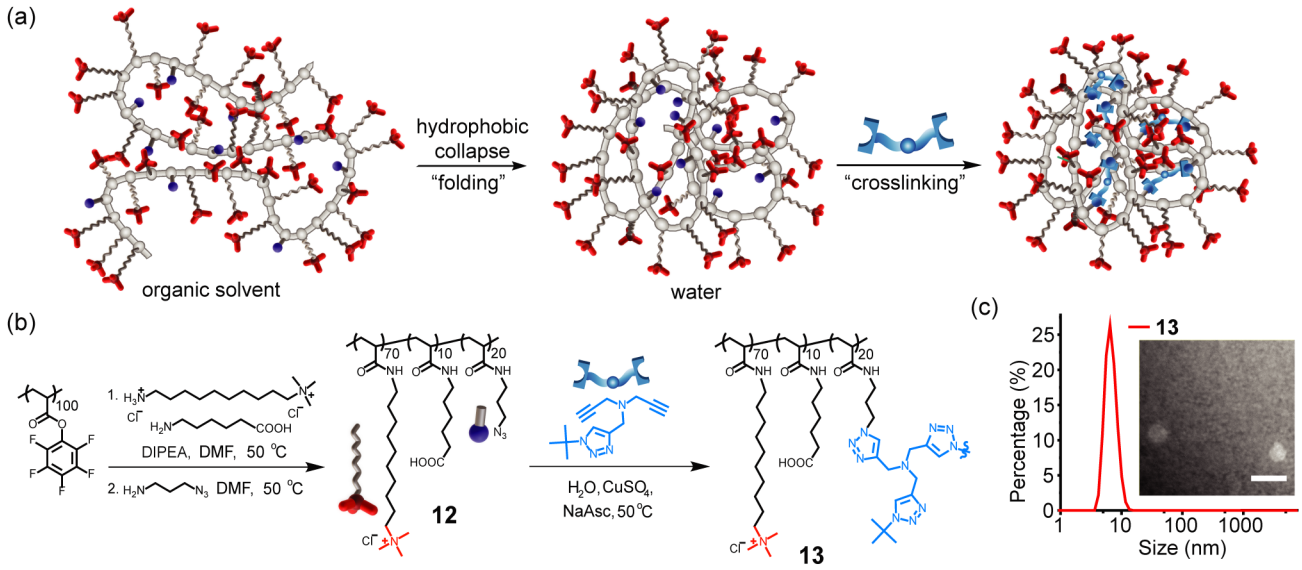


Figure 5. Preparation and characterization of SCNP **13**. (a) Illustration of SCNP preparation using a "folding and crosslinking" strategy. (b) Two-step conversion of poly(pentafluorophenyl acrylate) to SCNP **13**. (c) DLS data and TEM image (inset) of SCNP **13**. Scale bar = 20 nm. Adapted with permission from ref. 46. Copyright 2019 American Chemical Society.

catalyst at low concentration. The nanoparticle was made water-soluble by dehydroxylating the alkene groups using *N*-methyl-morpholine-*N*-oxide (NMO) and K_2OsO_4.[44] By varying the initiator to monomer ratio and using a chain-transfer agent to cap the end of the polymer, various sizes of SCNP could be prepared each carrying a single reactive functional group (see TEM, Figure 4). Likewise, the synthesis in Figure 4 was readily adapted to fluorophore-tagged monomers and the resultant fluorescent SCNP photobleached more slowly than free fluorophores.[45]

A major limitation in intramolecular cross-linking processes is the need for high dilution to reduce the probability of intermolecular reaction. In a recent effort to increase scalability we reported a new strategy called "folding and cross-linking" to prepare covalently cross-linked SCNP (Figure 5).[46] Polyacrylamide **12** was synthesized by post-functionalizing poly(pentafluorophenyl acrylate). In water, **12** adopts a unimolecular micelle-like structure through hydrophobic collapse. By using a hydrophobic diyne cross-linker, **12** was cross-linked using the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction. This strategy appears to inhibit aggregation and, thus, interparticle reaction and allows cross-linking at 0.2 mM which is nearly 100-fold higher than that used to prepare **10**.[41] An earlier, but related approach was applied to the RCM of dendrimers and involved moving the alkene groups inside the dendritic shell.[47] The intermolecular reactions are disfavored by the requirement for dendrimer interpenetration. Using this strategy RCM cross-linking reactions could be achieved at concentrations as high as 1 mM, which is 100-fold higher than that used with dendrimer **1**. Barner-Kowollik very recently reviewed progress in improving scalability in SCNP synthesis.[48]

