J|A|C|S COMMUNICATIONS

Published on Web 04/06/2009

Surpassing the Use of Copper in the Click Functionalization of Polymeric Nanostructures: A Strain-Promoted Approach

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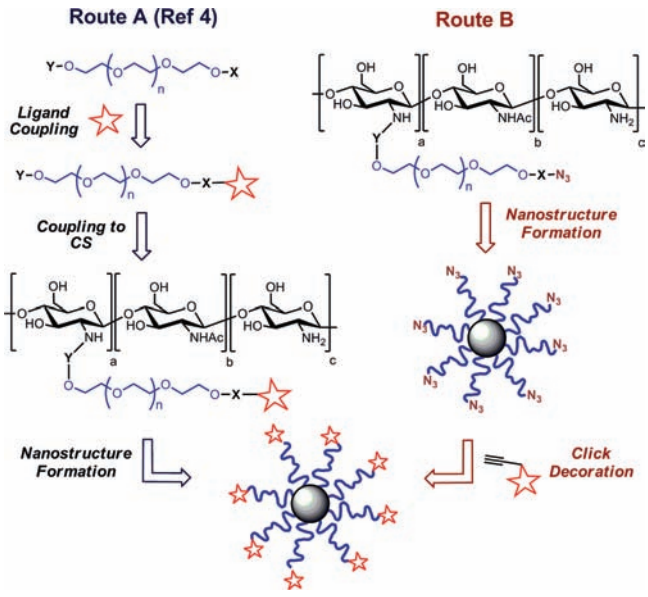
With the aim of developing stable polymeric carriers for drug delivery, we and others have recently turned our attention to chitosan-*g*-poly(ethylene glycol).[1] Chitosan (CS) is a linear polysaccharide comprising glucosamine and *N*-acetylglucosamine that is characterized by high biocompatibility and biodegradability.[2] The presence of poly(ethylene glycol) (PEG) in the resulting CS-*g*-PEG nanostructures is aimed at modifying their biodistribution and enhancing their biocompatibility, stability, and circulation times in the bloodstream.[3]

When CS-*g*-PEG nanostructures for active targeting were being designed, the necessity of localizing the ligands at the distal end of the PEG to ensure an effective ligand−receptor interaction was clearly recognized. With this purpose, we recently reported the grafting of various PEGylated bioactive molecules and probes to CS (Scheme 1, Route A).[4] The resulting functionalized CS-*g*-PEGs

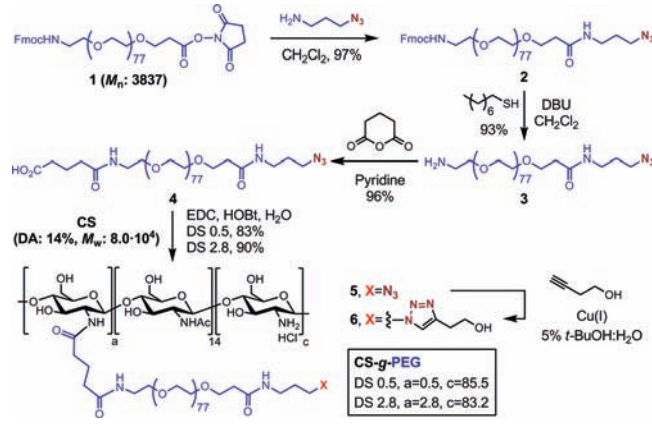
Scheme 1



have demonstrated their utility in brain delivery applications when engineered as nanoparticles (NPs).[4] However, the necessity of incorporating the bioactive molecule in the first step of the synthetic sequence made this strategy unfeasible for attaching biomacromolecular ligands [e.g., antibodies (Ab's) or RNA aptamers] and revealed the necessity of a more convergent approach, namely, the conjugation of the ligands to a preformed CS-*g*-PEG nanostructure (Scheme 1, Route B). As classical bioconjugation procedures are of limited application for this goal because of the low pK_a of CS amines (∼6.0−7.0), in the present work we relied on the orthogonality of click chemistry, in particular the Cu(I)-catalyzed[5] and strain-promoted[6] azide−alkyne [3 + 2] cycloadditions (CuAAC and SPAAC, respectively), which are coupling technologies that proceed in high yields under mild conditions. By incorporation of terminal azides at the distal end of the PEG, a highly convergent and selective functionalization of CS-*g*-PEG nanostructures with bioactive species carrying alkyne groups was expected.[7]

