DOI: 10.1002/chem.200601624

A Smart Surface in a Microfluidic Chip for Controlled Protein Separation

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Abstract: The smart surface created in a microfluidic chip has shown the capability of adsorbing and releasing proteins under electrical control. The inner surface of the chip channel was first coated by a thin layer of Au through sputtering and was subsequently modified with loosely packed self-assembled monolayers (SAMs) of thiols with terminal carboxylic or amino groups. Upon application of an external electric potential to the gold substrate, reversible conformational transformation between "bent" and "straight" states for the anchored mercapto chains could be modulated, through the electrostatic effect between the ionized terminal groups and the charged gold substrate. Thus, a hydrophobic or hydrophilic channel surface was established and could be reversibly switched electrochemically. Accordingly, the microchips prepared in this way can reversibly and selectively adsorb and release differently charged proteins under electrical control. Two model proteins, avidin and streptavidin, were demonstrated to be readily adsorbed by the smart chips under negative and positive potential, respectively. Also, more than 90% of the adsorbed proteins could be released upon an electrical command. Furthermore, these chips were applied to the controlled separation of avidin and streptavidin mixtures with 1:1 and 1:1000 molar ratios. Under specific applied potentials, the chips adsorbed a certain protein from the mixture whereas the other protein was allowed to flow out, after which the adsorbed protein could be released by switching the applied potential. Thus, two eluted protein fractions were obtained and the separation of the two proteins was achieved. For the former mixture, each eluted fraction contained up to ≈80–90% avidin or streptavidin. For the latter mixture, the resulting separation efficiency indicated that the molar ratio of avidin and streptavidin could be increased from 1:1000 to about 32:1 after five run separations.

Keywords: cyclic voltammetry • microfluidic chips • proteins • self-assembly • smart surfaces

Introduction

The development of a "smart surface" with reversible properties, for example, wettability,[1–6] has attracted substantial research interest recently. This is due to their enormous potential for applications such as self-cleaning surfaces,[7] intelligent interfaces or devices, bioseparation,[8] and easily-controlled cell culture.[9] Diverse external stimuli used to trigger reversible changes in the properties of smart surfaces have included photoillumination,[10,11] electric potential,[6,12,13] thermal driving,[14] magnetic field,[15] and surrounding media treatment.[16] These external stimuli usually trigger changes in the structure, conformation, or oxidation states of the molecules that modify the surface, and thus result in the simulation of an "on/off" switch in the macroscopic properties of the surface. Many kinds of materials have been employed to establish this kind of controlled switchable surface including inorganic metal oxides (e.g., zinc oxide), smart polymers (e.g., temperature-responsive poly(*N*-isopropylacrylamide) (PNIPAAm)), and self-assembled monolayers (SAMs), as reviewed by our previous work.[17] Recently, SAMs have become more and more attractive and are considered to be a novel and important candidate in this field.[18–21] The "on/off" switch of a SAM-modified smart surface is normally based on dissociation/reassociation,[22,23] conformational transition,[19,24] and reversible attachment[25–27] of the assembling molecules in response to the external stimulations. For example, Joerg Lahann and coworkers demonstrated a kind of low-density SAM (LD-SAM). This LD-SAM has sufficient spatial freedom for each molecule to undergo a conformational transition between "straight" and "bent" under applied electric potentials, which subsequently results in macroscopic changes in surface properties.[19] In our previous study, which had been

