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Technical Notes

Protein Recognition via Surface Molecularly Imprinted Polymer Nanowires

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In this paper, we present a technique for the preparation of polymer nanowires with the protein molecule imprinted and binding sites at surface. These surface imprinting nanowires exhibit highly selective recognition for a variety of template proteins, including albumin, hemoglobin, and cytochrome *c*. This recognition may be through a multistep adsorption, with the specificity conferred by hydrogen bonding and shape selectivity. Due to the protein imprinted sites are located at, or close to, the surface; these imprinted nanowires have a good site accessibility toward the target protein molecules. Furthermore, the large surface area of the nanowires results in large protein molecule binding capacity of the imprinted nanowires.

Molecularly imprinted polymers (MIPs) are artificial, template-made receptors with the ability to recognize and to specially bind the target molecule.[1] The stability, ease of preparation, and low cost of these MIPs have led to their assessment as substitutes for antibodies or enzymes in chemical sensors, catalysis, and separations.[2−5] Although creating a MIP against small molecules is straightforward now, imprinting of large structures, such as proteins and other biomacromolecules, is still a challenge. The major problem associated with the imprinting of such large structures lies in the restricted mobility of them within highly cross-linked polymer networks and the poor efficiency in rebinding. Early developments included the creation of protein imprinted cavities in a thin layer of acrylic polymer[6] or in conventional acrylamide gels,[7] but there was only limited success. Up to now, imprinting at the surface seems to be the most promising way to overcome such difficulties. Because the imprinted sites are situated at or close to the surface of MIPs, they are easily accessible to target protein molecules. Unfortunately, these surface imprinting MIPs are not universally adopted because their preparation requires specially adapted protocols;[8] and they always have a lower protein binding capacity compared to bulk imprinting MIPs.[9]

Mosbach and co-workers have introduced a remarkably clever protocol for creating surface imprinting based on oriented immobilization of the imprint molecule on porous silica beads prior to polymerization.[10] This protocol has been further developed by Sellergren for the imprinting of amino acids and peptides.[11,12] Recently, our group also reported a similar protocol for creating surface imprinting nanowires by immobilizing of the imprint molecule within the pores of the nanoporous alumina membrane. These nanowires are relatively monodispersed and have a moderately high imprinting surface area.[13]

In this paper, we present a technique for the preparation of polymer nanowires with the protein molecule imprinted and binding sites at surface (Figure 1). First, the template protein molecule is immobilized on the pore walls of the nanoporous alumina. The nanopores are then filled with a mixture of the functional monomer (acrylamide in this paper) and cross-linking monomer (*N*,*N*′-methylenebisacrylamide in this paper). Polymerization is then initiated via oxidation with ammonium persulfate. Following polymerization in the nanopores, the alumina membrane is subsequently removed by chemical dissolution, leaving behind polymer nanowires with protein molecule binding sites situated at the surface.

EXPERIMENTAL SECTION

Reagents. The alumina membranes were purchased from Whatman that had nominally 100-nm-diameter pores. (3-Aminopropyl)trimethoxysilane was purchased from Aldrich and used as received. Cytochrome *c* from horse heart, cytochrome *c* from bovine heart, human hemoglobin, bovine hemoglobin, human

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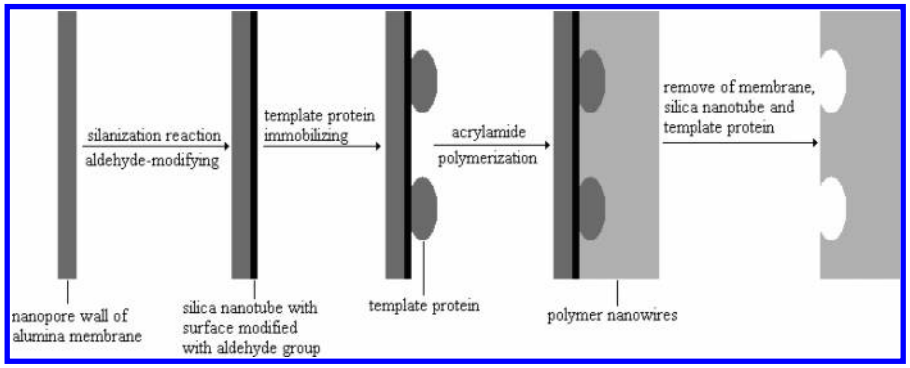

Figure 1. Schematic representation of the protein molecule imprinted approach employing immobilized template and a sacrificial support.

insulin, streptavidin, ribonuclease A, acrylamide and *N*,*N*′-methylenebisacrylamide were purchased from Sigma and used as received. Distilled, deionized water was used for preparation of all aqueous solutions.

