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Stealth Polymeric Vesicles via Metal-Free Click Coupling

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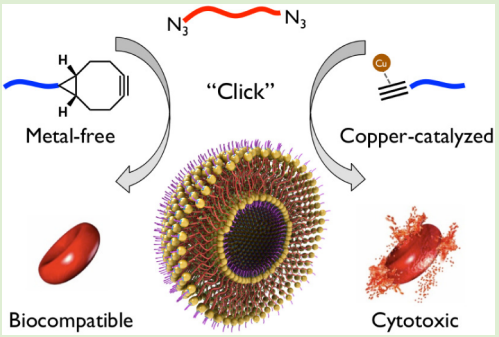
Supporting Information

ABSTRACT: The strain-promoted azide−alkyne cycloaddition represents an optimal metal-free method for the modular coupling of amphiphilic polymer blocks. Hydrophilic poly(oxazoline) (PMOXA) or poly(ethylene glycol) (PEG) A-blocks were coupled with a hydrophobic poly(siloxane) B-block to provide triblock copolymers capable of self-assembling into vesicular nanostructures. Stealth properties investigated via a complement activation assay revealed the superior in vitro stealth attributes of polymeric vesicles synthesized via a metal-free approach to those coupled via the widely used copper-catalyzed click method. Furthermore, the ability to change a single parameter, such as the hydrophilic block, allowed the direct comparison of the biocompatibility properties of triblock copolymers containing PMOXA or PEG. Our studies convincingly demonstrate the need for a metal-free approach, both in preventing cytotoxicity while imparting optimal stealth properties for potential biomedical applications.



INTRODUCTION

Amphiphilic block copolymers capable of self-assembling into vesicular nanostructures have garnered significant interest in the biomedical arena, especially as drug delivery vehicles.[1] Recently, the modular synthesis of block copolymers has been aided by click chemistry, with the copper-catalyzed azide−alkyne cycloaddition (CuAAC) emerging as the predominant method for polymer−polymer coupling.[2] However, the cytotoxicity of the copper(I) catalyst,[3] combined with its difficulty of removal,[4] has limited the biological applicability of CuAAC. Furthermore, copper contamination may diminish pivotal stealth attributes of a polymeric delivery vehicle, leading to detection by the immune system and rapid clearance.[5]

Clearly, a metal-free polymer−polymer coupling approach is desirable for biological applications. Several metal-free click methods have been reported for polymer coupling such as tetrazine-norbornene, aldehyde-hydrazine, and hetero-Diels−Alder.[6] However, these are not easily adapted to biomedically relevant siloxane-based polymers, since all these methods require polar end-groups to be introduced into the siloxane polymer which is challenging due to the low solubility of siloxanes in polar solvents. Additionally, direct end-group functionalization via the end-blocker method is infeasible since these end-group are incompatible with the cationic ring-opening polymerization (CROP) conditions used for siloxane polymerization. One of the most attractive metal-free approaches is the strain-promoted azide−alkyne cycloaddition (SPAAC), which couples an azide and cyclooctyne moiety without the need for a catalyst (Scheme 1).[7] A major advantage of azide−alkyne coupling is the ease of introducing azido groups for live-cell and in vivo applications.[8] SPAAC has been mainly applied to conjugating low-molecular weight compounds, and, to the best of our knowledge, the use of SPAAC for the coupling of polymer blocks has not been reported. Herein, we demonstrate for the first time the coupling of a hydrophilic A-block, poly(methyloxazoline) (PMOXA) or poly(ethylene glycol) (PEG), with a highly hydrophobic poly(dimethylsiloxane) (PDMS) B-block, to form well-defined amphiphilic ABA triblock copolymers using SPAAC. In addition, these ABA triblock copolymers were synthesized via CuAAC using a copper nanoparticle catalyst, and the resulting properties were compared to those clicked via SPAAC. The power of a modular click approach was demonstrated by the direct comparison of a single parameter change on the polymeric self-assembling dynamics and resulting in vitro properties. Polymeric vesicles (polymersomes) were formed in aqueous solution,[9] and the physical properties were analyzed to investigate how copper contamination and/or the identity of the A-block affects self-assembly.