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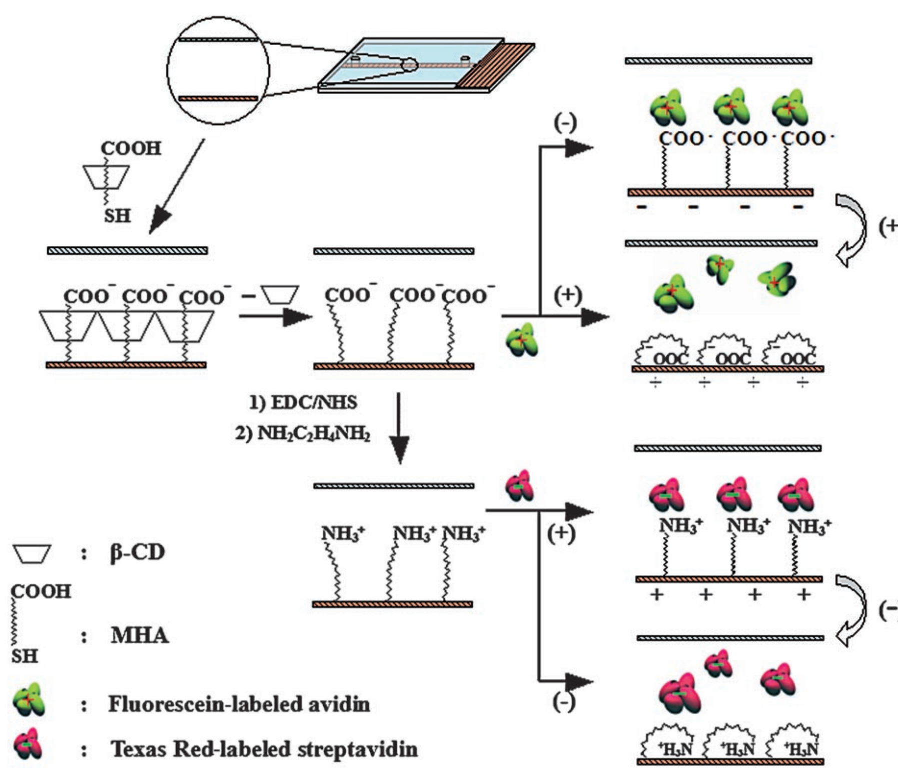
inspired by this result,[20,21] by employing cyclodextrin as a removable spacer, we successfully created a mercaptohexadecanoic acid (MHA) LD-SAM in which the individual MHA molecules can also undergo a conformational transformation under electrical control. The MHA LD-SAM-modified smart surface was proven to reversibly undergo switching between hydrophobic/hydrophilic properties in response to the external electric potential. Its application in selectively capturing a charged target protein, for example, avidin or streptavidin, was also studied.[21] In addition to significant advancements in developing smart SAMs,[28,29] efficient and high-throughput devices that could put the possible applications of SAMs into practice are also highly desirable.

Many efforts have been directed towards the development of a micro total analysis system (μ-TAS), which is regarded to be promising technology for biological separation and assays, medical diagnosis, and so forth.[30,31] In this context, the microchip is one of the most attractive and powerful tools that could be used for this purpose and that has been applied in diverse research fields, including sensors, and chemical and biological reactors, owing to its rapid and high-throughput analysis, minimized consumption of sample and reagent, as well as lower manufacturing cost.[32] Although intelligent microfluidic devices employing smart polymers, for example, PNIPAAm, as the smart agent have been reported,[33–37] the creation of a smart microfluidic system through use of smart SAMs has rarely been addressed. The difficulty to control the microcosmic dimension and structure of SAM molecules is believed to be the drawback of this field. However, the combination of these two concepts, that is, the smart SAM and microchip, could be very promising in protein separation, and is also the pursuit of this work. It could provide a new route to the separation of mixed proteins, especially for a sample that has high- and low-abundant proteins.

In this report we discuss the design, construction, and characterization of "smart" microchips, and their applications in controlled protein separation. Two kinds of smart microchips, the microchip modified with MHA LD-SAM (abbreviated as COOH-chip) and the microchip modified with LD-SAM that contains terminal amino groups (abbreviated as NH_2-chip), were fabricated in a similar way to that described in our previous procedures on solid electrodes.[20,21] The reversible conformational transformation of the SAM molecules that responded to the external applied electric potentials resulted in an alternation between hydrophobicity and hydrophilicity (also between electroneutrality and electronegativity or electropositivity) of the microchannel surface. These smart microchips could then selectively separate positively or negatively charged protein from their mixtures. In an attempt to find a possible alternative method for concentrating low-abundance proteins, which is very important for proteomics research, it was found that they could even separate low concentrations of protein from a protein mixture. In this study, two differently charged proteins, avidin and streptavidin (Scheme 1), the isoelectric points (IP) of which are 10.5 and 5.8, respectively, were chosen as the model proteins to demonstrate the feasibility of the idea. The resultant smart microchip was proven to be efficient, cost effective, and showed low energy consumption. The applied electric potential was only 0.3 or −0.3 V (vs. Ag/AgCl, the same hereafter), which suggests that simple hand-held battery operation is feasible and practical. It is believed that these SAM-modified smart microchips could supply a favorable alternative route to protein separation that could be employed in proteomics, medical diagnostics, and environmental surveillance.



Scheme 1. Illustration of the smart microfluidic chips and the electrically controlled adsorption/release of avidin and streptavidin.

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Results and Discussion

Electrically controlled protein adsorption/release in the smart microchips: As described in the Experimental Section, the COOH-chip and NH_2-chip were prepared and then characterized by cyclic voltammetric (CV) sweeping, FTIR-ATR (ATR: attenuated total reflectance), and X-ray photoelectron spectroscopy (XPS).[38] As shown in Figure 1, the CV peak currents (i_p) for the (IC)-modified channel (IC: inclusive complex between MHA and cyclodextrin) are much smaller than those for the bare (unmodified) channel, which reveals the presence of the assembled insulating IC SAM on the channel surface. The COOH-chip was prepared by removing β-cyclodextrin (β-CD) molecules from the SAM through thorough washing with absolute ethanol, as displayed by the increase of the i_p.[21] From both the FTIR and XPS spectra it can be seen that an NH_2-terminated surface was obtained by treating the COOH-terminated surface with 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) followed by ethylenediamine. The resultant microchips were then used in the following protein adsorption and release experiments.

