Porous Structures

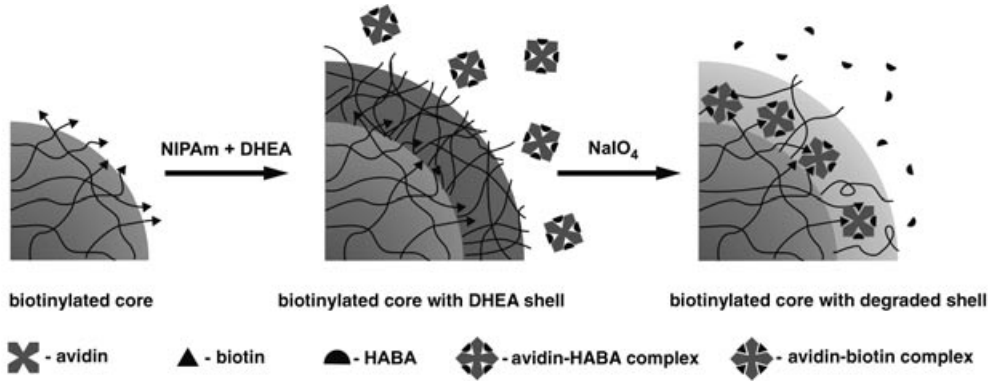
Ligand-Functionalized Core/Shell Microgels with Permselective Shells**

*Satish Nayak and L. Andrew Lyon**

Recently the field of nanotechnology has received immense attention, which in part is owed to its potential impact on biotechnology. Nanometric materials find application in many fields such as chemical sensing[1] and biosensing,[2] catalysis,[3] optics,[4] separations,[5] and drug delivery.[6] Nanomaterials offer advantages in such arenas owing to the potential for new or enhanced optical, chemical, mechanical, or electrical properties. Of particular interest is the creation of multifunctional nanoparticles, which contain multiple chemistries such that they interact with their environment in a more-complex fashion. Here we report the synthesis of a well-defined, yet chemically and topologically complex colloidal particle. This structure comprises a porous, thermosensitive hydrogel "core" particle that is modified with a ligand (biotin). This particle is contained within a porous, thermoresponsive hydrogel "shell" that sterically excludes the transport and binding of the protein avidin, for which biotin is a tight-binding ligand. However, by using a cross-linker that can be chemically cleaved in the shell, the ability of avidin to penetrate the particle and bind to the core can be chemically tuned with a high degree of fidelity.

The polymer used in the construction of these particles is mainly composed of poly(*N*-isopropylacrylamide) (pNIPAm), which is a classic stimuli-responsive polymer.[7] Materials fabricated from pNIPAm are often deemed to be "smart" because they can be tuned to respond to temperature,[8] pH,[9] light,[10] ionic strength,[11] or biomolecules.[12] pNIPAm can be cross-linked into nanoparticles by emulsion or precipitation polymerization processes with good size control over the range 10^{-8}–10^{-5} m.[13] We have developed precipitation polymerization methods to create a class of core/shell hydrogel particles derived from pNIPAm. The specific concept employed for the chemically and topologically complex particles presented herein is shown in Scheme 1. A biotinylated hydrogel core nanoparticle is coated with a hydrogel shell that contains the cleavable cross-linker *N*,*N*′-(1,2-dihydroxyethylene)bisacrylamide (DHEA). At the initial cross-link density, the pore size of the shell is too small to allow avidin molecules to pass through to the core where they bind biotin. Upon chemical cleavage of the cross-links, the pore size increases enough to allow avidin to pass through and bind biotin.

We have previously synthesized core/shell hydrogel nanoparticles by a multistage "seed and feed" method, whereby preformed thermoresponsive hydrogel nanoparticles are used as seeds for the addition of either chemically similar or distinct hydrogel shells.[14] In the present study, the core is predominantly composed of pNIPAm with acrylic acid (AAc) as a minor component (10 mol%). The acid groups are used for attaching biotin to the nanoparticles by carbodiimide coupling (see the Experimental Section in the Supporting Information). These biotinylated cores are then used as seed particles to which a shell is added. The shell also consists of pNIPAm, but it is cross-linked with the cleavable diol cross-linker DHEA. Traditionally, bulk gels that are cross-linked with DHEA have been used for electrophoretic protein



Scheme 1. A NIPAm/AAc core is biotinylated with biotin hydrazide and EDC, followed by addition of a NIPAm/DHEA shell. The cross-linker density of the shell is such that the pore size is smaller than the avidin–HABA complex which prevents the protein from passing through the shell. Upon treatment with periodate, the DHEA cross-linker degrades which subsequently increases the pore size to allow passage of avidin–HABA complex molecules. The K_d value for avidin–HABA (10^{-6}) is higher than the K_d value for avidin–biotin (10^{-15}). Therefore, HABA is released from avidin when biotin binds to avidin. The binding is detected by UV/Vis spectroscopy. NIPAm = *N*-isopropylacrylamide, AAc = acrylic acid, EDC = 1-ethyl-3-(3-dimethylaminopropryl)carbodiimide, DHEA = *N*,*N*′-(1,2-dihydroxyethylene)bisacrylamide, HABA = 2-(4′-hydroxyazobenzene)benzoic acid.

