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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

PAPER

Facile real-time evaluation of the stability of surface charge under regular shear stress by pulsed streaming potential measurement

Lei Zhao, Xianglu Peng, Shenghong Yang, Yuan Zhang, Jing Wu, Xuan Wei, Fengyun Li and Qiaosheng Pu*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Self-assemblies of polyelectrolytes have been widely used to tailor the surface charges, but their stability varies significantly due to the diversified properties of both polyelectrolytes and surfaces. Fast and reliable evaluation of the stability of the assembled layers is essential to optimize the assembling processes and select the proper working conditions. In this work, the applicability of the pulsed streaming potential measurement for real-time evaluation of stability of assembled layers was demonstrated. Polyethyleneimine (PEI) with a molecular weight of 1800 was selected as the model polyelectrolyte and the results confirmed the dependence of the stability of the assembled layers on the assembling conditions. A parameter S_R, which is defined as the relative zeta potential change rate, was adopted to reflect the difference of the positively charged layer. Effect of assembling conditions, including pH, polyelectrolyte concentration and contact time, on the change of the streaming potential were evaluated based on S_R values. The feasibility of the technique for the evaluation of the stability of adsorbed proteins, oligonucleotide and cells onto the formed PEI layer was also investigated and its reliability was confirmed by capillary electrophoresis.

Introduction

Surface modification has been widely used to immobilize proteins,[1] nucleotides and other bio-substances. It has become an essential step in many applications, including ELISA,[2] protein microarrays[3] and biosensors.[4] In most of cases, biomolecules cannot be directly attached to the surfaces of the carriers, one or more intermediate layers are frequently implemented to ensure stable anchoring and maintain good bio-activity of the attached biomolecules. Attachment of these intermediate layers is normally referred to as surface modification.

Surface modification can be roughly divided into two categories, physical adsorption and chemical coupling/transformation. The later could be achieved through chemical reactions between the surface groups and the modifiers,[5-7] or through high energy induced attachment or depletion, *i.e.*, photochemical grafting[8-10] and plasma etching.[11] The layers formed from these methods are thought to be permanent due to the nature of chemical bonds, but to perform chemical coupling/transformation is often complicated and might need harsh conditions. Chemically formed layers are frequently not as stable as expected too. Physical adsorption, including self-assembling of polyelectrolyte, which can be accomplished by simple contact of the modifiers with the surfaces, has been proved to be an indispensible alternative to the chemical coupling in many applications.[12, 13] The construction of self-assemblies of polyelectrolytes onto various surfaces is one of the commonly used physical modification methods nowadays.

More and more evidences prove that the stability of some polyelectrolyte layers can be comparable with chemically bonded molecules.[14-16] Layer-by-layer (LbL) sequential adsorption of oppositely charged polyelectrolytes or bipolar amphiphiles, which was proposed by Decher's group,[17-19] has been recognized as a standard way for stable polyelectrolyte layers. The polyelectrolyte layers formed with this method have been used for the immobilization of genes,[20, 21] proteins,[22, 23] cells,[24, 25] and even nanomaterials.[26] However, the stability of the assembled layers may vary significantly among different polyelectrolytes and surfaces. For the self-assembling of polyelectrolytes onto the oppositely charged surfaces, the electrostatic interaction is frequently the primary driving force. Surface charge plays a critical role in the process. In addition, surface charge may also be a direct indicator of functional groups on the very top of exposed surface. To evaluate surface charge of polyelectrolyte films and multilayers, zeta potential obtained with electrokinetic methods including streaming potential (SP) measurement could be used.[27, 28] Self-assemblies of polylelectrolytes have been carefully studied through SP measurement by Adamczyk's group, and many important conclusions have been drawn.[29-31] However, the operation of the traditional SP measurement suffers from some limitations in both measuring solutions and electrodes. Generally pure KCl solution and Ag/AgCl[28, 32] electrodes have to be used and the time needed for a measurement is relative long, typically dozens of minutes.[33, 34] The time resolution at this level might not be good enough for the fine examination of the quick adsorption/desorption of substances onto/from microchannels, where extremely short distances between the main streams and

the channel surfaces ensure the rapid adsorption/desorption processes. Pulsed SP measurement can avoid these limitations,[35] and has been used for monitoring biomolecule's adsorption process and characterizing surfaces[36].