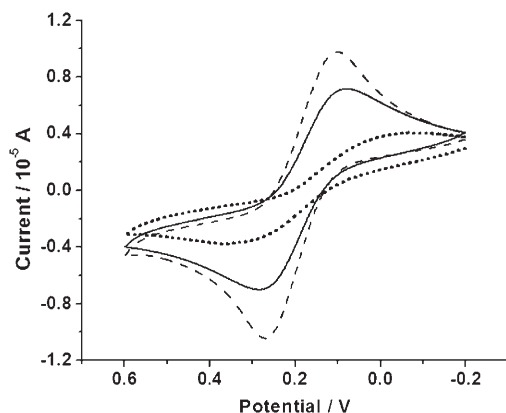

Figure 1. CV curves of bare (-----), IC-modified (•••••), and MHA LD-SAM-modified (——) microchannel surfaces. Electrolyte: KCl (100 mmol L^{-1}) containing $K_3Fe(CN)_6/K_4Fe(CN)_6$ (2.5 mmol L^{-1}); scan rate: 10 mV s^{-1}.

The controlled avidin (positively charged under the experimental conditions, pH 7.4) adsorption in the COOH-chip was investigated by fluorescence (FL) measurements. As shown in Figure 2, the FL emission intensities of the avidin solutions that have been adsorbed by the COOH-chip under different potentials vary. The avidin solution eluted from the COOH-chip after being adsorbed under 0.3 V exhibits the largest FL intensity, whereas that under −0.3 V exhibits the smallest, and the difference is up to about 85.76% of the total loaded protein. These results reveal that under 0.3 V, most of the fluorescein-labeled avidin molecules remained in the solution, but at −0.3 V, they were captured by the microchip. Because the ionized carboxyl groups were negatively charged and could thus be electrostatically attracted or repelled by the charged substrate, the above results can be attributed to the "bent"/"straight" state of the MHA molecules under positive/negative potential, which made the microchannel surface hydrophobic/hydrophilic and electronegative. Therefore, the channel surface was inclined to adsorb avidin under a negative potential. The FL intensity of the protein solution that had been adsorbed in the COOH-chip at 0 V is smaller than that under 0.3 V. We conclude that when no potential was applied, the MHA molecules oriented randomly, thus parts of the MHA molecules were straight and could adsorb some avidin molecules.

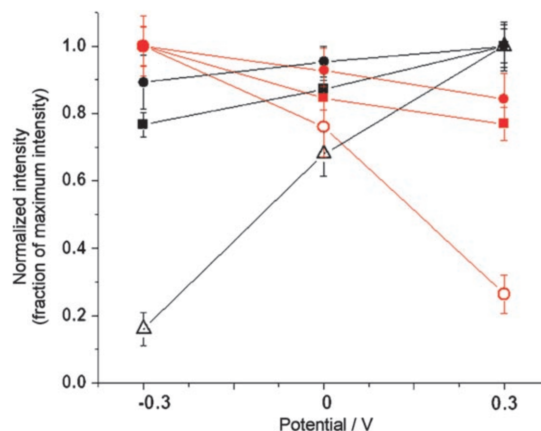

Figure 2. Normalized FL emission intensity (FL emission intensity of the phosphate buffer solution, PBS, was set as blank and had been deducted) of the eluted avidin solution from the HD-MHA-chip (●), bare microchip (■), and COOH-chip (△), and that of the eluted streptavidin solution from the HD-NH_2-chip (●), bare microchip (■), and NH_2-chip (○) after being adsorbed in the microchips under different potentials for 30 min. For each chip, the FL intensities of the outflows were normalized to the maximum one. The data were the average of three measurements. The excitation wavelength (λ_{ex}) was 494 nm (for avidin) or 595 nm (for streptavidin), whereas the emission wavelength (λ_{em}) was 518 nm (for avidin) or 615 nm (for streptavidin).

In order to further confirm that the different protein-adsorbing abilities of the COOH-chip under different applied potentials were derived from the conformational transformation of MHA molecules rather than the electrostatic force of the gold substrate, triggered by electric potential, two control experiments were performed. Avidin-adsorption experiments on a bare gold microchip and on a high-density MHA SAM-modified microchip (HD-MHA-chip, prepared by directly modifying the microchip channel surface with a solution of MHA in ethanol) under −0.3, 0, and 0.3 V were carried out. As shown in Figure 2, compared with the COOH-chip, there is little difference in avidin-adsorbing capability under different potentials when using the HD-MHA-chip. Because of the compact packing of the MHA molecules there was almost no conformational transformation upon applying external electric potentials, and accordingly, no change in surface properties. The avidin adsorption on the bare gold surface resulted solely from the electrostatic force of the substrate and showed only ≈30% of that in

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the COOH-chip when a negative potential was applied, which suggests that it was the MHA LD-SAM instead of the substrate that dominated the protein attachment. These results confirm that only the COOH-chip has absolute electrically controlled protein-adsorption capability, and that this controlled property comes from the conformational transformation of MHA molecules, triggered by the electric potential.