The required CS-*g*-PEG-N_3 (**5**) was prepared by grafting to CS [$M_w = 8.0 \times 10^4$ by size exclusion chromatography−multiangle laser light scattering (SEC-MALLS)] an azide-terminated PEG (**4**) carrying a carboxylic acid, as shown in Scheme 2. PEG **4** (M_n = 3723 by MALDI-TOF) was synthesized in three steps from a heterodifunctional PEG (**1**) in 87% overall yield. In order to ensure the purity of the end groups and to avoid complex polymeric mixtures, reactions with PEG were forced to proceed quantitatively. Graft copolymer **5** was obtained with various degrees of substitution (DS = 0.5 and 2.8) with excellent conversions and mass recoveries.

Scheme 2



The functionalization of **5** was first attempted in solution under CuAAC conditions, employing homopropargylic alcohol (2000 mol % per N_3) as a model alkyne (Scheme 2). Both Cu(II)/ascorbate and Cu(II)/Cu systems were used as the source of Cu(I). The pH of the reaction was set in the range 5.8−7.1: lower pH values hampered the reaction from proceeding, while higher pHs resulted in precipitation of the polymer. Quantitative conversions were typically obtained with Cu(II)/ascorbate and 70−90% conversions for Cu(II)/Cu under various conditions [see the Supporting Information (SI)]. Nevertheless, the utility of this Cu(I)-catalyzed approach proved to be very limited, as concomitant severe depolymerization of the CS backbone was observed by SEC (Figure S4 in the SI), resulting in poor mass recoveries (typically <50%). This depolymerization was rationalized as being mediated by the hydroxyl radical (•OH), the presence of which was detected in the reaction medium (see the SI). The most likely source of •OH is a Fenton reaction involving the reduced form of a transition metal, Cu(I) in our case, and H_2O_2:[8] $Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^-$.[9,10]

10.1021/ja8100243 CCC: $40.75

Reactive oxygen species (ROS), especially •OH, are well-known to bring about severe structural damage to biomolecules, such as polysaccharides, nucleic acids, and enzymes, in a process at the origin of many human diseases.[11,12] The mechanism involves an exceedingly rapid abstraction of a C-bonded H atom accompanied by the formation of new radicals, ultimately resulting, in the case of polysaccharides, in the scission of glycosidic bonds and a drastic decrease of molecular weight (MW).[9,12−14]

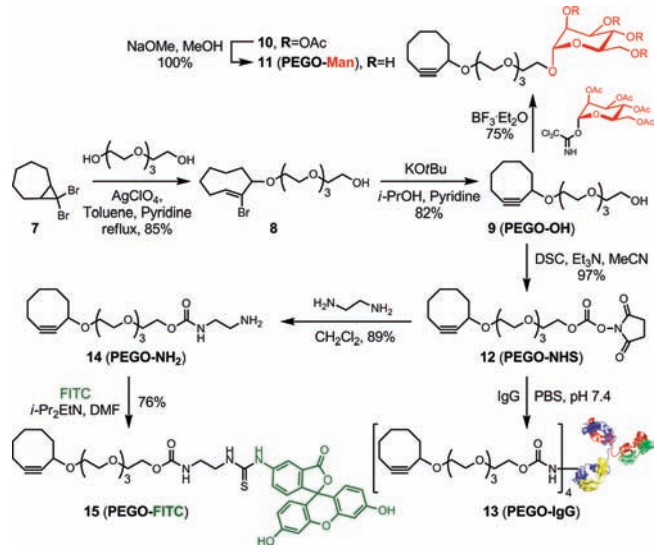
During the completion of this project, however, various reports on the application of CuAAC for the selective functionalization of polysaccharides (cellulose, Curdlan, starch)[15] and the preparation of hydrogels and microparticles from hyaluronic acid (HA) and dextran[16] have appeared in the literature. Unfortunately, in none of them has the possible adverse effect of Cu(I) on the MW of the resulting clicked polysaccharides been evaluated. For this reason, and with the aim of exploring the scope and limitations of CuAAC for the selective functionalization of polysaccharide-based systems, we performed a SEC-MALLS study of the depolymerizing effect of Cu(I) on CS and three other representative polysaccharides, namely, dextran, mannan, and HA. Under the least depolymerizing conditions found for CS-*g*-PEG [Cu(II)/Cu, pH 6.5−7.0, overnight (see the SI)], a decrease in MW was observed for the four polysaccharides in a process that parallels their Cu-complexing ability [polymer (% decrease in MW): mannan (3%), dextran (38%), CS (95%), HA (>99%)]. Accordingly, the process seems to be of general scope and agrees with previous reports on the enhanced degradation of highly Cu-complexing polysaccharides, as in these cases the short-lived •OH are produced closer to the polymer backbone.[17,18]