An effective method for investigating the stealth properties and biocompatibility of nanostructures in vitro is via complement activation.[10] The complement system is part of the innate immune response, and enables (or complements) the clearance of pathogens by phagocytes and antibodies.[11] Evading complement activation is the key to imparting stealth properties and increasing circulation times of nanoparticles,[12] which are critical attributes of drug delivery vehicles.[13] The stealth properties of polymersomes synthesized via SPAAC versus CuAAC were evaluated in vitro using a quantitative complement activation assay. In addition, we compared the effect of using PEG versus

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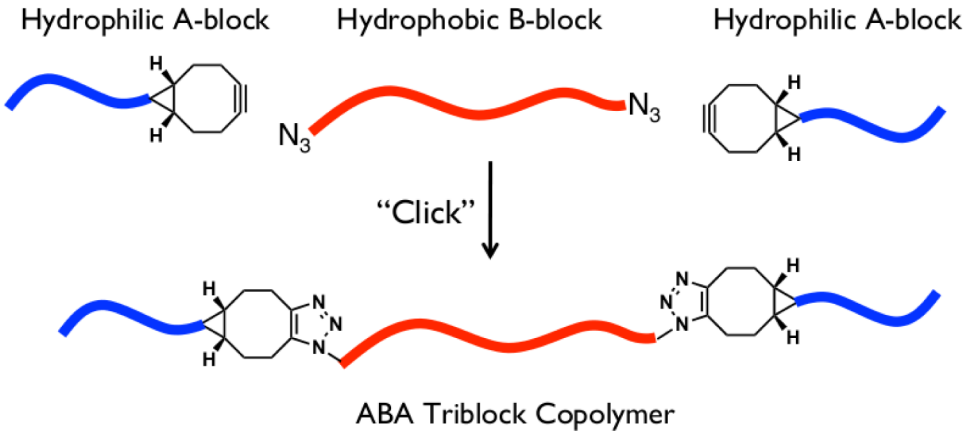
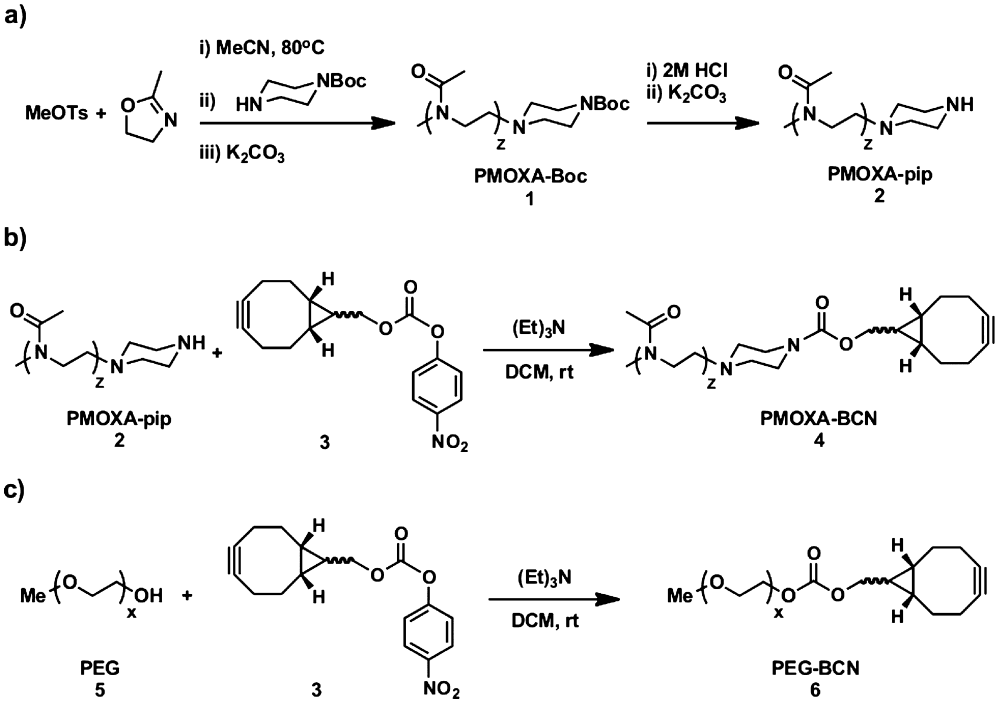
Scheme 1. Synthesis of ABA Triblock Copolymers via Strain-Promoted Azide−Alkyne Cycloaddition