Protein Immobilized on the Pore Wall of Alumina Membrane. A sol−gel template synthesis method was used to deposit silica nanotubes within the pores of the alumina membranes. First, the alumina membrane was modified with an aminopropylsilane. This was accomplished by immersing the membrane into a solution prepared by mixing 0.5 mL of (3-aminopropyl)trimethoxysilane with 3 mL of ethanol that containing 0.2 mL of sodium acetate buffer solution (50 mM, pH 5.0). The silanization reaction was terminated after 5 min by rinsing the membrane with ethanol. The membrane was then cured in an oven at 150 °C for 2 h. The freshly made aminopropyl group-modified alumina membrane was immersed for 120 min in 0.2 M glutaraldehyde containing phosphate buffer solution (pH 7.0). The resulting aldehyde-modified membranes were then rinsed with ethanol and dried for 12 h at 45 °C with N_2 flushing.

The aldehyde-modified membrane was added to a flask and placed under vacuum for 30 min. After the template protein solution (4 mg of protein/mL of phosphate buffer containing 120 mg of acrylamide and 21.5 mg of *N*,*N*′-methylenebisacrylamide) was added under vacuum, the mixture was incubated overnight at 4 °C and then for 1 day at room temperature. The protein-modified membrane was then mounted into a flow device and thoroughly rinsed with phosphate buffer solution (containing 120 mg of acrylamide, 21.5 mg of *N*,*N*′-methylenebisacrylamide and 5 mg of ammonium persulfate per 1 mL) before use.

Preparation of Polymer Nanowires with Protein Imprinting and Binding Sites at Surface. The protein-modified membrane was immersed in 4 mL of phosphate buffer solution (containing 480 mg of acrylamide, 86 mg of *N*,*N*′-methylenebisacrylamide, and 20 mg of ammonium persulfate) at 35 °C for 2 h and at 45 °C for 2 h with fast vibration. Then the membrane was rinsed with ethanol and dried for 5 h at 45 °C. After dissolving alumina membrane and silica nanotubes with 1 M NaOH at 4 °C, the resulting polymer nanowires were washed with a 10% (v/v) solution of acetic acid containing 10% (w/v) SDS for three times. It must be noted that ultrasonic vibration could not be used in the dissolving and washing processes because it would break the polymer nanowires.

RESULTS AND DISCUSSIONS

Nanoporous alumina membrane with a diameter of 100 nm was used in this study. A sol−gel template synthesis method was used to deposit silica nanotubes (with a wall thickness of several nanometers) within the pores of the alumina membrane (using (3-aminopropyl)trimethoxysilane).[14,15] The terminal amino groups then reacted with bifunctional glutaraldehyde to form free aldehyde ends. Aldehyde groups react spontaneously with free amino sites on protein, and this approach was used to attach the template protein molecule to the inside walls of the silica nanotubes. The same aminopropyl-derived alumina membrane was used for the preparation of the control polymer.

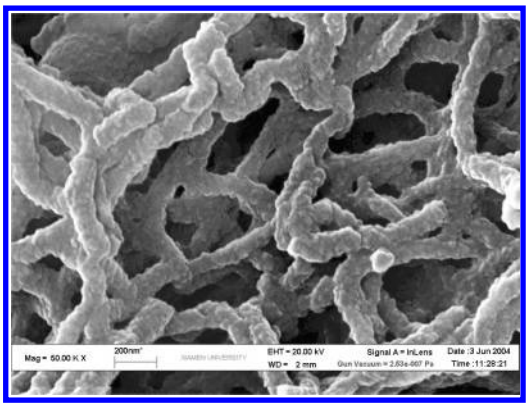

Figure 2. SEM of polyacrylamide nanowires.

The degree of cross-linking of imprinted polymer is very important in this experiment. When the mole ratio of cross-linking monomer to functional monomer used for polymerization was lower than 1:50, the imprinted polymer formation in nanopore was shapeless (no nanowire structure could be found by scanning electron microscopy, SEM). When this ratio was higher than 1:5, the capacity and selectivity of imprinted nanowires to template protein decreased dramatically. We think this may be due to the incomplete washing of the template protein and the poor efficiency in protein rebinding at such a high cross-link structure. So the mole ratio of cross-linking monomer to functional monomer used in the experiment was set at 1:12. Following the polymerization process, the alumina membrane was subsequently removed by NaOH dissolution. The SEM results (Figure 2) verify the formation of polymer nanowires with controlled size in the nanoporous alumina membrane.

The remaining imprinted protein molecules have a negative effect on the rebinding properties of the imprinted nanowires.