2.3. Noncovalently Cross-linked Polymers.

The use of non-covalent interactions is another appealing method to cross-link polymers. The typical interactions used are hydrogen bonding, host-guest interactions, metal coordination, as well as hydrophobic collapse in water. For example, Palmans and Meijer used the benzene-1,3,5-tricarboxamide moiety to fold single-chain polymers by hydrogen bonding in both organic solvent and water.[11,49] Scherman reported supramolecularly cross-linked SCNP by preparing poly(*N*-hydroxyethylacrylamide) polymers with naphthalene- and viologen-containing side-chains, guest molecules that form a tight heterocomplex with cucurbit[8]uril.[50] Compared to dynamic covalent cross-linking, non-covalent interactions are typically highly reversible with low barriers to complexation. The folding conformation is therefore dependent on the external conditions, allowing controlled folding and unfolding and, thus, the development of stimuli responsive systems.

Inspired by the work of Lemcoff,[12] in 2016 we reported a new type of catalytic SCNP that is cross-linked by the copper ion coordination.[51] Polymer **14** bearing water solubilizing imidazolium groups and copper-coordinating amino acid groups was synthesized through ROMP polymerization followed by deprotection and copper coordination (Figure 6). The amino acids are known to form stable coordination complexes with both Cu(I) and Cu(II) in a 2:1 stoichiometry. Therefore, polymer **14** was cross-linked in water at 5 μM by adding 0.5 eq. of $CuSO_4$ relative to the amount of amino acid units. The coordination between the amino acids and copper ions intramolecularly cross-linked the polymer and afforded nanoparticle

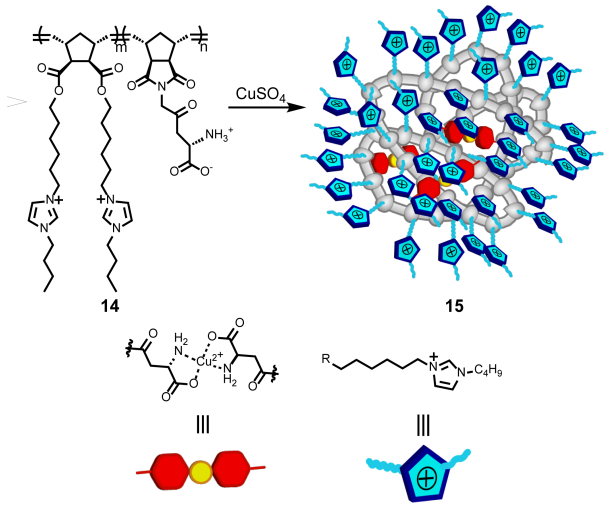


Figure 6. Schematic illustration of the synthesis of copper crosslinked SCNP. Adapted with permission from ref. 80. Copyright 2018 American Chemical Society.