On the other hand, use of microchannels will be a main theme in biological applications due to the benefits they provided[37, 38]. However, because of their tiny sizes, modification of microchannels can be much harder than other kinds of surfaces. The stability of the modification layers may be a serious concern due to the significant shear force within microchannels.[39] Pulsed SP measurement can be a convenient way to evaluate the stability of the adsorbed layers within microchannels based on the surface charge.

In this work, the feasibility of using pulsed SP measurement to monitor both the formation and stability of positively charged surfaces was illustrated. A simple parameter was proposed to describe the stability of the positive charge of adsorbed layers. Factors that may affect the stability of the layers were systematically evaluated. The adsorption behavior of the proteins with different isoelectric points (pIs) and their stability was investigated to show the possibility of the method for fine examination of the formation of protein layers inside microchannels. The results were confirmed by capillary electrophoresis. The applicability of monitoring the adsorption stability of oligonucleotide, serum components and bacteria adsorbed onto PEI layers through pulsed SP measurement was also demonstrated.

Experimental

Chemicals

Polyethyleneimine (PEI, 99%, MW 1800) and (3-aminopropyl) triethoxysilane (APTES, 99%) were purchased from Aladdin (Shanghai, China). Bovine serum albumin (BSA, purity ≥ 98%, MW 66.3 kDa), bovine γ-globulins (BGG, purity ≥ 98%, MW 150 kDa), and lyszome (LYZ, ≥ 5000 units/mg dry weight, MW 16.4 kDa) were purchased from Sinopharm chemical Reagent Co., Ltd. A oligonucleotide strand (20 base pairs, TTTTTGGTTGGTGTGGTTGG, purity ≥ 90%) was supplied by Sangon Biotech (Shanghai) Co., Ltd. Analytically pure KH_2PO_4, Na_2HPO_4, and NaOH were obtained from Guangfu chemical (Tianjin, China). Analytically pure anhydrous methanol and ethanol were purchased from Lee Longbo Pharmaceutical Chemical Co., Ltd. (Tianjin, China). The bare fused silica capillary (i.d. 75 μm) was purchased from Sino Sumtech Co., Ltd. (Handan, China). Serum was isolated from blood collected from a healthy volunteer following general procedure.[40] *Escherichia coli* (E. coli) was prepared with LB broth following the standard procedure.[41] Deionized water from a Milli-Q water purification system (Millipore) was used for all solution preparation.

Pulsed SP measurement

The device for SP measurement was similar to that described earlier.[35] Its operation is briefly described as follows: A piece of capillary was inserted into a small vial containing measuring buffer (or coating solution). The vial was connected to a vacuum source through a 3-way solenoid valve. The other end of the capillary was placed in another vial containing the same solution. Each vial was equipped with a piece of platinum electrode to measure the potential drop across the capillary. The potential between two electrodes was fed into a USB-6009 data acquisition card (National Instruments, Austin, TX, USA) through a voltage follower built with an opamp TL082cp (Texas Instruments, Dallas, TX, USA). To get rid of the interference of interfacial potentials at the electrodes, the applied pressure was pulsed by switching the solenoid valve intermittently. When the valve was switched to the vacuum position (vacuum ON) for a short period (normally 5 s), the solution was forced flowing through the capillary. When the valve was switched to the vent position (vacuum OFF), there was no flow in the capillary. The difference of potential between the vacuum ON and OFF periods was taken as the SP. The operation of the valve was controlled through the digital output of the same USB-6009 card (National Instruments) and the pulsing of the vacuum was repeated multiple times so that the change of the SP along time could be continually recorded. The switching process eliminates the liquid-electrode interfacial and other interfering potentials, makes it suitable for online monitoring the change of the SP in real-time. The operation of the solenoid valve and the data acquisition were realized through a program written in Labview (National Instruments). All measurements were performed at room temperature.

Although it is proved that the zeta potential of the porous membrane may be overestimated by Smoluchowsky equation with classical theories, it has been suggested that when the double-layer's thickness can be negligible, the classical theories can be used to calculate the zeta potential of the layer.[42] In the present work, the diameter of the capillaries is 75 μm, far larger than the thickness of double layer.