In the case of CS-*g*-PEG, the great tendency of this depolymerization to proceed rendered unfruitful any attempt to reduce it: various pH values, the absence of O_2, cysteine as an •OH scavenger, and $[Cu(MeCN)_4][PF_6]$/sulfonated bathophenanthroline as the source of Cu(I). In addition, the greenish/brown appearance of the resulting clicked products **6** (Figure S3) revealed a high content of cytotoxic Cu even after workup with EDTA, Amberlite IR-120, or Dowex M-4195 [>200 ppm by flame atomic absorption spectroscopy (FAAS); Table S1 in the SI], which severely compromises their use in biomedical applications.

At this stage, it was therefore clear that for the azide−alkyne coupling to become a useful tool in the functionalization of CS-*g*-PEG nanostructures, an alternative to Cu(I) for lowering the activation barrier of the reaction was required. In this regard, Bertozzi and co-workers[6] have recently reported a tremendous acceleration of this cycloaddition by making use of strained cyclooctynes within the bioorthogonal chemical reporter strategy (i.e., the SPAAC approach). In this way, the efficient labeling of proteins and cell-surface glycans in live cells, as well as the in vivo imaging of glycans in developing zebrafish embryos, have been reported.[6] Such benign Cu-free conditions were envisaged as an environmentally friendly[19] alternative for the orthogonal functionalization of polymeric nanostructures.

With this aim, we prepared cyclooctyne **9** (PEGO-OH, two steps, 70% overall yield) carrying a short PEG linker for increased solubility in water (Scheme 3). The incorporation of various functional groups at the distal end of the PEG (active carbonate **12** and amine **14**) facilitated the bioconjugation of PEGO to relevant ligands and probes, such as mannose (**11**), an anti-BSA rabbit immunoglobulin G (IgG) (**13**), and fluorescein (**15**), in very good yields. Moreover, PEGO proved to be rather stable for many months at −20 °C, and no decomposition was observed after prolonged heating in aqueous media (80 h, 80 °C).

Scheme 3



The SPAAC coupling of CS-*g*-PEG-N_3 **5** (DS = 2.8) was first evaluated in solution with **9** (250 mol % per N_3) as a model system (25, 55, and 80 °C; 5% DMSO/H_2O; Figure 1). The progress of the click functionalization was monitored (presat ^{1}H NMR)[20] by the disappearance of the methylene protons γ to the azide group at 3.3 ppm (Figure 1 and Figures S4−S6). Complete functionalization was observed in all cases after 4 h (80 °C), 13 h (55 °C), and 86 h (25 °C), leading to **16** as a ∼2:1 mixture of regioisomers (as determined by ^{1}H NMR), with typical mass recoveries of >95% (no depolymerization by SEC). When more sophisticated mannose and FITC PEGO derivatives (**11** and **15**) were used, complete functionalization was also noticed (55 °C), affording graft copolymers **17** and **18** with excellent mass recoveries.