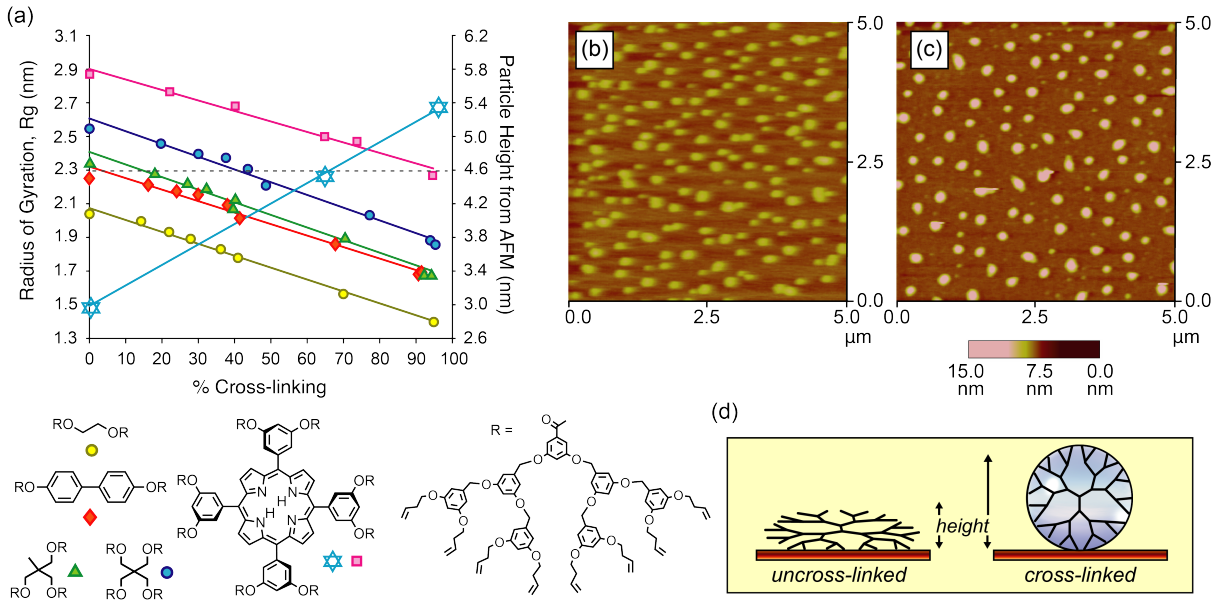


Figure 7. (a) Structure of five dendrimers used and plots showing controllable level of RCM linearly correlates with R_g. Particle height measured by AFM for porphyrin dendrimer increases with crosslinking. Horizontal line indicated dendrimers with same R_g but different degrees of crosslinking. (b) AFM images of porphyrin dendrimer before crosslinking. (c) After crosslinking. (d) Schematic model of how crosslinking affects AFM height measurement. Adapted with permission from ref. 55. Copyright 2004 American Chemical Society.

15, which was subsequently characterized using atomic force microscopy (AFM) and dynamic light scattering (DLS).

3. PHYSICAL PROPERTIES OF CROSS-LINKED POLYMERS

The intramolecular cross-linking of polymers significantly changes the chain arrangement, rigidifies the interior scaffold structure, and, as predicted by Kuhn decades ago, leads to a marked decrease in size.[3,4] Our group was one of the first to study the size reduction and, indirectly, the increased rigidity. Various groups have also studied the physical properties of intramolecularly cross-linked polymers. For example, Meijer and Guan utilized AFM to nanomechanically unfold a single SCNP to understand the physical forces operating during the noncovalent folding and unfolding process.[52] Diesendruck reported that covalently cross-linked polyacrylate SCNPs underwent mechano-chemically induced scission analogous to linear polymers, but with significantly lower loss in molecular weight.[53] Indeed, at 10 mol% cross-linking the polyacrylate with M_n 132 kD was nearly inert to a 100 min sonication suggesting enormous practical applications for minimizing polymer fragmentation (degradation) and thereby improving long-term polymer performance. The same group extended this work to Rh-π bonds showing mechanical breaking of the cross-links and their subsequent thermal repair.[54]

In 2004, we reported an extensive investigation of the controlled cross-linking of dendrimers via the RCM reaction.[55] Thus, various dendrimers were synthesized with different molecular weights and chemical structure, and intramolecularly cross-linked to different degrees by controlling the RCM reaction time. After the cross-linking, the radius of gyration (R_g) was found to decrease linearly ($R^2 > 0.98$) with increasing percentage of cross-linking (Figure 7a). Assuming a spherical shape and that the R_g values approximate the molecular radii, the polymer volumes after 95% cross-linking were calculated to be between just 33% and 39% of the original dendrimer volume. Given a less than 10% mass reduction in the RCM reaction, this size reduction indicates a significant increase in polymer density and presumably rigidity.

Mechanical property changes, in particular altered rigidity, were studied indirectly by AFM. As shown in Figure 7, the height of dendrimers was found to increase with increasing degree of cross-linking. The apparent inconsistency between the observed increase in AFM height and observed decrease in R_g is explained by the model shown in Figure 7d. Thus, the uncross-linked dendrimers are relatively flexible and flatten on the mica surface, whereas the highly cross-linked dendrimers behave as more rigid globular particles. We observed similar AFM results with the copper cross-linked SCNP described above, where it was found that the polymers adopt a more rigid, compact and uniform structure after the cross-linking.[51]

4. MOLECULAR IMPRINTED DENDRIMERS

The development of synthetic hosts that bind guest molecules with high binding affinity and selectivity continues to be an important goal of supramolecular chemists.[56,57] Synthetic hosts can be used to construct self-assembling materials and achieve selective recognition for chemical and biological sensing.[58] Among the various methods to create synthetic hosts, molecularly imprinting is one of the most conceptually appealing and extensively studied.[59,60] The pioneering report by Wulff used a template that was covalently linked to the reactive monomers.[61] Interest in molecularly imprinted polymers (MIPs) rapidly increased when Mosbach reported a simpler method using methacrylic acid and a crosslinking agent.[62] The overall process involves polymerizing around a template to create, following template removal, cavities with shapes and functional groups complementary to the template.