In contrast, the NH_2-chip showed an entirely opposite trend with respect to the adsorption ability for streptavidin (negatively charged under the experimental conditions, pH 6.6) upon change in potential. As shown in Figure 2, the outflow from the NH_2-chip after being adsorbed under 0.3 V shows the smallest FL emission intensity, whereas that under −0.3 V shows the largest, and the difference is up to about 73.54%. Because under positive potential the molecules on the channel surface of the NH_2-chip achieved a "straight" state owing to the fact that the amino groups were charged positively, accordingly, the negatively charged streptavidin molecules were trapped by the microchip. These amino-terminated molecules exhibited a "bent" conformation under negative potential, so the microchannel surface switched to be hydrophobic and was unable to adsorb streptavidin. These results demonstrate that the microchip can be electrically controlled to adsorb negatively charged proteins.

Electrically controlled consecutive adsorption and release of avidin (streptavidin) in and from the COOH-chip (NH_2-chip) were studied by in situ laser-induced fluorescence (LIF, Figure 3) and FL measurements (Table 1). As shown in Figure 3, after −0.3 V had been applied to the COOH-chip for 30 min, the FL signals of the outflow from the COOH-chip were recorded. The first observed peak represents unbound avidin flowing past and out of the chip channel. After four minutes into the experiment, the applied potential was converted to −0.3 V and another elution peak was observed, corresponding to avidin captured by the chip. This experiment was repeated three times, and the similar results confirmed that the avidin adsorbed in the COOH-chip under −0.3 V can be released under the opposite potential. This can be attributed to the conformational transformation of the MHA molecules from "straight" to "bent" which make the channel surface hydrophobic with almost no affinity for the avidin molecules. Table 1 exhibits the quantitative results from the FL measurements both for avidin and streptavidin. It shows that the COOH-chip has 85.99/90.58% adsorption/release efficiency for avidin under −0.3/0.3 V, whereas the adsorption/release efficiency of the NH_2-chip is 74.60/94.64% for streptavidin under 0.3/−0.3 V.

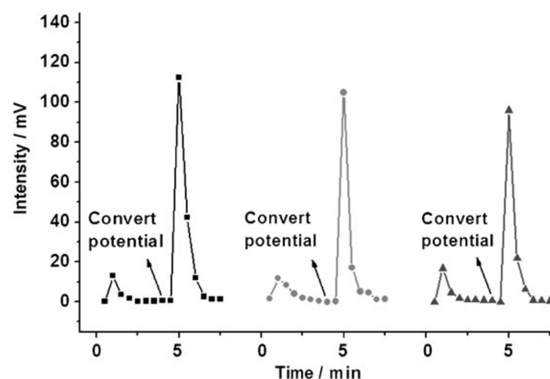

Figure 3. FL emission intensity (against time) of the eluted avidin solutions from the COOH-chip after −0.3 and 0.3 V were applied consecutively. The ■, ●, and ▲ symbols represent values for the first, second, and third runs of the experiments, respectively. $\lambda_{ex}/\lambda_{em}=494/518$ nm. Injection sample: PBS (10 μL) containing avidin (0.1 g L^{-1}, pH 7.4).

To investigate the reproducibility of these smart microchips, multiple adsorption/release experiments were carried out on one single COOH-chip. These switching processes were repeated for up to 40 cycles with a ≈30% decrease in the amount of adsorbed avidin.

Electrically controlled protein separation: Because the COOH-chip and the NH_2-chip exhibit the capability to electrically control the adsorption and release of the positively or negatively charged proteins, such as, avidin and streptavidin, it was natural to work out a concept to exploit these smart microchips in the controlled separation of differently charged proteins. Therefore, the separation of an avidin and streptavidin mixture by using the smart microchips was investigated.