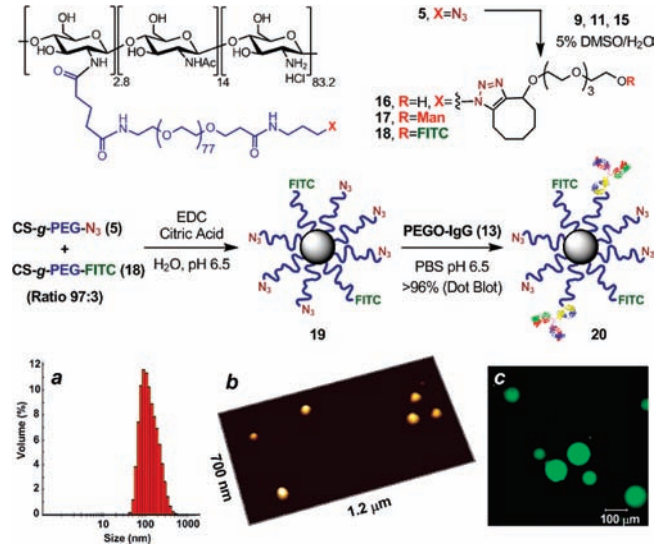


Figure 1. SPAAC conjugation of CS-*g*-PEG **5** and NPs **19**. (a) DLS histogram of **19** (H_2O, 25 °C). (b) Tapping mode AFM of **19**. (c) LSCM image of BSA-agarose beads stained by immuno-NPs **20**.

Having established the SPAAC conditions for the complete functionalization of **5** in solution, we decided to check its applicability for the decoration of CS-*g*-PEG nanostructures. With this purpose, clickable fluorescent NPs **19** were prepared by cross-linking **5** (DS = 0.5) and CS-*g*-PEG-FITC **18** (DS = 2.8) with citric acid/EDC (Figure 1).[21] The dynamic light scattering (DLS)

histogram of **19** revealed a hydrodynamic size of 91 nm and a polydispersity index (PDI) of 0.20. Visualization of **19** in the solid state was also possible by atomic force microscopy (AFM) and transmission electron microscopy, revealing a spherical shape and a mean diameter of ~50 nm (Figure 1 and Figure S7). From these data, the MW of **19** was estimated as 4.6×10^7, yielding an average of 1025 PEG-N_3 and 115 PEG-FITC chains per NP (see the SI).

A demanding biomacromolecular ligand such as PEGO-IgG **13** was selected for the SPAAC functionalization of NPs **19**. Thus, although the conjugation of Ab's to nanostructures has been largely known, conventional methods often rely on activation steps and reactive coupling reagents that limit their efficiency.[22] Our decoration of **19** with **13** (0.3 mol % of **13** per N_3, **19/13** mass ratio = 100:1) was carried out in PBS (pH 6.5) and monitored by Dot Blot (Figure 1 and Figure S9). After 7 h of incubation at 25 °C, more than 96% of the starting PEGO-IgG was incorporated onto the surface of the resulting immuno-NP **20**, affording an average of 3.0 IgG per NP (see the SI).

The mildness of the conditions reported herein was evidenced by DLS, as the immuno-NPs **20** had identical size and PDI as NPs **19** without any sign of larger aggregates (Figure S9). In addition, the bioactivity of the anti-BSA rabbit IgG was retained during the SPAAC conjugation, according to laser scanning confocal microscopy (LSCM) experiments. Thus, BSA-agarose beads were fluorescently stained by immuno-NPs **20** but not by nonfunctionalized fluorescent NPs **19** used as control (Figure 1 and Figure S10).

In conclusion, we have shown the limitations (depolymerization and contamination by Cu) in the use of CuAAC as a general strategy for the selective functionalization of polysaccharide-based systems as well as the use of SPAAC as an efficient alternative that eliminates the requirement of Cu(I). In this context, the application of SPAAC for the efficient decoration of CS-*g*-PEG nanostructures with an IgG under physiological conditions represents a step forward in the development of environmentally friendly bioconjugation technologies for the preparation of immunonanoparticles. The very recent enhancement of the SPAAC rate to values comparable to that of CuAAC by appending propargylic fluorine atoms or fused phenyl rings to the cyclooctyne probe[6] suggests a rich future for SPAAC in nanotechnology and materials science.

Acknowledgment. This work was supported by MICINN and XUGA. The authors thank Prof. A. Moreda, Prof. F. Dominguez, and F. C. Ceballos for assistance with FAAS and Dot Blot.