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They occupy the binding sites and thus prevent the adsorption of other molecules. Thus, the influence of the washing procedure (i.e., the residual quantities of imprint protein molecule occupying the binding sites) on the rebinding properties of the bovine hemoglobin (Hb) imprinted nanowires was evaluated. Prior to each rebinding test, the imprinted polymer was washed with a 10% (v/v) solution of acetic acid containing 10% (w/v) SDS. The rebinding was performed using 2 mL of bovine Hb solution (100 μg/mL bovine Hb in pH 7.0 PBS solution containing 0.01% Tween-20) added to 2 mg of bovine Hb-imprinted nanowires. The results showed that as the number of washing steps increases, the amount of bovine Hb rebound by the nanowires increases. The quantity of the protein rebound reached a maximum value after three washing cycles, and then the rebinding capacity stayed unchanged afterward.

After the removal of the embedded protein, the protein-imprinted sites are created on the nanowire surface. These sites have a physical shape complementary to the protein molecule and also might have a precisely positioned amide group. We think that these imprinted sites can specifically recognize their template protein molecules. When a protein molecule binds to a complementary binding site, a multitude of simultaneous hydrogen bonds can be formed between the oriented amide groups within the binding site and the protein surface polar residues. These cooperative, multivalent hydrogen bonds lead to a significantly increased overall protein binding affinity. Furthermore, van der Waals forces may also play an important role in specific recognition because of steric complementarity between the target protein molecule and the imprinted site.

Based on the above supposition, bovine Hb imprinted nanowires were used as a model to study the template protein binding ability of such kind of surface imprinting MIPs. Dry imprinted nanowires or control nanowires were mixed with 1 mL of bovine Hb solution (100 μg/mL bovine Hb in pH 7.0 PBS solution containing 0.01% Tween-20) in a 1.5-mL microtube. The microtube was oscillated in an oscillator at 25 °C for 3 h. After the nanowires were removed by centrifugation, the concentration of bovine Hb in the supernatant was determined using a spectrophotometer at 405 nm. The results showed that the imprinted nanowires have a higher binding capacity for bovine Hb than those of the control nanowires (Figure 3). At a nanowire concentration where the imprinted nanowires bind 50% of the bovine Hb, the control nanowires bind only 6.8%. Imprinted nanowires were also prepared with different protein molecules to investigate the universality of applicability of such surface imprinting protocol. Imprinted nanowires prepared with bovine serum albumin (BSA), bovine cytochrome *c*, and horseradish peroxidases (HRP) were checked for their ability to rebind template proteins (Table 1). These imprinted nanowires were also found to have a higher template protein binding capacity than the control nanowires.

Competitive binding of a template protein in respect to a nontemplate protein was studied (Figure 4). The selective nanowires were prepared with bovine Hb as template and assayed for their capability of rebinding with a mixture made of a fixed concentration of the template protein and a series of increasing concentrations of the competing protein. The competitors chosen were BSA (M_r 66 000, isoelectric point 4.7), streptavidin (M_r 60 000, isoelectric point 5.0), human insulin (M_r 5800, isoelectric point 5.3), HRP (M_r 44 000, isoelectric point 7.2), human IgG (M_r 150 000, isoelectric point 9.0), and ribonuclease A (M_r 13 700, isoelectric point 9.6). Figure 4 shows the quantity of template protein (bovine Hb) bound to the imprinted nanowires plotted as its dependence on the concentration of competing protein. It

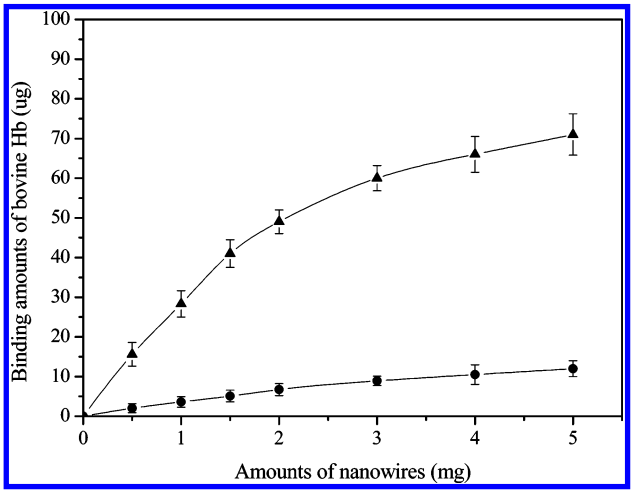

Figure 3. Binding profiles of bovine protein as a function of the nanowires concentration. (▲) Imprinted nanowires and (●) control nanowires. The points represent mean values of three measurements.