Several obstacles have limited the widespread application of MIPs, including the difficulty of template removal, slow mass transfer in and out of the polymer, and, most importantly, heterogeneity of binding sites resulting in a broad range of binding affinities. Efforts to improve mass transfer have involved multiple strategies, including making MIPs smaller.[63] The site heterogeneity remains a challenge and has been studied carefully, leading to a better understanding of

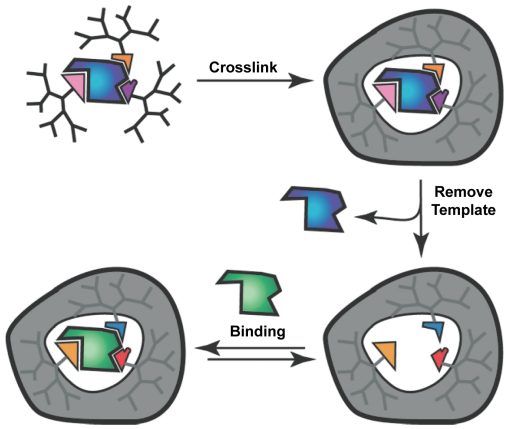


Figure 8. Schematic illustration of the monomolecular imprinting process, a potential strategy for preparing artificial antibodies. Adapted with permission from ref. 67. Copyright 2003 American Chemical Society.

the differences between covalent and noncovalent imprinting with regard to the range of binding affinities and populations.[64,65]

The intramolecularly cross-linked dendrimers described above are chemically homogeneous and exhibit rigid structures. These properties and the conceptual similarity between the chemistry and covalent polymer imprinting, inspired us to develop synthetic hosts by molecular imprinting inside dendrimers. The overall process is shown schematically in Figure 8. Beyond the imprinted dendrimer being soluble, there were multiple perceived advantages relative to traditional polymer imprinting. Perhaps most importantly, the single-polymer single-imprint approach means that separation or fractionation could solve the heterogeneity issue; well-formed imprints could be separated from poor ones.

In 2002 we reported a proof of concept approach to imprinting porphyrin structures into Fréchet-type dendrimers.[66,67] The synthetic procedure is illustrated in Figure 9a. The porphyrin cored dendrimer **16** was synthesized by esterifying the tetrakis-meso(3,5-dihydroxyphenyl)porphyrin unit with eight third-generation Fréchet-type dendrons[25] containing homoallyl end-groups. Dendrimer **16** was then intramolecularly cross-linked by RCM reaction by using Grubbs 1st generation catalyst, and the porphyrin core was subsequently removed quantitatively through ester hydrolysis. The hydrolysis resulted in eight carboxylic acid groups around the porphyrin cavity inside dendrimer **18,** and they enabled the hydrogen bonding to the guest molecules.

Imprinted dendrimer **18** did not bind the template but did complex the 2,6-dihydroxy isomer suggesting the hydrolysis reaction made the cavity too small to accommodate the 3,5-isomer. Dendrimer **18** also bound pyridylporphyrins, the apparent association constants, K_{app}, shown in Figure 9b. Overall, dendrimer **18** showed relatively strong and selective binding towards porphyrin derivatives with enough hydrogen bonding groups. The K_{app} values for H_2T(3-pyridyl)P and H_2T(4-pyridyl)P were found to be higher than for other guests, and this could be attributed to shape selectivity from the imprinting.

In the above effort, the porphyrin served as the photometric probe of the host-guest interaction. It would be more useful if the imprinted dendrimer could itself signal binding. To this end, we developed a molecularly imprinted dendrimer with chromogenic properties.[68,69] Cross-linked dendrimer **19** used a diamino butane template covalently but reversibly linked to two trifluoro-acetylazobenzene units, which in turn were linked to the same dendrons used in **18**. The azo dye is known to serve as a chromogenic reporter by reversibly forming carbinolamine adducts with amines.[70] After the removal of the diamine template, the molecular imprinted dendrimer **20** was tested with a library of amine and alcohol containing guest molecules, and the UV-vis absorbance was collected 30 s after the mixing. The significant selectivity observed was entirely consistent with imprinting (Figure 10). However, a careful kinetic study revealed that instead the selectivity arose from the faster binding of linear diamines that can undergo intramolecular base catalyzed addition.[71] Zhao has developed a very