Scheme 2. Synthesis of (a) PMOXA A-Block, (b) Cyclooctyne-Terminated Blocks PMOXA-BCN, and (c) PEG-BCN



PMOXA as the hydrophilic A-block, since PMOXA is a known biocompatible alternative to PEG.[14] Our studies convincingly demonstrated the need for a metal-free approach over a copper-catalyzed method to ensure optimized stealth and biocompatibility properties for polymersomes in vitro.

RESULTS AND DISCUSSION

We began with the synthesis of a PMOXA A-block containing a piperazine end-group, which was modified with a clickable cyclooctyne moiety post-polymerization. Methyl tosylate was used as the initiator for the CROP of 2-methyl-2-oxazoline in acetonitrile at 80 °C (Scheme 2). After ^{1}H NMR demonstrated complete initiation and monomer consumption, the polymerization was terminated with 1-Boc-piperazine. The resulting piperazine salt was deprotonated with potassium carbonate to provide PMOXA-Boc (**1**) in quantitative yield, with $M_n \sim 1500$ as demonstrated by ^{1}H NMR using end-group analysis. Trifluoroacetic acid is a popular reagent for the acidic cleavage of the Boc group;[15] however, this method did not result in complete Boc cleavage. Alternatively, an approach using 2 M aqueous HCl resulted in quantitative Boc cleavage to provide the secondary amine.[16] Following deprotonation of the polymer chain with potassium carbonate, PMOXA-pip (**2**) was provided with the desired piperazine end-group.

Although several clickable cyclooctyne groups are found in the literature,[17] bicyclo[6.1.0]nonyne (BCN) was chosen due to its ease of functionalization, commercial availability, and rapid cycloaddition kinetics with azides via SPAAC.[18] BCN was reacted with 4-nitrophenyl chloroformate to give the electrophilic BCN-nitrophenyl carbonate (**3**) (Supporting Information). PMOXA-pip (**2**) was coupled with BCN-nitrophenyl carbonate (**3**) in the presence of triethylamine to give PMOXA-BCN (**4**), as confirmed by ^{1}H NMR. After removal of the *p*-nitrophenol byproduct, followed by precipitation into cold Et_2O, PMOXA-BCN (**4**) was obtained as an off-white solid.

The synthesis of a clickable PEG A-block was achieved by coupling a cyclooctyne group to the polymer chain end postpolymerization. BCN-nitrophenyl carbonate (**3**) was

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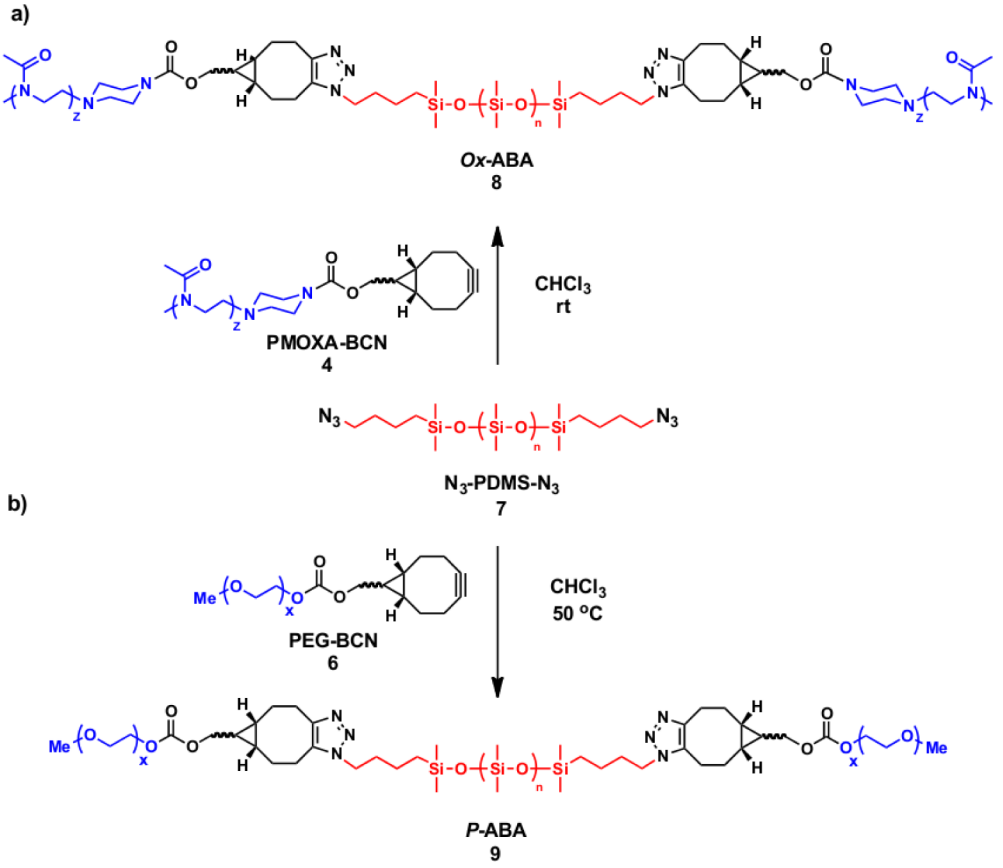
Scheme 3. The Synthesis of Triblock Copolymers (a) *Ox*-ABA (8) and (b) *P*-ABA (9) via Strain-Promoted Azide−Alkyne Cycloaddition with Bis-Azide Poly(dimethylsiloxane) B-Block N_3-PDMS-N_3 (7)