As shown in Figure 4a, after the protein mixture containing 0.1 g L^{-1} avidin and streptavidin in PBS (pH 7.4) was subjected to the COOH-chip under −0.3 V for 30 min, the eluted solution from the microchip showed a small FL emission intensity at $\lambda=518$ nm when excited at $\lambda=494$ nm (corresponding to avidin), whereas strong emission at $\lambda=615$ nm occurred when excited at $\lambda=595$ nm (corresponding to streptavidin). From the FL intensities, we estimated that the solution consisted of 79.77 and 20.23% streptavidin and avidin, respectively. The streptavidin isolated in this procedure was

Table 1. The adsorption/release properties for COOH-chips and NH_2-chips.[a]

	Normalized FL intensity[b] [%]		E_a[c] [%]	Γ[d] [mg cm^{-2}]	E_r[e] [%]
COOH-chip/avidin[f]	14.01 (−0.3 V)	77.89 (0.3 V)	85.99	0.008	90.58
NH_2-chip/streptavidin[f]	25.40 (0.3 V)	70.60 (−0.3 V)	74.60	0.007	94.64

[a] All the data are the average of 3 measurements with relative standard derivatives (RSD) <10%. [b] All the FL intensities were normalized to that of the control protein samples (see the Experimental Section). [c] The adsorption efficiency (E_a) was determined according to the equation: $E_a=(I_0-I_1)/I_0\times100\%$; I_1: the FL intensity of the eluted solution after −0.3/0.3 V had been applied to the chips for 30 min; I_0: FL intensity of the initial protein sample. [d] The protein coverage (Γ) on the channel surface was determined according to the equation: $\Gamma=c\times V\times E_a/S$, in which c and V are the concentration and volume of the protein sample, S is the area of the channel surface, calculated with the relative data in the Experimental Section. [e] The release efficiency (E_r) was determined according to the equation: $E_r=I_2/(I_0-I_1)\times100\%$; I_2: FL intensity of the eluted solution after 0.3/−0.3 V has been applied. [f] The adsorption/release for avidin and streptavidin were performed in the COOH-chip and NH_2-chip, respectively.

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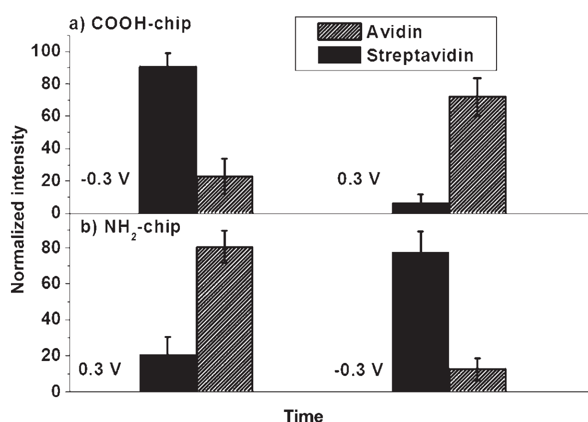

Figure 4. Normalized FL emission intensities (both at 518 and 615 nm, corresponding to avidin and streptavidin, respectively) of the eluted solutions from a) the COOH-chip containing protein mixture (0.1 $g L^{-1}$ avidin and streptavidin, pH 7.4) after −0.3 V (for 30 min) and 0.3 V were applied in turn, and b) the NH_2-chip containing protein mixture after 0.3 V (for 30 min) and −0.3 V were applied in turn. All FL intensities were normalized to that of the control protein mixture.

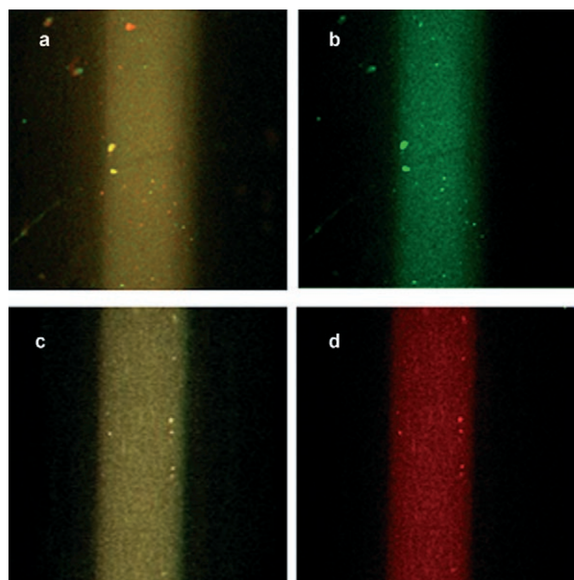

Figure 5. Confocal fluorescence microscopy images of the microchannels in a) the COOH-chip filled with a mixture of 0.1 $g L^{-1}$ avidin and streptavidin, b) the COOH-chip after adsorbing the mixture (pH 7.4) under −0.3 V for 30 min and being washed with PBS, c) the NH_2-chip filled with a mixture of 0.1 $g L^{-1}$ avidin and streptavidin, and d) the NH_2-chip after adsorbing the mixture (pH 6.6) under 0.3 V for 30 min and being washed with PBS. The images are the superposition of both the Fluorescein ($\lambda_{ex}/\lambda_{em}$ = 494/518 nm) and Texas Red ($\lambda_{ex}/\lambda_{em}$ = 595/615 nm) detection channels.