Table 1. Binding of Proteins to MIP and Blank Nanowires[a,b]

	protein binding amounts (μg)		
nanowires	BSA	bovine cytochrome *c*	horseradish peroxidases
MIP	25.1	19.7	21.9
blank	5.7	3.1	4.3

[a] Experiment was conducted by the addition of 1 mg of nanowires in 100 μg/mL protein solution (1 mL) pH 7 at room temperature. [b] $n = 3$.

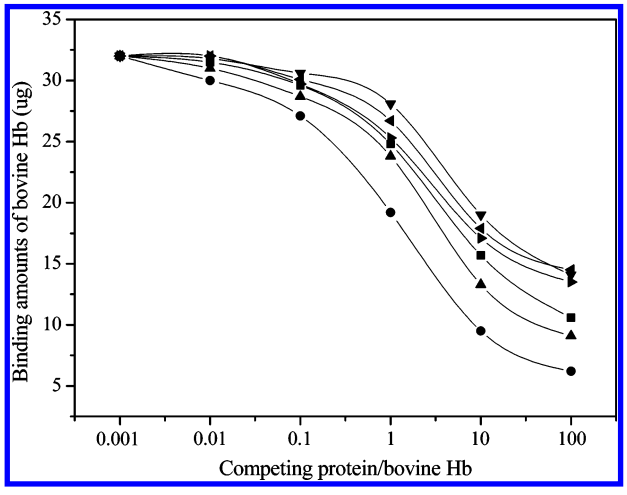

Figure 4. Competitive rebinding of template protein (bovine Hb) in respect to a nontemplate protein. Rebinding was done at pH 7 for 3 h, with a fixed concentration of bovine Hb and increasing concentration of competing protein. Competing proteins: (●) BSA, (▲) streptavidin, (■) HRP, (▲) human IgG, (▲) ribonuclease A, and (▼) human insulin.

Table 2. Specificity of the Binding of Homologous Proteins to MIP Nanowires[a,b]

	protein binding amounts (μg)			
imprinted nanowires	bovine Hb	human Hb	bovine cytochrome *c*	horse cytochrome *c*
bovine Hb MIP	27.2	7.1		
human Hb MIP	6.9	25.9		
bovine cytochrome *c*			19.7	18.6
horse cytochrome *c*			18.1	17.5

[a] Experiment was conducted by the addition of 1 mg of nanowires in 100 μg/mL protein solution (1 mL) pH 7 at room temperature. [b] $n = 3$.

clearly shows that the addition of the competing protein displaces the binding of bovine Hb. The more competing protein added, the less quantity of bovine Hb bound to the imprinted nanowires. In both cases, the high selectivity of the imprinted nanowires toward its template protein was demonstrated. And isoelectric points of the proteins seem to have no obvious influence on competitive binding.

To further investigate the specificity of these imprinted nanowires in protein recognition, we imprinted two kinds of homologous proteins (Table 2). It is interesting to note that the imprinted nanowires were able to discriminate between bovine Hb and human Hb, notwithstanding that these two Hb proteins have a similar amino acid sequence (differing in only 15% of the 574 amino acid positions) and three-dimensional structure. This result confirms the high specificity of the imprinted nanowires (Table 2). Surely, the reason for this remarkably high selection should lay in their difference in cooperative, multivalent hydrogen bonds. And it should also be noted from Table 2 that the imprinted nanowires were unable to discriminate between bovine cytochrome *c* and horse cytochrome *c*. We propose it is due to the exceedingly similar amino acid sequence of these two cytochrome *c* proteins.

CONCLUSIONS

The results presented here demonstrate that the surface imprinted nanowires can be use for the recognition of template protein molecules. This recognition may be through a multistep adsorption, with the specificity conferred by hydrogen bonding and shape selectivity. Because the protein imprinted sites are located at, or close to, the surface, these imprinted nanowires have a good site accessibility toward the target protein molecules. Furthermore, the large surface area of the nanowires results in large protein molecule binding capacity of the imprinted nanowires. These imprinted nanowires can be well dispersed in aqueous media and their applications should therefore be compatible with procedures where biological antibodies might otherwise be used, such as affinity chromatography and biochemical sensor. The surface imprinting protocol reported in this paper may also be used to imprint other biomacromolecules, such as DNA and virus. But it may be unfitted to imprint other larger biomacromolecules such as cells and bacteria, because these biomacromolecules are too large to enter the nanopores of nanoporous alumina membrane. We propose that the imprinting of smaller peptides corresponding to a unique surface amino acid sequence of a target cell or bacterium on the nanowire surface may be an effective way to imprint these larger biomacromolecules. Some work in this direction is already in progress and will be communicated in due course.

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