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COMMUNICATIONS

Published on Web 08/01/2006

Selective Sensing of Metalloproteins from Nonselective Binding Using a Fluorogenic Amphiphilic Polymer

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Development of new biosensors for the detection of proteins is an active area of research due to the implications in proteomics, medical diagnostics, and pathogen detection.[1] Designing artificial scaffolds for this purpose has been of interest, because of their inherently robust characteristics that make them suitable for device fabrication. Conjugated polyelectrolytes have received particular attention for sensing proteins, due to their water solubility and fluorescence properties.[2,3] More specifically, the possibility of energy or electron transfer from the polymer to the biological analyte, resulting in a fluorescence change, renders conjugated polymers useful for sensing metalloproteins. However, it has been shown through a set of careful experiments that such a change in fluorescence behavior could also be achieved through binding of non-metalloproteins.[4] The planarization/deplanarization of the conjugated polyelectrolyte backbone upon interaction with the target analyte results in a chromic change of the polymer.[5] We conceived that covalent incorporation of a fluorophore in a nonconjugated amphiphilic homopolymer nanoassembly, introduced by us in the literature recently,[6] provides a unique solution to circumvent this issue: (i) The polymers form micelle-type assemblies with sizes of about 40 nm and afford optically clear assemblies in water. (ii) The proximity of the hydrophobic fluorophore to the solvent-exposed carboxylate units to which the proteins bind should aid efficient energy- or electron-transfer events. (iii) Since any possible conformational change associated with the binding event is unlikely to change the inherent fluorescence characteristics of a pendent chromophore, the response will be specific to metalloproteins. With these characteristics, we hypothesized that, even if the polymers bind nonspecifically to the positively charged surface of the proteins, response in terms of a fluorescence change will occur only with proteins that contain the appropriate energy or electron-donating/accepting functionalities, i.e., metalloprotein cofactor. We disclose here that our polymer was indeed selective in its fluorescence response to metalloproteins.

The structure of our target polymer is represented by **4** in Scheme 1.[7] Polymer **4** is designed so that the hydrophobic fluorescent anthracene present at the core of the nanoparticle[6] will act as a transducer for sensing, whereas the carboxylate groups presented at the solvent-exposed periphery of the assembly act as ligands for protein binding. The absorption and emission spectrum of polymer **4** reveals that the anthracene functionality is efficiently incorporated onto the polymer backbone and that incorporation into the polymer or aggregation in water does not alter the inherent electronic properties of the anthracene moiety.[7] The ability of polymer **4** to sense proteins was studied by analyzing the change in the anthracene emission with increasing concentration of the protein.

To test our hypothesis regarding the selective response to metalloproteins, we compared the fluorescence response of polymer **4** to that of cytochrome *c* (Cc, a metalloprotein) and lysozyme (a non-metalloprotein). The results are shown in Figure 1. It is clear that the fluorescence from the anthracene moiety in **4** reduced with the increasing concentration of Cc, while addition of similar concentrations of lysozyme to the solution had no effect on anthracene fluorescence. The p*I*'s of Cc and lysozyme are 10.2 and 11.0, respectively. Therefore, we assumed that the nature of the interactions of our amphiphilic polymer assembly with these two proteins would be similar. A fluorescence decrease occurs with Cc, since the porphyrin functionality in this protein is capable of quenching the excited state of anthracene by energy or electron transfer. However, lysozyme does not have a photoactive or electroactive functionality to access anthracene's excited-state energy.