According to Smoluchowsky equation[43]

$$\Delta E = \frac{\varepsilon\varepsilon_0\zeta\Delta P}{\eta\kappa} \qquad (1)$$

where ΔE is the measured streaming potential, ΔP is the pressure to drive the flow, ζ is zeta potential, κ is the solution conductivity, η is the solution viscosity, ε is the dielectric constant and ε_0 is the vacuum permittivity. In the present work, ΔP, κ and η were maintained same, ζ was calculated from the equation.

Preparation of positively charged surface inside bare fused silica capillaries

The bare fused silica capillary (15 cm long) was rinsed with deionized water, 1 M NaOH and deionized water sequentially for 5, 15 and 5 min, respectively. The surface with positive charge was formed by flushing the PEI solutions through the bare fused silica capillary for given periods of time under a pressure drop of 50 kPa. To monitor the assembling process, the flushing was performed following the same procedure for the pulsed SP measurement as mentioned in the previous section. In this case, the PEI solutions were also used as the measuring buffers. To evaluate the layer stability, the capillary with an assembled layer was first washed with deionized water for 3 min, and dried with Ar flow for 5 min, then SP was measured with blank phosphate buffers with different pHs.

Adsorption and stability evaluation of proteins and other biomaterials

Adsorption processes of three proteins and an oligonucleotide strand both on PEI coated layer and bare fused silica capillaries

were investigated using the proposed method following the same procedure described above. For this purpose, the PEI layers were prepared under the optimum PEI assembling condition (50 mg/mL PEI in 0.01M NaCl. Proteins and the oligonucleotide strand were dissolved in phosphate buffer at pH 7.1, the conductivities of these solutions were maintained at 290 ± 10 μS/cm. The stability of the adsorbed layers was measured with a blank phosphate buffer at the same concentration and pH. Adsorption of serum was evaluated using a procedure same as for proteins. E. coli (in 3 mL of LB borth) was first centrifuged to remove the broth and re-dispersed in phosphate buffer of pH 7.1 with a conductivity of 290 ± 10 μS/cm for the measurement.

Capillary electrophoresis of proteins

Capillary electrophoresis was performed with a high voltage module (DW-P203-1C1A, Dongwen High-Voltage Power Supply, Tianjin, China) and a UV absorbance detector (Linear UVIS 200, Linear Instruments, Reno, NV, USA) at 214 nm. Forced air flow was used for capillary cooling. Sample injection was performed by raising the sample vial 10 cm above the buffer vial for 5 s. The running buffer used for BSA was 30 mM PB at pH 7.0, for LYZ was 30 mM PB at pH 4.5. The length of capillary was 39 cm with an effective length of 31 cm. High voltage used for the separation was 14000 V.

Results and discussion

In-situ monitoring of the assembling process

PEI as positively charged polycations can be used as a surface modifier.[44, 45] Normally higher the molecular weight the better the stability of the formed layers, but low molecular weight PEI is widely used as gene carriers[46] and other purposes. In the present work, in order to illustrate the time resolution of the pulsed SP measurement, PEI with relatively low molecule weight (1800) was selected. As shown in Fig. 1a, gradual change of zeta potential along the time is evident at lower PEI concentrations. At higher concentrations, PEI covered the surface very quickly, the adsorption equilibrium was attained in a period that is less than one cycle of pressure pulsing (10 s) (Fig. 1b). Zeta potentials maintained constant in the following pulsing cycles and showed pH dependence as expected.[47] At lower pHs, the surface was more positive (+50.22 mV at pH 3.1), but even at pH 10.6, a positive zeta potential of 2.62 mV was still obtained.

Real-time evaluation of the stability of positive surface charge

With this low-molecule PEI, gradual change of surface charge under a fixed flow rate (that means a fixed shear force) is detectable with pulsed SP measurement (Fig. 2). The shear force is for each pulse, the calculated zeta potential should indicate the change of the surface.