coupled to commercially available PEG (**5**) ($M_n \sim 2000$) in the presence of triethylamine at 60 °C, as confirmed by ^{1}H NMR (Scheme 2). Unlike PMOXA-pip (**2**), which is terminated with a highly nucleophilic secondary amine, PEG (**5**) contains a weakly nucleophilic primary alcohol end-group; therefore an elevated temperature was required. The *p*-nitrophenol by-product was removed, and PEG-BCN (**6**) was obtained after solvent precipitation.

With clickable hydrophilic A-blocks PMOXA-BCN (**4**) and PEG-BCN (**6**) in hand, we aimed to synthesize two triblock copolymers using a hydrophobic bis-azide poly(dimethylsiloxane) (N_3-PDMS-N_3) (**7**) B-block via SPAAC (Scheme 3). The synthesis and characterization of N_3-PDMS-N_3 ($M_n \sim 5300$) was described previously by our group.[19] The coupling of PMOXA-BCN (**4**) with N_3-PDMS-N_3 (**7**) proceeded smoothly at room temperature in a solution of chloroform, providing ABA triblock copolymer PMOXA−PDMS−PMOXA (*Ox*-ABA) (**8**), as confirmed by ^{1}H NMR. Furthermore, the click coupling was quantitative, requiring 1 equiv of N_3-PDMS-N_3 (**7**) and 2 equiv of PMOXA-BCN (**4**), thus circumventing the need for an excess of either block. The coupling of PEG-BCN (**6**) and N_3-PDMS-N_3 (**7**) proceeded at 50 °C in a solution of chloroform using 2 equiv of PEG-BCN (**6**) and 1 equiv of N_3-PDMS-N_3 (**7**). Thus, PEG-PDMS-PEG (*P*-ABA) (**9**) was provided in quantitative yield as confirmed by ^{1}H NMR. ABA triblock copolymers were synthesized via CuAAC by coupling either a PMOXA or PEG A-block terminated in an alkyne, with an N_3-PDMS-N_3 (**7**) B-block using a copper nanoparticle catalyst. The synthesis of *Ox*-ABA via CuAAC is discussed in our previous publication,[19] while the synthesis of *P*-ABA via CuAAC is detailed in the Supporting Information.