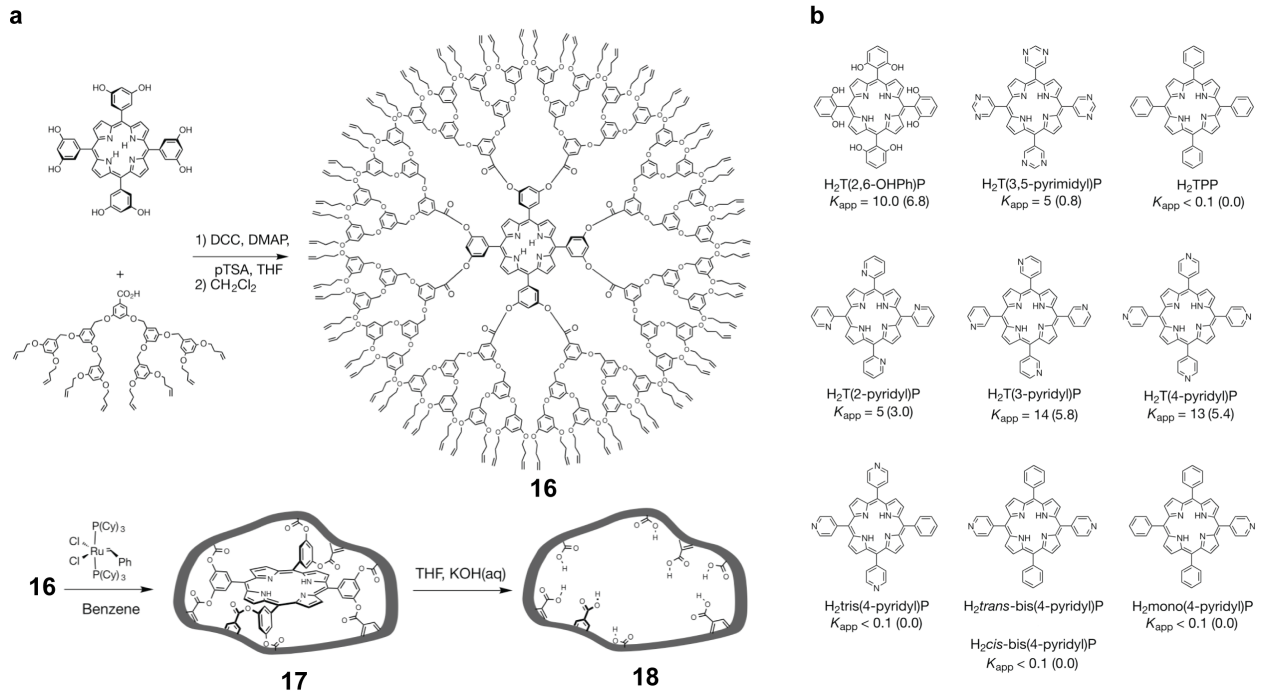


Figure 9. (a) Preparation of porphyrin imprinted dendrimer **18**. (b) Porphyrin guests used to probe binding pocket of **18** and binding constants in toluene or 5% (v/v) ethyl acetate/toluene. Adapted with permission from ref. 66. Copyright 2002 Nature Publishing Group.

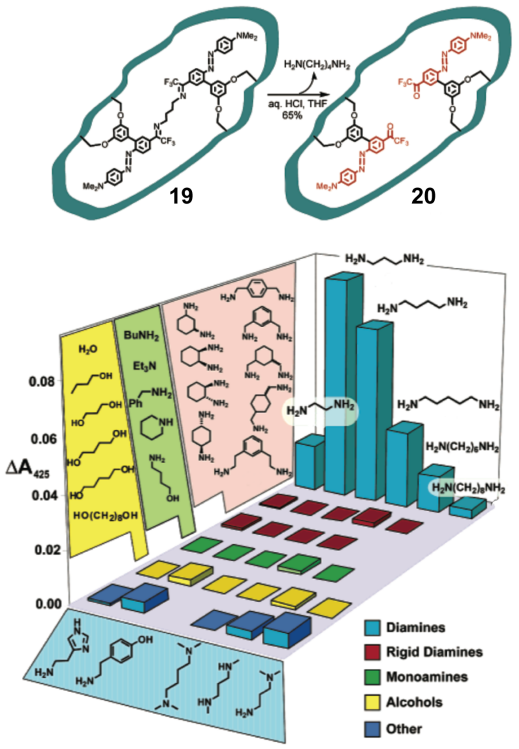


Figure 10. Synthesis of diamine imprinted dendrimer **20** and its selectivity profile. Adapted with permission from ref. 68. Copyright 2003 American Chemical Society.