However, due to the absolute value of the zeta potential may be affected by many factors, it is impractical to make a batch of capillaries with exactly same zeta potential values (*i.e.* exactly same surface status). To define the stability of the assembled

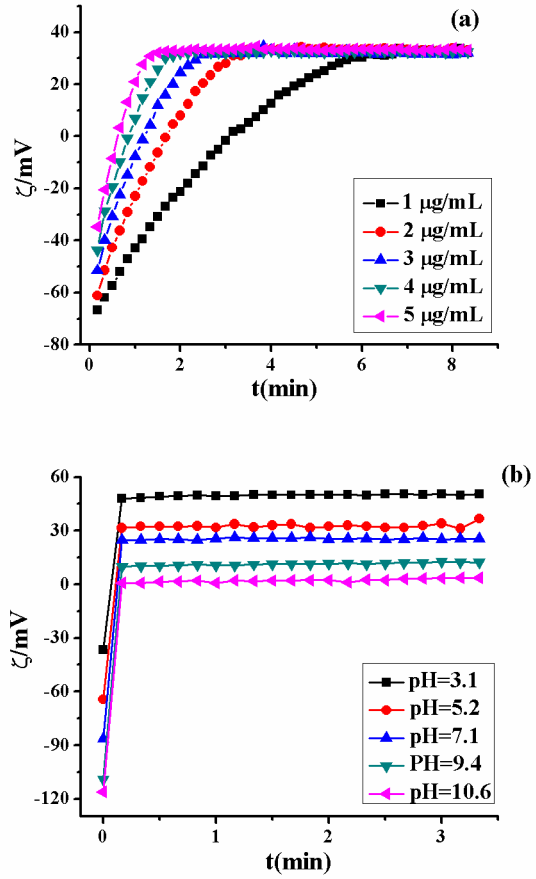

Fig. 1 (a) Zeta potentials measured with lower concentrations of PEI in PB, pH = 7.1, (b) Zeta potentials measured with higher concentration of PEI (0.50 mg/mL PEI in 2 mM phosphate buffer) solutions at different pHs.

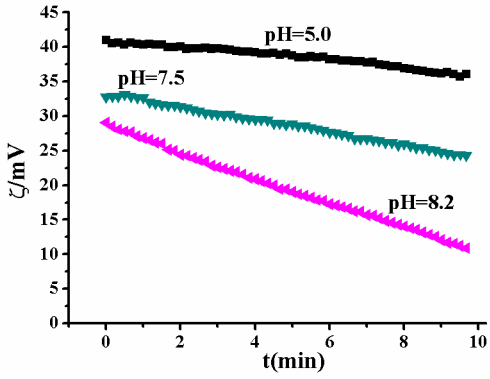

Fig. 2 Typical zeta potential change for PEI layers formed at different pHs. PEI layers formed with 20 mg/mL PEI in 0.01 M NaCl for 10 min.

layer directly with zeta potential drift is not accurate. Therefore, the slopes (S) of the lines obtained at a given pH were used to define the stability of the polyelectrolyte layers formed inside microchannels. Relative zeta potential change was used to

compare the stability under different experimental conditions (Equation 2):

$$S_R = \frac{S}{\zeta_{FC} - \zeta_B} \qquad (2)$$

where S_R is the relative zeta potential change, S is the slope of the zeta potential change along the time, ζ_{FC} is the zeta potential of the fully coated layer and ζ_B is the zeta potential of the bare channel measured with the same buffer that used for the coated channel. The physical meaning of S_R can be thought of as the reciprocal time to completely wash the assembled layer away if the initial degradation speed is maintained. S_R value can be used for evaluating both strongly and weakly adsorbed layers. For weakly adsorbed ones, as long as 3-5 initial points of zeta potential can be measured, S_R can be determined. Based on our measurement, layers can be thought to be stable when $S_R \leq 1 \times 10^{-2}$ min^{-1}.