The effects of copper contamination on the self-assembling dynamics of *Ox*-ABA and *P*-ABA triblock copolymers synthesized via SPAAC or CuAAC were investigated. Vesicles were prepared using the film rehydration method (see Supporting Information), and the resulting aqueous vesicular solution was analyzed by dynamic light scattering (DLS) (Table 1). Interestingly, despite similar block lengths and hydrophilic/hydrophobic ratios, DLS demonstrated that

Table 1. Physiochemical and Surface Properties of Polymersomes Synthesized and Purified via Various Methods

polymersome composition/ synthetic method	treatment/ purification	size DLS (nm)	zeta potential (mV)
Ox-ABA-CuAAC	filtration of copper	180	−0.145
Ox-ABA-CuAAC	Na_2S treated[a]	169	1.50
Ox-ABA-CuAAC	$Na_2S/MgSO_4$ treated[a]	190	1.05
Ox-ABA-SPAAC	nonmetal click[b]	110	−1.31
P-ABA-CuAAC	filtration of copper	159	0.859
P-ABA-CuAAC	Na_2S treated[a]	156	0.472
P-ABA-CuAAC	$Na_2S/MgSO_4$ treated[a]	146	−1.99
P-ABA-SPAAC	nonmetal click[b]	96	−1.12

[a]Treatments were done consecutively after copper click chemistry. [b]No treatments were done post polymer synthesis.

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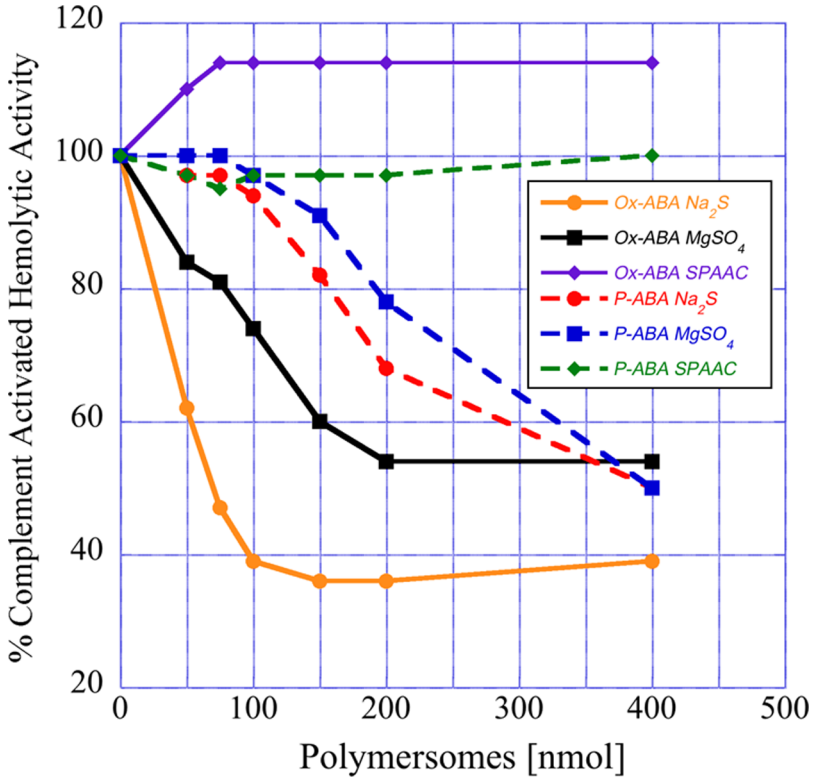

Figure 1. Polymersome-induced complement activation in the presence of human serum as a function of concentration. A decrease in the percent complement activated hemolytic activity indicates an increase in complement activation. Polymersomes coupled via SPAAC demonstrate negligible complement activation. CuAAC-coupled polymersomes purified with Na_2S induce complement activation due to the presence of copper, and subsequent $MgSO_4$ treatment reduces complement activation.

vesicles formed from polymers coupled via SPAAC are substantially smaller than those formed from polymers coupled via CuAAC. This is most probably due to the chelation of copper metal ions in the hydrophilic block, by the ether oxygens in the PEG blocks, or the amide nitrogens in the PMOXA blocks.

Subsequent treatment with sodium sulfide and/or magnesium sulfate, which serves to reduce the amount of copper contaminate, resulted in smaller vesicle sizes. The inclusion of copper in polymersomes was substantiated by the decrease in the diameter of the vesicle with each subsequent purification step. Polymersomes formed from metal-free clicked block copolymers had the smallest diameter due to the absence of copper in the hydrophilic backbone. Additionally, the size ratio of polymersomes formed via CuAAC and SPAAC were the same for both *P*-ABA and *Ox*-ABA triblock copolymers, indicating similar mechanisms for the change in diameter, namely copper chelation. Although small variations in the zeta potential were observed, the polymersomes were close to neutral charge, as expected. The vesicular morphology of the nanostructures was confirmed by transmission electron microscopy (TEM) (Supporting Information).