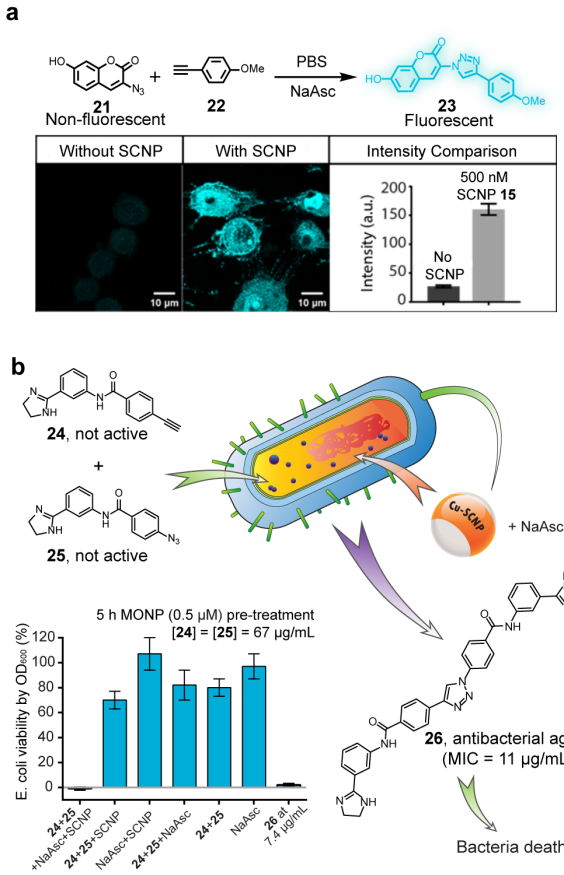


Figure 11. Intracellular CuAAC reactions. (a) Fluorogenic reaction inside H460 cells. The cells were incubated with 500 nM of **15**, and then **21** (100 μM) and **22** (100 μM). (b) Intracellular toxic agent synthesis inside *E. coli*. Adapted with permission from refs. 51 and 88. Copyright 2016 American Chemical Society and 2018 Royal Society of Chemistry.

promising alternative approach to nanoscale covalent molecular imprinting using cross-linked micelles.[72-74]

5. ENZYME-LIKE CATALYTIC SCNP

In nature, enzymes achieve their remarkable catalytic efficiency and selectivity using a precisely folded macromolecular peptide structure to bind substrates. Considerable progress has been made in combining transition-metal catalysts with macromolecular scaffolds including both proteins[75] and synthetic polymers.[76-78] Numerous researchers have drawn the analogy between the cross-linking of SCNP and protein folding as well as the enzyme-like behavior of SCNP catalysts. However, very few demonstrations of Michaelis-Menten kinetics had appeared[79] when we began to develop the catalytic SCNP described in the next section for bioapplications.

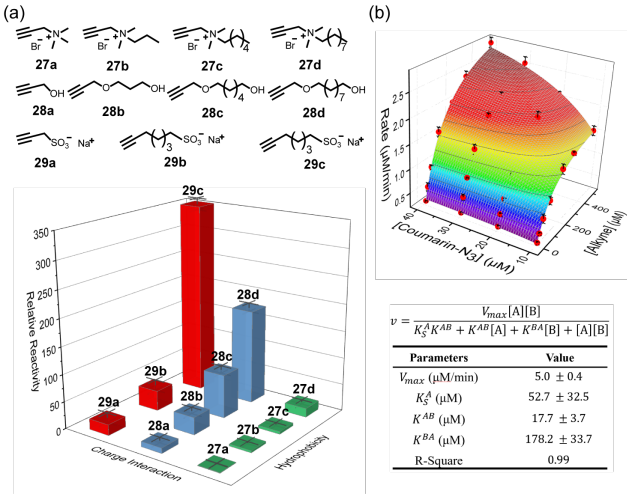


Figure 12. Catalysis profile of **15**. (a) Structures of alkyne substrates and their fluorogenic rates. (b) Random-sequential two-substrate enzyme kinetics fitting of **15** kinetics data and fitting parameters. Adapted with permission from ref. 85. Copyright 2018 American Chemical Society.

5.1. SCNP Catalyze the CuAAC Reaction Inside Living Cells

The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) or click reaction has emerged as one of the most useful and widely used methods of conjugation.[80,81] Copper-cross-linked SCNP **15** described above in Figure 6 was shown to perform a range of click reactions in >90% yield with just 50-100 ppm of copper, indicating the nanoparticle is highly efficient with a high turnover number.[51] Thus, using the known fluorogenic click reaction of 3-azido-7-hydroxycoumarin (**21**),[82,83] we showed triazole formation with *p*-ethynylanisole (**22**) not only occurred at micromolar concentrations, but also within live NCI-H460 and MDA-MB-231 cells. As shown in Figure 11a, strong fluorescence was observed inside cells provided **15**, both substrates and sodium ascorbate were present.

To demonstrate the potential for intracellular synthesis of a bioactive agent, the non-toxic alkyne- (**24**) and azide-containing (**25**) amidine compounds were incubated with *E. coli* after treating the cells with SCNP **15**.[51] The click product is a bisamidine-based antimicrobial agent (**26**).[84] Cell viability was 0% with all four components present (**15**, **24**, **25**, NaAsc), whereas in control experiments lacking one or more of the four, the bacteria showed activity.