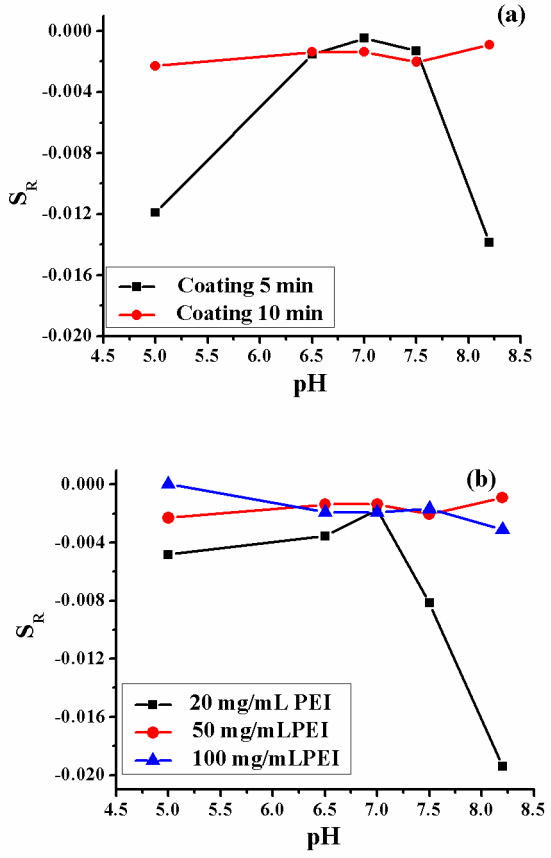

Fig. 3 (a) S_R values of positively charged surface self-assembled by PEI (50 mg/mL, with 10 mM NaCl) coated for 5 and 10 min at different pHs, (b) S_R values of positively charged surface at different concentrations. Coating time was 10 min.

The stability variation of PEI layers at different pHs was clear as shown in Fig. 3. Fine examination of the influence of experimental conditions on the layer stability is therefore practical. This technique can help to clarify some important issues. For example, PEI coating processes used by other investigators exhibit quite large variation, the coating time was in a range of 10-30 min.[48, 49] Is there any justification to use such long coating time? Pulsed SP measurement results gave clear clue on this. Although SP attained constant values almost at the first cycle of the vacuum pulsing (Fig. 1b) with higher concentration of PEI, the stability of the obtained layer represented by S_R was quite different. S_R of the formed layers is dependent on the coating (flushing) time, which confirms the necessity of using longer coating time even the adsorption equilibrium can be attained in less than 10 s.

As shown in Fig. 3a, 10 min of flushing gives more stable layers than that with 5 min, further increase the flushing time gave no obvious change on S_R. Because the PEI layers were formed rapidly, the effect of coating time on the stability of the layers was related to the defects on the layers. The most probable origin of these defects is the loosely adsorbed PEI molecules, particles or other contaminants. Upon the action of pulsed shear flow during the measurement, these spots gradually exposed to the solution and led to a larger S_R.

The effect of PEI concentration on the stability of the layers was also examined (Fig. 3b). At a PEI concentration of 20 mg/mL, the formed layer showed much quicker degradation when the measuring medium was basic. However, when the concentration of PEI increased to 50 mg/mL, S_R values were almost same in the whole pH range. Further increase of PEI concentration to 100 mg/mL gave similar results to that of 50 mg/mL. This result indicates that the surface defects are also dependent on the coating solution. A larger concentration is necessary to get PEI layers with fewer defects. It should be also mentioned that this process was also dependent on the molecular weight of PEI.

Application in the evaluation of stability of adsorbed biomaterials

Norde's group has studied Protein's adsorption onto various surfaces with traditional SP measurement.[32, 50-53] Parallel plates to form laminar flow in these works. Adsorption of biomolecules and even bacteria in microchannels can be more easily evaluated by pulsed SP measurement.

Proteins are typical polyelectrolytes, elucidation of their adsorption onto the different surfaces is important for many biological applications. Because PEI layers carry positive charge within a wide range of pH, it can absorb any proteins with negative charge.

The adsorption behavior of different proteins was quite different. In our experiment, three proteins with different pIs were selected, which include BSA, BGG and LYZ, their pIs are 4.8, 6.6 and 11.0, respectively (Table 1).