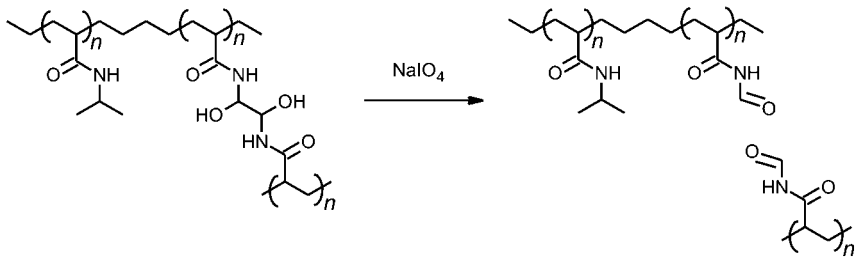
[*] S. Nayak, Prof. L. A. Lyon
School of Chemistry and Biochemistry
Georgia Institute of Technology
Atlanta, GA 30332-0400 (USA)
Fax: (+1) 404-894-7452
E-mail: lyon@chemistry.gatech.edu

[**] L.A.L. gratefully acknowledges financial support from the National Science Foundation, Division of Materials Research (DMR-0203707). Support through a Blanchard Foundation Fellowship is also acknowledged.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200461090

separation.[15] As the vicinal diols in the cross-linker can be cleaved by stoichiometric amounts of sodium periodate, the pore size of the gel can be varied quantitatively by degradation of the cross-linker (Scheme 2). Figure 1 shows the



Scheme 2. Structure of the cleavable polymer. Sodium periodate cleaves the 1,2-glycol bond of DHEA through oxidation of the vicinal diols to aldehydes.

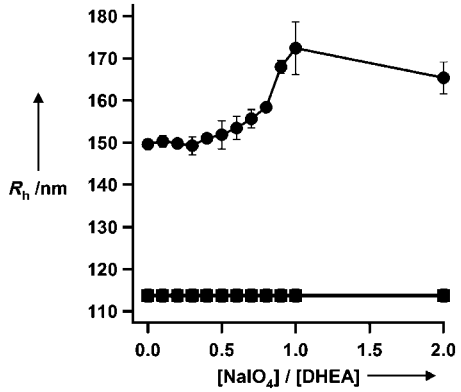


Figure 1. Hydrodynamic radius (R_h) of the core/shell particles (●) as a function of the ratio of periodate:DHEA. The R_h of the core is represented by ■. The error bars represent one standard deviation about the average of five separate measurements.

variation in the hydrodynamic radius (R_h) of the core/shell particle as a function of cleavage of the cross-links in the shell measured at 25 °C (filled circles). The filled squares represent the R_h values for the core. From this we see that the initial R_h value of the core is $\approx$115 nm, whereas that of the core/shell particle prior to the addition of periodate is $\approx$150 nm. Upon addition of periodate to the solution, the shell swells as suggested by the increase in the R_h values of the core/shell particle as a function of periodate concentration. This is expected, as a decrease in the concentration of cross-links in the network should result in a larger equilibrium swelling volume. Ultimately, the R_h value of the core/shell particle increases by $\approx$20 nm following complete cleavage of the periodate cross-links.

To detect the sieving of avidin through the shell we used the colorimetric 2-(4′-hydroxyazobenzene)benzoic acid (HABA) assay.[16] Four equivalents of HABA bind weakly to avidin at the biotin binding site ($K_d = 10^{-6}$ M),[17] and the resultant complex shows an absorption band at $\lambda = 500$ nm. In the presence of biotin, HABA is displaced from avidin ($K_d = 10^{-15}$ M for the avidin–biotin system),[18] and the intensity of the absorbance band at $\lambda = 500$ nm decreases. Figure 2 shows the absorption spectra of the avidin–HABA complex in the presence and absence of biotinylated core particles. The amount of biotin conjugated to the core particle was determined by titration of the acid groups before and after conjugation. Subtracting the number of moles of acid in the biotinylated sample from that in the non-biotinylated sample reveals that $\approx$160 mg of biotin is conjugated to one gram of core particles. From the avidin–HABA assay, only $\approx$25 mg of biotin was determined to be available for binding to avidin in one gram of core particles. This means that only $\approx$16% of avidin is accessible from the total amount of biotin conjugated to the polymer.