5.2. Enzyme-Like Behavior of SCNP

Given the high activity of copper-containing SCNP **15**,[51] we sought to study it in more detail and especially to document potential

enzyme-like catalysis.[85] The fluorogenic click reactions between **21** and 11 different alkyne substrates were conducted in PBS buffer with low substrate concentration (10^{-5} M). As shown in Figure 12a, SCNP **15** exhibits considerable selectivity with two clear trends observed. First, there is a charge matching component with anionic alkynes reacting faster than neutral alkynes, which in turn, are faster than cationic alkynes. Overlaid on this electrostatic effect is a chain-length dependence consistent with faster click reactions for the more hydrophobic substrates. The importance of hydrophobic binding was further supported by pyrene uptake measurements and molecular dynamics (MD) simulations where the extent of guest binding within cationic SCNP **15** correlated with the reaction rate.

Direct experimental evidence for substrate binding was observed by saturation transfer difference (STD) spectroscopy and two-dimensional nuclear Overhauser effect spectroscopy (2D-NOESY). The alkyne substrate was found to bind to the aliphatic side-chain and imidazolium groups, not to the hydrophobic ROMP backbone. The binding and kinetic parameters were found by varying both substrate concentrations and fitting to a random-sequential two-substrate enzyme kinetics equation.[86] Saturation kinetics in both substrates and the excellent fit seen in Figure 12b provide strong evidence for enzyme-like behavior for SCNP **15**.

5.3. SCNP-Mediated Bioconjugation Using Proteins

The ROMP backbone of SCNP **15** was expected to contribute to the hydrophobic binding, but the NOESY data suggested a minimal role.[85] For this reason, we developed the polyacrylamide-based SCNP **13**, described above (Figure 5).[46] The backbone is more flexible allowing better chain folding. More importantly, the covalent cross-linking installed a tris(triazolylmethyl)amine ligand for Cu^{I}, which is analogous to the BTTAA ligand, developed by Marlow, Liu, and Wu.[87] BTTAA is arguably the fastest tris(triazolylmethyl)amine ligand developed for the CuAAC reaction. When conducting reactions with substrate **21** and **22**, the new nanoparticle was about ten times faster compared with **15** and the most efficient small molecule catalysts.[46] In addition, MD simulation and NMR supported the conclusion that **13** exhibited similar substrate binding behavior compared to **15**.

Transition metal catalysts are important tools for protein modification, however, their effectiveness is often poor at the low concentrations typically used for protein modification (μM).[88,89] Strikingly, Cu^{I}-SCNP **13** proved to be exceptionally effective in the click reaction between coumarin azide **21** and a series of alkynylated proteins.[46] Indeed, for multiple proteins with negative surface charge (ζ-potential ≤ -20), **13** proved to be significantly faster than BTTAA and BTGTA (Figure 13a). Other proteins, especially avidin with a postive surface charge underwent slower reaction suggesting that electrostatic binding between **13** and the negatively charged proteins led to the rate enhancement .

A combination of STD-NMR and MD simulations confirmed that **13** can complex protein through electrostatic and hydrobic interactions.[46] The MD simulations were performed with a single molecule of **13** and BSA, the free energy calculated as the center of mass distance (CMD) was increased from 2.8 to 8.8 nm. As shown in Figure 13b, the SCNP is flexible, initially flattening as pushed against the BSA surface. As it pulls away, multiple trimethyl-ammonium-carboxylate ion pairs and hydrophobic contacts were formed and an energy minimum is reached at 3.1 nm CMD. The apparent complex between SCNP **13** and proteins suggests an "attach mode" is operating for biocatalysis. As an application, we showed that **13** effectively "clicked" small molecules and proteins to azide- and alkyne-containing cell surface glycans, demonstrating its potential applications for cell surface bio-conjugation.