BSA carries more negative charge at pH 7.1, its adsorption onto PEI layer is rapid, a stable zeta potential value was attained in 10 s of flushing during measurement period (Fig. 4a). While its adsorption on bare fused silica capillary is rather slow, equilibrium was not attained until 200 s. The stability of the immobilized BSA layer is quite different. S_R measured following the procedure described above for BSA was $(2.33 \pm 0.28) \times 10^{-2}$ min^{-1} on bare fused silica capillary and $(7.84 \pm 0.10) \times 10^{-4}$ min^{-1} on the PEI layer, the later is much more stable. The pI of BGG was 6.6, close to the solution pH 7.1. The adsorption of this protein was slow for both bare fused silica capillary and the PEI layer because of its weak negative charge (Fig. 4b). Within the measuring period, equilibrium was not attained for both kinds of

surface. Immobilized BGG was stable, the measured S_R was (2.49 ± 0.51) × 10^{-3} min^{-1} for bare fuse silica capillary and (2.42 ± 0.22) ×10^{-3} min^{-1} for the PEI layer. These results indicate that the charge is not the only factor determining the stability of the immobilized protein layers. The overall charge of LYZ is positive at pH 7.1, assembling of LYZ onto the bare fused silica capillary was quick, the S_R value was (1.79 ± 0.35) ×10^{-2} min^{-1}, equilibrium was attained in 100 s (Fig. 4c). However, flushing LYZ over PEI layer did not cause any apparent change of zeta potential. The changes of the zeta potentials due to the adsorption of these proteins were summarized in Fig 4d, the adsorption tendencies of different proteins at different surfaces can be easily distinguished.

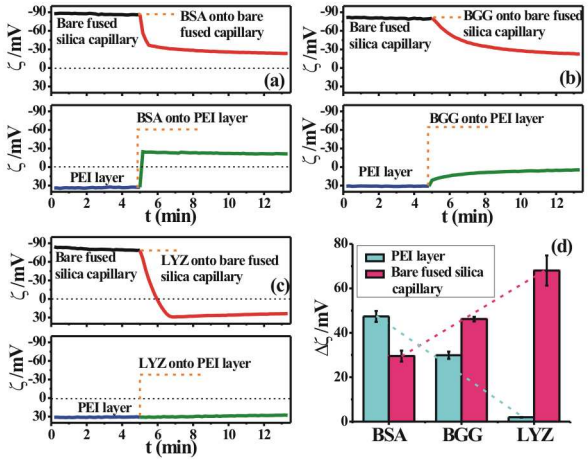

Fig. 4 Adsorption of proteins onto positively charged PEI layers and the surface of bare fused silica capillary (15 cm long), (a) 604 μg/mL BSA in PB buffer, (b) 12.8 μg/mL BGG in PB buffer, (c) 17.6 μg/mL LYZ in PB buffer, (d) the absolute value of the change of zeta potential for three proteins. (PB: pH=7.1, κ=290 ± 10 μS/cm).

Table1. Protein's pI and S_R values under different surfaces.

	BSA	BGG	LYZ
pI	4.8	6.6	11.1
S_R(Bare fused silica capillary) /min^{-1}	(2.33 ± 0.28) ×10^{-2}	(2.49 ± 0.51) ×10^{-3}	(1.79 ± 0.35) ×10^{-2}
S_R(PEI layer) /min^{-1}	(7.84 ± 0.10) ×10^{-4}	(2.42 ± 0.22) ×10^{-3}	—

The value is calculated by n times test, n=2~4.

Because the separation efficiency of capillary electrophoresis can be seriously affected by the analyte adsorption, electrophoretic analysis of BSA and LYZ was performed to verify the results obtained by the pulsed SP measurement (Fig. 5a). Theoretical plate number of BSA obtained on bare fused silica capillary was higher than that on PEI coated one (11499 vs 3853), which indicates stronger adsorption of BSA on PEI coated capillary. For LYZ, PEI coating gave quite good electrophoretic efficiency (plate number of 20387), while bare fused silica capillary gave no peak of LYZ at all (Fig. 5b). This result matches quite well with the SP data, where flushing of LYZ over PEI layer gave tiny zeta potential change (1.94 ± 0.094 mV), but flushing of LYZ through bare fused silica capillary gave a zeta potential change of 68.02 ± 6.78 mV, completely reversed the polarity of the surface (Fig. 4c). Since the measurement of the zeta potential change before and after flushing of proteins or other components can be rather quick (< 1 min), the results obtained here imply a possibility of fast pre-screening of capillary coating and buffer composition to completely get rid of unwanted analyte adsorption and achieve efficient separation.