90.72 % of the total streptavidin in the original mixture. As shown in confocal fluorescence microscopy (CFFM) images (Figure 5a, b), initially the channel of the microchip containing the protein mixture exhibits both red and green fluorescence when excited at λ = 494 and 595 nm (the superposition image of the two detection channels is yellow), but it only fluoresces strongly in the green region after the separation process (the superposition image is green). This result confirms that the chip almost only adsorbed avidin, whereas most of the streptavidin was eluted. The applied potential was changed to 0.3 V, and conversely, the FL emission intensity of the outflow at λ = 518 nm was much stronger than that at λ = 615 nm. It can be estimated that this eluted solution consisted of 91.98 % avidin and 8.02 % streptavidin. The total avidin isolated in this procedure is 72.01 % of the avidin in the original mixture. This demonstrates that upon the electrical command, that is, on converting the applied potential to 0.3 V, the adsorbed avidin was released from the chip. The resultant fractions were treated repeatedly as in the above separation process, and the avidin and streptavidin were separated more completely (data not shown here). This is attributed to the fact that no matter whether the MHA molecules were straight or bent, which made the microchannel surface hydrophilic and electronegative or hydrophobic, there was no affinity between the microchannel surface and the negatively charged streptavidin molecules, but the surface property switching can control avidin attachment. So these two kinds of protein in the mixture can flow out of the chip in turn and are separated under the electrical control.

Similar experiments were performed by using the NH_2-chip. To the NH_2-chip containing an avidin and streptavidin mixture (pH 6.6), 0.3 V was applied first and was then changed to −0.3 V. As shown in Figure 4b, under 0.3 V, the eluted solution consisted of 20.20 % streptavidin and 79.80 % avidin, which was 80.45 % of the avidin in the original mixture. After −0.3 V was applied, an eluted solution containing 13.92 % avidin and 86.08 % streptavidin (which was 77.64 % of the streptavidin in the original mixture) flowed out of the chip. The separated fractions could also be treated again for a more thorough separation. These results prove that the NH_2-chip behaved oppositely to the COOH-chip. The former selectively adsorbed negatively charged streptavidin molecules under 0.3 V and released them under −0.3 V, whereas it had almost no effect on avidin. Accordingly, most avidin can flow out of the chip first; this was also confirmed by CFFM images (Figure 5c, d).

Another mixture containing 0.0001 $g L^{-1}$ avidin and 0.1 $g L^{-1}$ streptavidin (molar ratio, 1:1000) in PBS (pH 7.4) was also separated by the COOH-chip. The outflow was detected by LIF (for avidin) and FL (for streptavidin). A similar separation efficiency was observed. After being subjected to −0.3 V in the microchip for 30 min, the eluted solution showed only 17.67 and 88.32 % FL intensity for avidin and streptavidin, respectively. After the potential was changed to 0.3 V the outflow showed a clear FL signal for avidin (78.88 % of its initial amount), but only 10.23 % was observed for streptavidin. It can be anticipated and estimated that if the latter eluted solution is treated with the chip five times, the molar ratio of avidin and streptavidin would be increased up to about 32:1. These results demonstrated the ability of the COOH-chip to separate a low-concentration protein from a protein mixture. In principle, the molar ratio

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of the target protein in the eluted solution could be further improved by repeating the cycle.

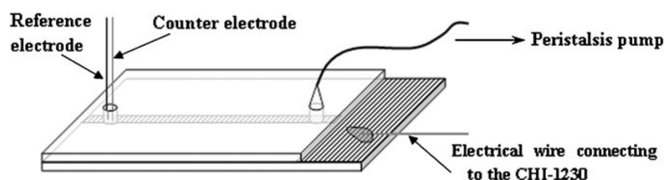
Conclusion

This work gives a successful example of the combination of the microfluidic chips and smart SAM surfaces. The smart SAM-modified microchip was shown to be capable of controlling protein adsorption and release under electrical modulation and thus achieved intelligent separation of proteins with different IPs in response to an external applied electric potential. Furthermore, this microchip also exhibited the potential of separating low-concentration avidin from the avidin/streptavidin mixture, which provides a novel alternative for preconcentrating low-abundance protein. This microfluidic system would be useful for controlled "on-chip" capture of target proteins directly from a complex mixture and would provide a new method to fabricate miniaturized bioanalytical devices for proteomic research, clinical diagnosis, or medical bioanalysis.

Experimental Section

Materials and chemicals: The chip materials were polymethyl methacrylate (PMMA) Plexiglas pieces (Shanhu Chemical Factory, Shanghai, China). MHA, EDC, and NHS were acquired from Sigma–Aldrich and used as received. β-Cyclodextrin, ethylenediamine, and all the other chemicals were purchased from Shanghai Lingfeng Chemical Reagent Co. (Shanghai, China). The fluorescent-labeled proteins, avidin (Fluorescein labeled, IP ≈ 10.5) and streptavidin (Texas Red labeled, IP ≈ 5.8), used in the study were purchased from Invitrogen Co.