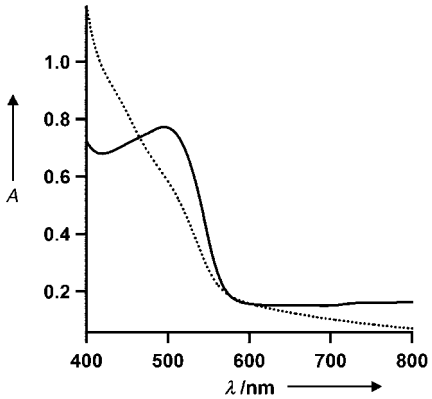


Figure 2. Absorption spectra of avidin–HABA complex in the presence (•••••) and in the absence (——) of biotinylated core particles.

Figure 3 shows the variation in the absorbance of the avidin–HABA complex at $\lambda = 500$ nm as a function of the ratio of the concentrations of periodate/DHEA for particles with 20, 15, and 2% cross-linked shells. For the 20% cross-

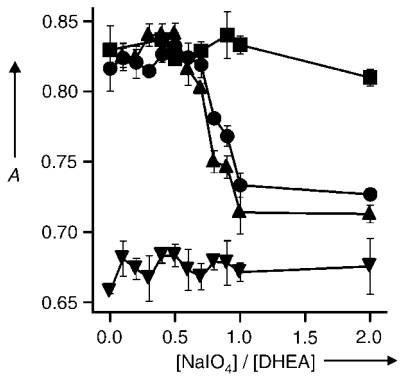


Figure 3. Absorbance of the avidin–HABA complex at $\lambda = 500$ nm as a function of the ratio of periodate:DHEA for 20% cross-linked shell particles (●), 15% cross-linked shell particles (▲), 2% cross-linked shell particles (▼) and biotin-free control particles (■). The error bars represent one standard deviation about the average of three different samples.

linked shell, the absorbance at $\lambda = 500$ nm remains essentially constant with increasing concentrations of periodate until approximately 70% of the diol groups are cleaved. The absorbance decreases precipitously beyond that concentration which is an indication of the displacement of HABA from avidin by the core-bound biotin. This clearly implies that until $\approx 70\%$ degradation, the pore size of the shell is not big enough to allow the passage of avidin–HABA. At $> 70\%$ degradation, the pore size becomes large enough to allow avidin–HABA to pass through the shell and bind biotin. Figure 3 also shows the variation in absorbance for control particles that do not have a biotinylated core. No substantial changes in the absorbance are observed for the control samples which further confirms that the changes in the absorbance observed for the biotinylated samples indeed arise from the binding of biotin to avidin. The 15% cross-linked shell shows the same trend as the 20% cross-linked sample, but the decrease in absorbance starts slightly earlier. This is presumably a result of the larger initial pore size in the 15% cross-linked shell relative to that in the 20% cross-linked shell. For 2% cross-linked shells, the absorbance for 0% degradation is already lower than that for the 20% and 15% cross-linked samples, which were treated with a twofold excess of periodate. This result indicates that the pore size for this cross-linking density is so large that it renders the shell permeable and thereby allows avidin to bind biotin in the core without any degradation of the shell. The increased degree of binding also suggests that more biotin molecules are accessible to avidin, as the more-open network structure of the 2% cross-linked shell imposes less steric hindrance to the transport of the protein. Also, further degradation does not lead to any substantial change in the absorbance which indicates that almost all of the biotin molecules are already accessible to avidin prior to any degradation.

To evaluate how a change in the radius of the protein impacts transport through the particle shell, we used an avidin–HRP (horseradish peroxidase) conjugate in which two moles of HRP were conjugated to one mole of avidin. The molecular weight of this conjugate is ≈ 154 kDa (compare to regular avidin: ≈ 66 kDa). Avidin–HRP binds both biotin and HABA with the same affinity as free avidin. Figure 4 shows the change in the absorbance at $\lambda = 500$ nm of the avidin–HRP–HABA complex as a function of the periodate/DHEA ratio for the 20% cross-linked shell particles. No significant decrease in absorbance is observed until an approximately twofold excess of periodate is added to the sample. Again, normal avidin begins to sieve through the shell at $\approx 70\%$ degradation. This indicates that the avidin–HRP complex is too big to sieve through the shell until all of the cross-links are cleaved. Presumably, the excess periodate is needed to drive the reaction in more highly cross-linked (less accessible) regions of the particle shell; the reaction proceeds under stoichiometrically equivalent conditions in free solution.