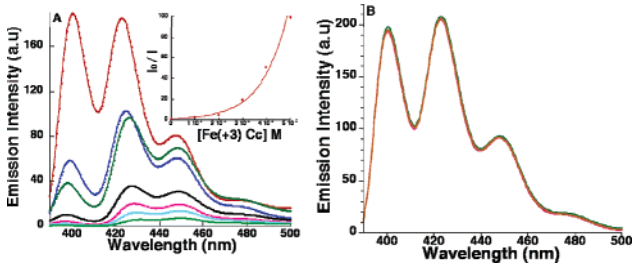
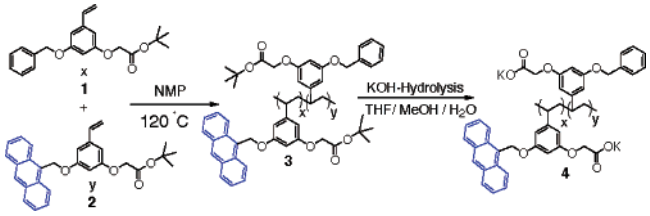


Figure 1. (A) Fluorescence spectra of polymer **4** with various concentrations of metalloprotein (Cc) in 5 mM sodium phosphate buffer (pH 7.4).[7] Inset: Stern−Volmer plot of the fluorescence spectra shown in panel A. (B) Fluorescence spectra of polymer **4** with various concentrations of non-metalloprotein (lysozyme).[7]

Scheme 1. Synthesis of Polymer



Concentration-dependent quenching was used to generate a Stern−Volmer plot (Figure 1A, inset). The slope obtained from the plot of the concentration of the protein *vs* the ratio of fluorescence peak intensities (I_0/I) is the Stern−Volmer quenching constant (K_{SV}). For Cc, this value was 2.0×10^5. The higher value of K_{SV} and the nonlinear behavior indicate a binding-induced fluorescence quenching, *i.e.*, a static quenching.

To test the generality of this sensing mode, we tested the fluorescence response of polymer **4** in water to 12 different proteins (Figure 2). Four of these proteins are metalloproteins, while the other eight proteins are non-metalloproteins. Independent of the p*I* of the protein, none of the non-metalloproteins affect fluorescence changes in the polymer, and thus the K_{SV} value is close to 0 (Figure 2). On the other hand, all metalloproteins exhibited K_{SV} values ranging from 10^4 to 10^6, illustrating the selective response of our nonconjugated, fluorescent polymer to metalloproteins. We have shown that polymers of type **4** can bind to proteins such as chymotrypsin.[8] Therefore, it is clear that the selectivity is in the response and not in binding. It is important to recognize that the

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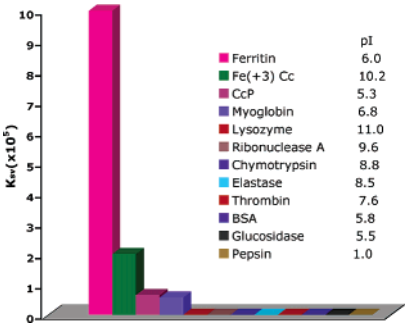

Figure 2. K_{SV} values of different proteins: ferritin ($K_{SV} = 1.0 \times 10^6$), Cc ($K_{SV} = 2.0 \times 10^5$), myoglobin ($K_{SV} = 5.7 \times 10^4$), CcP ($K_{SV} = 6.6 \times 10^4$), and all other non-metalloproteins ($K_{SV} \cong 0$).

K_{SV} value itself represents not only the binding affinity of the metalloproteins with the polymer but also the relative ability of the protein to quench the excited state of anthracene. For example, ferritin exhibits the highest K_{SV}; this is likely to be due to the fact that a single protein binding event brings hundreds of bound Fe^{2+}, the key electron-accepting functionality, close to the otherwise fluorescent anthracene moiety. On the other hand, the greater K_{SV} of Cc, compared to those of cytochrome *c* peroxidase (CcP) and myoglobin, could arise from the difference in binding affinities due to the differences in p*I*. Note, however, that p*I* alone is not a good indicator of the ability of a protein to bind to a charged polymer surface.[9] Moreover, this simple p*I*-dependent binding affinity explanation, and therefore its impact on K_{SV}, is complicated by the fact that shifts in electronic spectra of porphyrins are possible (and therefore changes in redox potentials and energy-accepting capabilities) upon binding to a species with a complementarily charged surface.[10]