Fabrication of the chips: The microchip consisted of two layers of PMMA pieces. One piece had bored inlet and outlet holes (called the cover sheet), and the other contained the microchannel (called the channel plate). The cover sheet was 3.5×2.5 cm, on which the punched inlet and outlet holes of 2.5 mm diameter were 3 cm apart (so the effective length of the microchannel was about 3 cm). The channel plate began as a 4×2.5 cm PMMA substrate and was hot-imprinted with a micromachined silicon template to introduce a microchannel with about 50 μm depth and 135 μm width to the middle of the substrate.[39,40] Then, a sputtering process was implemented to cover the microchannel with gold as the base of the surface modification, and also the gold-modified microchannel surface would act as the working electrode in the subsequent electrochemistry experiments. Before the sputtering process, a plastic mask was positioned on the channel plate to only expose the desired regions including the microchannel and a 0.5×2.5 cm region at one end of the channel plate (the shadow regions in Scheme 2). Then a 10 nm adhesion layer of titanium followed by 400 nm of gold were sputtered over the entire channel plate. After that the mask was removed carefully without damaging the gold layer.[41] At last, the two components, cover sheet and channel plate, were thermally bound together.[39,40]



Scheme 2. Illustration of the microchip with a three-electrode system coupled with the CHI-1230 and the peristaltic pump.

Synthesis of the IC and modification of the microchannel surface: The IC between MHA and β-CD was synthesized by following the method described in the literature.[20,21] MHA (20 mg) was added to an aqueous solution of β-CD (315 mg) in purified water (20 mL) with a molar ratio of 1:4 (excess β-CD was used to ensure that the majority of MHA in solution was bound by CD molecules). The resulting mixture was kept in an oil bath (40 ± 0.1 °C) with stirring for more than 48 h, and was then cooled to RT and filtered.

After being washed thoroughly with deionized water, the microchip was filled with the IC solution and kept at 4 °C for 24 h. Then the loading solution was removed, and the chip was washed with deionized water, followed by absolute ethanol to sufficiently remove the β-CD molecules and to produce the MHA LD-SAM-modified microchip.[20,21] The resultant microchip, the COOH-chip, was washed with deionized water again and was prepared for the subsequent experiments. The input of the deionized water, absolute ethanol, and the loading solution was performed by using a peristaltic pump connected to the output hole.

The NH_2-chip was prepared on the basis of the COOH-chip by using a carbodiimide activation protocol. An EDC/NHS mixture (containing 0.2 mol L^{-1} EDC and 0.05 mol L^{-1} NHS) freshly prepared in PBS (0.01 mol L^{-1}, pH 7.4) was pumped into the microchip to activate the carboxyl groups for 30 min. After the EDC/NHS mixture was removed and the microchip was washed thoroughly with deionized water, an aqueous solution containing ethylenediamine (1 mol L^{-1}) was then added into the microchip, and the chip was incubated at 4 °C for 30 min under buffer humidity. Then the microchip was washed with deionized water to remove excess ethylenediamine.

CV measurements for the COOH-chip: An aqueous solution containing $K_3Fe(CN)_6/K_4Fe(CN)_6$ (2.5 mmol L^{-1}) and KCl (100 mmol L^{-1}) was used as the electrolyte. It was freshly prepared and was purged with nitrogen for 15 min before use. Electrolyte (10 μL) was pumped into the microchannel. The reference electrode (Ag/AgCl wire) and the counter electrode (Pt wire) were hung up with their ends placed in the inlet hole carefully. It must be ensured that the electrodes were rinsed in the electrolyte without touching the surface of the microchannel or contacting each other. An electrical wire was attached to the gold-modified 0.5× 2.5 cm region of the channel plate by conducting resin. This wire connected the microchannel surface to the CHI-1230 Electrochemical Workstation (Shanghai Chenhua Co.) (Scheme 2), so that the bare, IC-, and MHA LD-SAM-modified gold microchannel surface could behave as the working electrode (the electrochemically controlled protein adsorption, release, and separation were all carried out with this three-electrode system and the CHI-1230).

Characterization of the NH_2-chip: An MHA LD-SAM-modified gold substrate, before and after being treated with ethylenediamine, was studied both by FTIR-ATR and XPS. FTIR-ATR spectra were recorded by using an FTIR apparatus with an ATR attachment (Nexus 470, Nicolet Co., USA): $\tilde{\nu}$ = 1674 ($\vartheta_{C=O}$), 3455 (ϑ_{N-H}), 1527 (δ_{N-H}), 1260 cm^{-1} (ϑ_{C-N}). XPS analyses were performed by using an RBD-upgraded PHI-5000C ESCA system (Perkin–Elmer) with a base pressure of 1×10^{-9} torr by using $Al_{K\alpha}$ radiation (1486.6 eV). Binding energies were calibrated by using the containment carbon (C1s = 284.6 eV). The resulting XPS spectra reveal the presence of the peak belonging to nitrogen (N1s at 399.8 eV). The atomic percentages derived from the photoemission peak areas are 81.9, 4.9, 8.6, and 4.6 % for C, O, N, and S, respectively.