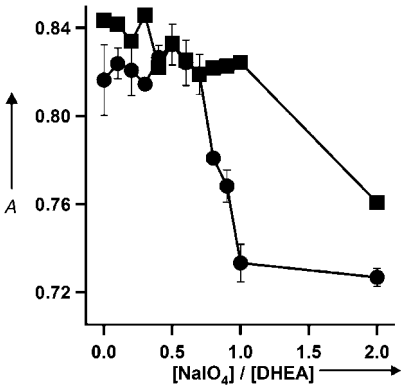


Figure 4. Absorbance of the avidin–HABA complex (●) and avidin–HRP–HABA complex (■) as a function of the percentage of degradation of 20% cross-linked shell particles. The error bars represent one standard deviation about the average of three different samples.

In conclusion, we have demonstrated the synthesis of complex hydrogel nanoparticles that bind to a protein through both native protein–ligand interactions as well as through steric sieving. These core/shell nanoparticles are formed by a simple "seed and feed" polymerization method, in which the shell has cleavable cross-links. The sieving of proteins through the shell to the core is simply controlled by the manipulation of the pore size of the shell through cleavage of those cross-links. At a particular cross-linker density, only avidin is allowed to sieve through the shell, whereas larger protein structures are excluded from binding. Such nanoparticles could find applications in protein separation processes—the shell could essentially behave as a molecular-weight cut-off membrane to eliminate the binding of macromolecules and assemblies that are larger than the protein of interest. Affinity-based sensors and assays that are required to work in complex biological fluids could also benefit from size-selecting structures. Finally, the specific biotin–avidin ligand/protein system used here has become important for a wide range of surface modification and bioconjugation methods. Thus, one could imagine applying this system to the preparation of a wide range of microgel bioconjugates in which access to the biomacromolecule can be reversibly tuned.

Received: June 25, 2004
Revised: August 27, 2004

Keywords: colloids · microporous materials · nanotechnology · polymerization · proteins

[1] S.-Y. Lin, S.-W. Liu, C.-M. Lin, C.-H. Chen, *Anal. Chem.* **2002**, *74*, 330.
[2] R. C. Bailey, J.-M. Nam, C. A. Mirkin, J. T. Hupp, *J. Am. Chem. Soc.* **2003**, *125*, 13541.
[3] S. Shen, K. Hidajat, L. E. Yu, S. Kawi, *Adv. Mater.* **2004**, *16*, 541.
[4] C. D. Jones, L. A. Lyon, *J. Am. Chem. Soc.* **2003**, *125*, 460.
[5] E. Guihen, J. D. Glennon, *Anal. Lett.* **2003**, *36*, 3309.
[6] I. Brigger, C. Dubernet, P. Couvreur, *Adv. Drug Delivery Rev.* **2002**, *54*, 631.
[7] C. Wu, X. Wang, *Phys. Rev. Lett.* **1998**, *80*, 4092.
[8] T. Tanaka, D. J. Fillmore, *J. Chem. Phys.* **1979**, *70*, 1214.
[9] T. Tanaka, D. J. Fillmore, S.-T. Sun, I. Nishio, G. Swislow, A. Shah, *Phys. Rev. Lett.* **1980**, *45*, 1636.
[10] A. Suzuki, T. Tanaka, *Nature* **1990**, *346*, 345.
[11] D. Duracher, F. Sauzedde, A. Elaissari, C. Pichot, L. Nabzar, *Colloid Polym. Sci.* **1998**, *276*, 920.

[12] T. Miyata, N. Asami, T. Uragami, *Nature* **1999**, *399*, 766.
[13] R. Pelton, *Adv. Colloid Interface Sci.* **2000**, *85*, 1.
[14] C. D. Jones, L. A. Lyon, *Macromolecules* **2000**, *33*, 8301.
[15] P. B. H. O'Connell, C. J. Brady, *Anal. Biochem.* **1976**, *76*, 63.
[16] N. M. Green, *Biochem. J.* **1965**, *94*, 23c.
[17] H. Hofstetter, M. Morpurgo, O. Hofstetter, E. A. Bayer, M. Wilchek, *Anal. Biochem.* **2000**, *284*, 354.
[18] O. Livnah, E. A. Bayer, M. Wilchek, J. L. Sussman, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5076.
[19] D. Gan, L. A. Lyon, *J. Am. Chem. Soc.* **2001**, *123*, 7511.
[20] C. D. Jones, L. A. Lyon, *Macromolecules* **2003**, *36*, 1988.