Supporting Information Available: Complete ref 4b, methods, experimental procedures, and characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Prego, C.; Torres, D.; Fernandez-Megia, E.; Novoa-Carballal, R.; Quiñoá, E.; Alonso, M. J. *J. Controlled Release* **2006**, *111*, 299. (b) Bhattarai, N.; Ramay, H. R.; Gunn, J.; Matsen, F. A.; Zhang, M. *J. Controlled Release* **2005**, *103*, 609. (c) Park, I. K.; Kim, T. H.; Kim, S. I.; Park, Y. H.; Kim, W. J.; Akaike, T.; Cho, C. S. *Int. J. Pharm.* **2003**, *257*, 103.

(2) Ravi Kumar, M. N. V.; Muzzarelli, R. A. A.; Muzzarelli, C.; Sashiwa, H.; Domb, A. J. *Chem. Rev.* **2004**, *104*, 6017.

(3) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. *Adv. Drug Delivery Rev.* **2003**, *55*, 217.

(4) (a) Fernandez-Megia, E.; Novoa-Carballal, R.; Quiñoá, E.; Riguera, R. *Biomacromolecules* **2007**, *8*, 833. (b) Aktas, Y.; et al. *Bioconjugate Chem.* **2005**, *16*, 1503.

(5) (a) Lutz, J.-F. *Angew. Chem., Int. Ed.* **2007**, *46*, 1018. (b) Moses, J. E.; Moorhouse, A. D. *Chem. Soc. Rev.* **2007**, *36*, 1249. (c) Fournier, D.; Hoogenboom, R.; Schubert, U. S. *Chem. Soc. Rev.* **2007**, *36*, 1369. (d) Wu, P.; Fokin, V. V. *Aldrichimica Acta* **2007**, *40*, 7.

(6) (a) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664. (b) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486. (c) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253. (d) Johnson, J. A.; Baskin, J. M.; Bertozzi, C. R.; Koberstein, J. T.; Turro, N. J. *Chem. Commun.* **2008**, 3064. (e) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793. (f) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046.

(7) For the use of CuAAC in the functionalization of various nanostructures, see: (a) Hein, C. D.; Liu, X.-M.; Wang, D. *Pharm. Res.* **2008**, *25*, 2216. (b) Polito, L.; Monti, D.; Caneva, E.; Delnevo, E.; Russo, G.; Prosperi, D. *Chem. Commun.* **2008**, 621. (c) Nicolas, J.; Bensaid, F.; Desmaële, D.; Grogna, M.; Detrembleur, C.; Andrieux, K.; Couvreur, P. *Macromolecules* **2008**, *41*, 8418. (d) Opsteen, J. A.; Brinkhuis, R. P.; Teeuwen, R. L. M.; Löwik, D. W. P. M.; van Hest, J. C. M. *Chem. Commun.* **2007**, 3136. (e) Li, B.; Martin, A. L.; Gillies, E. R. *Chem. Commun.* **2007**, 5217. (f) Brennan, J. L.; Hatzakis, N. S.; Tshikhudo, T. R.; Dirvianskyite, N.; Razumas, V.; Patkar, S.; Vind, J.; Svendsen, A.; Nolte, R. J. M.; Rowan, A. E.; Brust, M. *Bioconjugate Chem.* **2006**, *17*, 1373. (g) O'Reilly, R. K.; Joralemon, M. J.; Hawker, C. J.; Wooley, K. L. *J. Polym. Sci., Part A: Polym. Chem.* **2006**, *44*, 5203.

(8) Two possible mechanisms for the in situ production of H_2O_2 could operate: (i) In the presence of ascorbate, O_2 can be reduced to H_2O_2 in a process catalyzed by traces of Cu(II) or other transition metal ions [see ref 9 and: Halliwell, B.; Gutteridge, J. M. C. *Arch. Biochem. Biophys.* **1990**, *280*, 1. (ii) In the absence of ascorbate, Cu(I) can promote the reduction of O_2 to H_2O_2 through a two-step process involving the superoxide anion radical ($O_2^{\bullet-}$) [see ref 11 and: (a) Bar-Or, D.; Rael, L. T.; Lau, E. P.; Rao, N. K. R.; Thomas, G. W.; Winkler, J. V.; Yukl, R. L.; Kingston, R. G.; Curtis, C. G. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 856. (b) Mi, L.; Zuberbühler, A. D. *Helv. Chim. Acta* **1991**, *74*, 1679]. For an overview of the degradation of CS and other polysaccharides by $^{\bullet}$OH, see the SI.