The in vitro stealth attributes and biocompatibility of polymersomes synthesized via SPAAC versus CuAAC were investigated using a standard complement activation assay. The assay uses sheep red blood cells (RBCs) coated with rabbit antisheep erythrocyte IgM antibodies. When exposed to human serum, the rabbit antibodies activate the complement system. This leads to a cascade reaction resulting in the formation of the membrane attack complex, which assembles in the membrane of the sheep RBCs, leading to lysis of the cell and release of hemoglobin.[20] If additional components capable of activating the complement system are present, such as nanoparticles, complement protein binding to the sheep RBCs decreases. This results in less sheep RBCs being lysed, leading to a decrease in hemoglobin absorption. Thus, the complement activation due to the nanoparticle or polymersome can be quantitatively determined by monitoring the absorption of the released hemoglobin.

Biocompatibility of polymersomes coupled via SPACC was found to be vastly superior to those formed via CuAAC. In absence of human serum, lysis of sheep RBCs occurred when treated with copper-clicked polymersomes purified by simple filtration of the copper nanoparticle catalyst. PEG containing polymersomes (*P*-ABA) demonstrated up to 2% cell lysis, while their PMOXA counterparts (*Ox*-ABA) demonstrated up to 28% cell lysis (Supporting Information). PMOXA chelates copper more effectively than PEG due to the increased chelating properties of the amide nitrogens of PMOXA compared to the ether oxygens of PEG, leading to increased cell lysis. Importantly, lysis of RBCs did not occur with polymersomes synthesized via SPAAC, or when purification methods for copper removal are utilized for those synthesized via CuAAC. Complement activation of polymersomes coupled via SPAAC was found to be significantly smaller than those formed by CuAAC following purification (Figure 1). CuAAC-clicked *Ox*-ABA polymersomes demonstrated increased complement activation over their *P*-ABA counterparts, presumably due to the stronger copper chelation properties of the PMOXA block versus PEG. Purification of CuAAC-clicked polymers with sodium sulfide and/or subsequent magnesium sulfate treatment reduced complement activation, indicating a decrease

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of copper ions chelated in the polymer. The reason for the decrease in complement activation by each purification step is probably due to the change in steric properties of the surface.[21] Copper chelation effectively reduces the free volume excluded by the hydrophilic group lowering the shielding offered by steric hindrance.

The initial increase in complement activation seen with *Ox*-ABA polymersomes synthesized via SPAAC may be due to the presence of trace *p*-nitrophenol from the synthesis of **4** (Figure 1). This was inferred by comparing the hemoglobin release due to polymersomes alone to release in the presence of human serum. An increase in absorption is possible only if there is cell lysis, which did not occur in the absence of human serum. Another possibility is the oxidation of Fe(II) to Fe(III) by *p*-nitrophenol causing an increase in optical density (OD).[22] This is the most likely mechanism since the OD only increases in the presence of both polymersomes and human serum. Unlike previously established studies showing the cytotoxicity of copper-catalyzed click methods, our results demonstrate that even minute amounts of copper play a deleterious role in the stealth properties of polymersomes, despite being noncytotoxic. Thus, a metal-free approach is a necessity if polymers clicked via a modular method are to be useful in biomedical applications.

In conclusion, we have demonstrated the superior in vitro stealth attributes of polymersomes synthesized via a metal-free click methodology versus copper-catalyzed methods. SPAAC was utilized as a metal-free polymer−polymer coupling strategy for the synthesis of amphiphilic triblock copolymers, which self-assemble into vesicular nanostructures under appropriate conditions. In addition, PMOXA was shown to be a viable alternative to PEG in imparting stealth properties in ABA polymeric vesicles. The further development of novel metal-free polymer−polymer coupling methodologies, along with their applications in drug delivery is currently being investigated in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, spectroscopic data, TEM, DLS, and graph of Cu-induced cell lysis. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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