We were interested in identifying the possible modes of fluorescence quenching in these polymers. There are mainly two limiting mechanisms of fluorescence quenching: energy transfer and electron transfer. Among the metalloproteins studied, the mechanism of quenching for ferritin is likely to be based on electron transfer, since there is no chromophore that could accept the excited-state energy from anthracene. The other three proteins have porphyrin cofactors, the absorbance spectra of which overlap well with the emission spectra of anthracene. Therefore, a Förster-type energy transfer is possible. But, the redox potentials of these proteins are such that an electron-transfer-based quenching is also viable. Usually, the fluorescence emission from the energy acceptor upon exciting the energy donor is considered to be a clear evidence for energy transfer. However, in our case, such a distinction is difficult, since Fe−porphyrins are inherently poor fluorescence emitters.

To investigate whether both energy- and electron-transfer quenching is possible with this polymer, we have carried out the following model studies. We have shown before that our homopolymer-based polyelectrolyte assemblies are capable of binding small molecules with complementary charges.[11] Methyl viologen (MV^{2+}) is a cationic molecule that can accept an electron from the excited state of anthracene but does not have the possibility of accepting its energy. We found that MV^{2+} was able to quench the fluorescence of anthracene with a K_{SV} of 9.3×10^3 M^{-1} (Figure 3A). On the other hand, proflavin is a cationic dye which has an absorption spectrum that overlaps perfectly with the emission spectrum of anthracene. Therefore, the latter molecule can accept energy from anthracene. If this molecule does accept the energy, it should be observable through an emission from proflavin upon excitation of anthracene. Indeed, when anthracene was excited at 373 nm, a significant amount of fluorescence arose from proflavin with a concomitant decrease in fluorescence from anthracene (Figure 3B). Control experiments showed that directly exciting

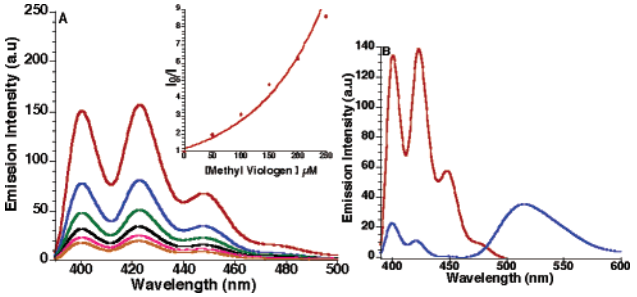

Figure 3. (A) Fluorescence spectra of polymer **4** in the presence of various concentrations of methyl viologen.[7] Inset: Stern−Volmer plot of the fluorescence spectra shown in panel A. (B) Fluorescence spectra of polymer **4** in the absence (red line) and in the presence (blue line) of proflavin.[7]

proflavin at 373 nm, by itself, at the same concentration does not afford a significant fluorescence. The K_{SV} value for this process was found to be 4.6×10^4 M^{-1}. These results suggest that both energy- and electron-transfer mechanisms could operate in this sensing event. We could not resolve which mechanism is dominant in the metalloprotein binding studies due to the inherent lack of fluorescence from protein cofactors; this will, however, be a focus of our studies using time-resolved spectroscopy.

In summary, we have shown that a nonconjugated, fluorescent, amphiphilic polymer can recognize proteins nonspecifically but responds only to metalloproteins. The reasons for this selective response are that (i) the cofactors in metalloproteins can quench the excited state of the fluorescent polymer by an energy- or electron-transfer process and (ii) there are no complications that arise from fluorescence response to binding-induced conformational changes. While these polymers are selective to metalloproteins, these results are not specific to a particular metalloprotein at this time. Designing amphiphilic polymer surfaces that exhibit such features is a part of the ongoing efforts in our laboratories.

Acknowledgment. The authors thank the National Institutes of Health (GM-65255), Office of Naval Research, and the NSF-supported Center Hierarchical Manufacturing for financial support.

Supporting Information Available: Detailed procedure for the synthesis of monomers and polymer and fluorescence spectra of polymer **4** with different proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA063544V