(9) (a) Tabbì, G.; Fry, S. C.; Bonomo, R. P. *J. Inorg. Biochem.* **2001**, *84*, 179. (b) Fry, S. C. *Biochem. J.* **1998**, *332*, 507.

(10) Biaglow, J. E.; Manevich, Y.; Uckun, F.; Held, K. D. *Free Radical Biol. Med.* **1997**, *22*, 1129.

(11) Halliwell, B.; Gutteridge, J. M. C. *Methods Enzymol.* **1990**, *186*, 1.

(12) Soltés, L.; Mendichi, R.; Kogan, G.; Schiller, J.; Stankovská, M.; Arnhold, J. *Biomacromolecules* **2006**, *7*, 659.

(13) (a) Ulanski, P.; von Sonntag, C. *J. Chem. Soc., Perkin Trans. 2* **2000**, 2022. (b) Crescenzi, V.; Belardinelli, M.; Rinaldi, C. *J. Carbohydr. Chem.* **1997**, *16*, 561. (c) Nordtveit, R. J.; Varum, K. M.; Smidsrod, O. *Carbohydr. Polym.* **1994**, *23*, 253. (d) Uchiyama, H.; Dobashi, Y.; Ohkouchi, K.; Nagasawa, K. *J. Biol. Chem.* **1990**, *265*, 7753. (e) Uchida, K.; Kawakishi, S. *Agric. Biol. Chem.* **1986**, *50*, 367. (f) Wong, S. F.; Halliwell, B.; Richmond, R.; Skowroneck, W. R. *J. Inorg. Biochem.* **1981**, *14*, 127.

(14) In the case of CS, the high efficiency of the process has been exploited for its controlled depolymerization (see ref 17) as well as for preventing oxidative damage of DNA (see: Harish Prashanth, K. V. Dharmesh, S. M. Jagannatha Rao, K. S. Tharanathan, R. N. *Carbohydr. Res.* **2007**, *342*, 190).

(15) (a) Pohl, M.; Schaller, J.; Meister, F.; Heinze, T. *Macromol. Rapid Commun.* **2008**, *29*, 142. (b) Bernard, J.; Save, M.; Arathoon, B.; Charleux, B. *J. Polym. Sci., Part A: Polym. Chem.* **2008**, *46*, 2845. (c) Tankam, P. F.; Müller, R.; Mischnick, P.; Hopf, H. *Carbohydr. Res.* **2007**, *342*, 2049. (d) Hasegawa, T.; Numata, M.; Okumura, S.; Kimura, T.; Sakurai, K.; Shinkai, S. *Org. Biomol. Chem.* **2007**, *5*, 2404. (e) Liebert, T.; Hänsch, C.; Heinze, T. *Macromol. Rapid Commun.* **2006**, *27*, 208.

(16) (a) De Geest, B. G.; Van Camp, W.; Du Prez, F. E.; De Smedt, S. C.; Demeester, J.; Hennink, W. E. *Chem. Commun.* **2008**, 190. (b) Crescenzi, V.; Cornelio, L.; Di Meo, C.; Nardecchia, S.; Lamanna, R. *Biomacromolecules* **2007**, *8*, 1844.

(17) Chang, K. L. B.; Tai, M.-C.; Cheng, F.-H. *J. Agric. Food Chem.* **2001**, *49*, 4845.

(18) (a) Tanioka, S.; Matsui, Y.; Irie, T.; Tanigawa, T.; Tanaka, Y.; Shibata, H.; Sawa, Y.; Kono, Y. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 2001.

(19) Anastas, P.; Horváth, I. T. *Chem. Rev.* **2007**, *107*, 2169.

(20) Under these NMR conditions, not only was the water solvent peak saturated, but also the intensities of all of the CS resonances were mostly reduced, leading to spectra with improved signal-to-noise ratio (Figure S1).

(21) Bodnar, M.; Hartmann, J. F.; Borbely, J. *Biomacromolecules* **2005**, *6*, 2521.

(22) For an alternative approach to immuno-NPs by means of Diels−Alder and a survey of other traditional coupling methods, see: Shi, M.; Wosnick, J. H.; Ho, K.; Keating, A.; Shoichet, M. S. *Angew. Chem., Int. Ed.* **2007**, *46*, 6126.

JA8100243