Electrically controlled protein adsorption, release, and separation in the modified microchips: For the controlled protein adsorption, a solution of avidin or streptavidin (10 μL, 0.1 g L^{-1}, ≈17 μM) in PBS buffer (10 mmol L^{-1}, pH 7.4 for the COOH-chip and 6.6 for the NH_2-chip, respectively)[42]) was pumped into the microchannel. The three-electrode system was constructed on the microchip, then −0.3, 0, and 0.3 V electric potentials were each applied for 30 min[43] to ensure the achievement of the adsorption equilibrium (the applied potentials were chosen above the lower limit for transitions and within the "window of stability").[20] Then the protein solution in the microchannel was pumped out slowly and carefully by using a peristaltic pump. At the same time, PBS buffer was

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added into the chip to keep the applied electric potential on the microchannel surface. This "wash" operation was stopped after 200 μL PBS buffer had been added into the chip, which ensured that the protein solution was properly removed. The eluted solution was collected into a quartz container for fluorescence (FL) measurements. The wash process was repeated until there was no notable FL signal due to avidin (or streptavidin). Then the measured FL intensities were added up. Control experiments were carried out with a pristine gold microchip and a HD-MHA-chip that was prepared by directly modifying the microchip with a solution of MHA in ethanol (or HD-NH_2-chip prepared by treating the HD-MHA-chip with EDC/NHS and ethylenediamine). The FL spectra were recorded by using a Cary Eclipse fluorescence spectrophotometer (Varian, USA), $\lambda_{ex}/\lambda_{em}=494/518$ nm for avidin and 595/615 nm for streptavidin.

After the adsorption process described above, the external electric potential was converted to 0.3 V for the COOH-chip (−0.3 V for the NH_2-chip). Then the microchannel was washed with another 200 μL PBS and the outflow was collected and measured. This process was repeated until there was no detected FL signal, and the measured FL intensities were added up. The whole adsorption/release process of avidin was also recorded by in situ detection of the outflow with LIF (the instrument was fabricated by Shanghai Institute of Optics and Fine Mechanics, Chinese Academy of Sciences, $\lambda_{ex}/\lambda_{em}=494/518$ nm). Avidin or streptavidin (10 μL, 0.1 gL^{-1}) solution was diluted to 200 μL with PBS and used as the control sample.

For the protein separation, a 10 μL mixture solution containing both avidin (0.1 gL^{-1}) and streptavidin (0.1 $mgmL^{-1}$) in PBS buffer (pH 7.4 and 6.6 for the COOH-chip and NH_2-chip, respectively) was applied to the COOH-chip (or NH_2-chip) and the outflows were measured by using FL in the same way as described above. The microchip before and after this separation process was studied by CFFM (TCSNT, Leica). Then the opposite electric potential (0.3 and −0.3 V for the COOH-chip and NH_2-chip, respectively) was applied to the microchip, and the solution in the microchip was pumped out and measured by using FL. 10 μL of the initial mixture solution was diluted to 200 μL with PBS and was used as the control sample.

For the separation of another 10 μL of protein mixture containing both avidin (0.0001 gL^{-1}) and streptavidin (0.1 gL^{-1}) in PBS buffer (pH 7.4), LIF ($\lambda_{ex}/\lambda_{em}=494/518$ nm) was used to detect the FL signal of the avidin because of its low concentration, and the FL signal of the streptavidin was detected by using FL measurements ($\lambda_{ex}/\lambda_{em}=595/615$ nm).

Acknowledgements

This work was supported by NSFC (20335040, 90606014, 20605005, 20525519) TRAPOYT, and Fudan University (985-II project).

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[42] The pH was optimized by carrying out the protein adsorption experiments under different pH conditions: for the COOH-chip the pH was 7.0–9.0 (which can ensure that avidin and streptavidin are oppositely charged and the carboxyl groups ionize), and for the

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NH_2-chip the pH was 6.0–7.0 (which can ensure that avidin and streptavidin are oppositely charged and the amino groups ionize). Finally, pH 7.4 and 6.6 were chosen because the adsorption and selectivity were the best under these pH conditions for the COOH-chip and NH_2-chip, respectively.
[43] The time for protein adsorption was optimized by carrying out the experiments for 5, 10, 15, 20, 25, 30, 35, and 40 min, respectively. The adsorption efficiency exhibited little increase after the adsorption time was more than 30 min, so we chose 30 min as the adsorption time considering both the adsorption efficiency and time usage.

Received: November 14, 2006
Published online: April 3, 2007

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