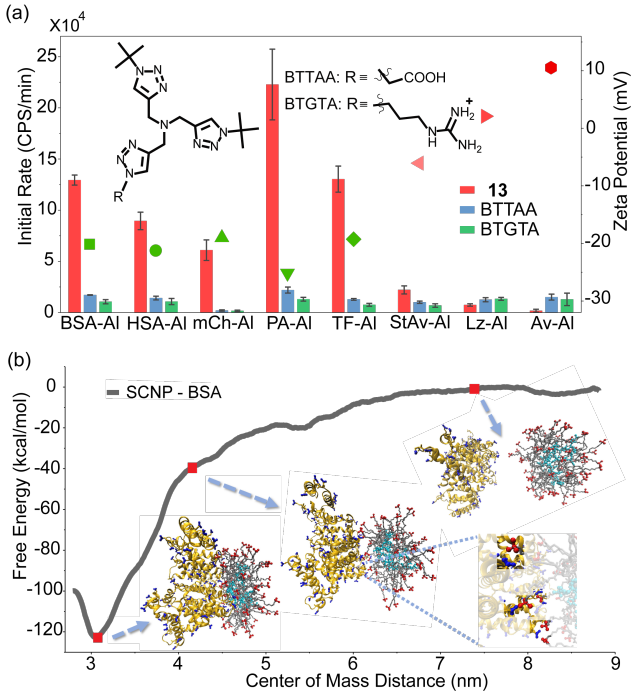


Figure 13. (a) Initial rates of CuAAC reaction with different alkynylated proteins using **13**, and the zeta potentials of proteins in PBS buffer. (b) MD simulation between a single **13** (blue) and BSA (yellow) and the calculated free energy versus CMD. Adapted with permission from ref. 46. Copyright 2019 American Chemical Society.

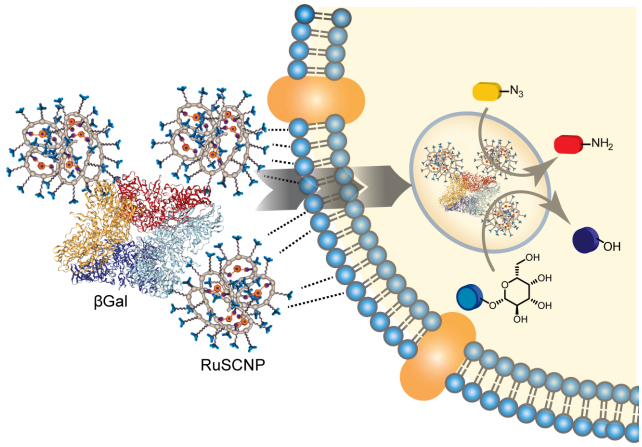


Figure 14. Illustration of RuSCNP-enzyme co-delivery and intracellular dual catalysis. Adapted with permission from ref. 90. Copyright 2020 American Chemical Society.

5.4. Concurrent and Tandem Intracellular Catalysis by Combining a SCNP and an Enzyme

There remains considerable interest in coupling synthetic organic chemistry with biocatalysis. One remaining challenge is performing such reactions inside cells where the low efficiency of synthetic catalysts combined with poor membrane permeability of extracellular enzymes is a major limitation. We recently reported a $Ru(bpy)_3$ containing SCNP (RuSCNP) that performs photo-catalytic azide reduction reactions. RuSCNP was shown to bind and deliver beta-galactosidase (βGal) into cells (Figure 14).[90] Thus, the cationic surface of RuSCNP binds through a combination of electrostatic and hydrophobic interactions, exhibiting a K_{app} ~ 31 nM.

The SCNP-enzyme complex was shown to enter cells by endocytosis.

Because the RuSCNP and βGal remained catalytically active within endosomes, the net effect was the engineering of "artificial organelles" that can serve as factories to produce small molecules with addition of appropriate precursors. Thus, RuSCNP and βGal performed both concurrent and tandem catalysis intracellularly, generating fluorophores and converting cancer pro-drugs into active agents. The results demonstrate the potential to combine natural and artificial enzymes for intracellular synthesis of active agents.

CONCLUSION AND OUTLOOK

In this account, we traced our efforts over the past two decades to intramolecularly cross-link a broad range of polymers. The combined results should convince the reader of the considerable potential for practical applications, particularly in the area of binding and catalysis. However, many challenges remain. Although general information about the rigidification and contraction of the polymers upon cross-linking has been obtained, beyond the preferences for long-range cross-linking in dendrimers, little is known about the preference or structure of other cross-linked polymers. Currently the cross-linking reactions are fully random. It may be that with greater precision in cross-link positioning, for example, by using sequence specific and discrete polymers, the SCNP may better mimic their natural counterparts. This knowledge is certainly important for predicting or tuning properties.

Whether cross-linked polymers will ever rival the performance of natural enzymes and proteins is a fair question, but even if the answer is no, they do offer numerous potential advantages. Most importantly, they can perform a much wider array of reactions. Indeed, only a small number of catalytic SCNP have been explored to date so this is an important area for future investigations. Another advantage of cross-linked polymers is their greater longterm stability in comparison to peptide-based biomacromolecules. Many of the intracellular catalysts developed exhibit relatively low turnover numbers in part because they can be demetallated in competitive biological environments. More robust metal complexes are needed or SCNP that offer better protection. Future advances will need to focus on gaining a better understanding of how the cross-linked polymers interact with biomolecules, cells, or organs. This information will be critical to moving toward in vivo applications.

The efforts described herein began with the question of whether one could extensively perform RCM reactions on alkene terminated dendrimers. Perhaps a more basic message to be taken from this Account is that asking fundamental questions can lead to important paths that would not otherwise be taken.

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