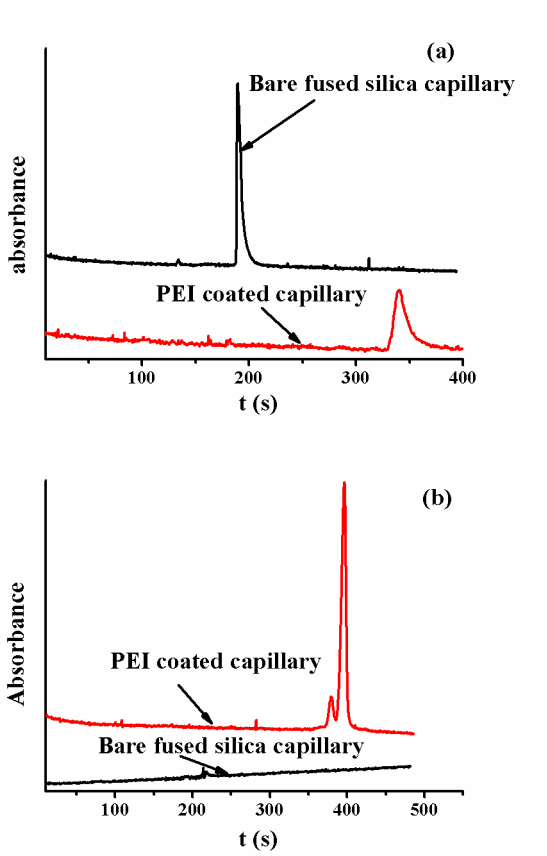

Fig. 5 Electropherogram of proteins on bare fused silica capillary and PEI coated capillary. (a) 248 μg/mL BSA in PB, (b) 220 μg/mL LYZ in PB. Capillary i.d. is 75 μm, length is 39 cm, effective length is 31 cm, Separation buffer (a): 30 mM PB, pH 7.0, for (b): 30 mM PB, pH 4.5; Separation voltage for bare fused silica capillary is +14 kV, for PEI coated capillary is -14 kV.

Oligonucleotide's adsorption on PEI layer was also tested by pulsed SP measurement. As shown in Fig. 6a, the oligonucleotide fragment gradually attached to the PEI layer and zeta potential drops from 33.41 to -54.56 mV. The assembled layer is stable, S_R measured with blank phosphate buffer is (7.74 ± 0.22) × 10^{-3} min^{-1}.

Serum's adsorption process on PEI layer was tested because it might provide a good bio-compatible surface. As shown in Fig. 6b components of serum could attach to the PEI surface rapidly, zeta potential changed from 34.80 to -9.20 mV at the first pressure pulse, and maintained stable thereafter. S_R was (1.14 ± 0.64) × 10^{-3} min^{-1}, which was as stable as PEI layers obtained at the optimum assembling condition. For serum adsorbed onto bare fused silica capillary, S_R was (2.47 ± 0.38) × 10^{-3} min^{-1}.

E. coli can be uniformly adsorbed on the PEI layer too, as shown in Fig. 6c, zeta potential changed from 31.16 to -31.98 mV in 8 min. Perhaps because of its bulky size, the stability of E. coli layer was lower than that of proteins, S_R was 0.012min^{-1}. But it is

still stable enough for the situations that without strong shear force caused by high velocity flows (6.27×10^{-4} mL/s). The change of zeta potentials caused by adsorption of biomaterials onto positively charged PEI layer was summarized in Fig. 6d.

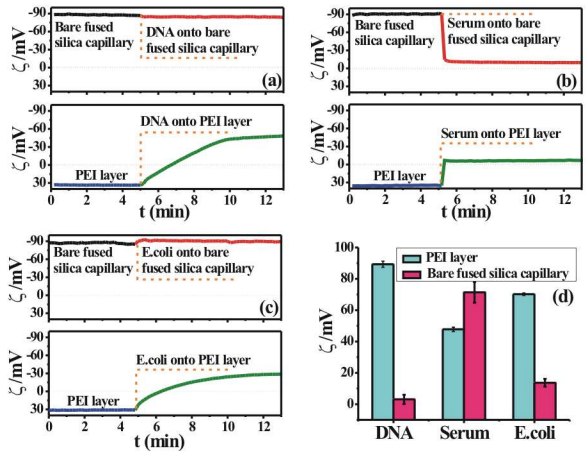

Fig. 6 Adsorption of biomaterials onto positively charged PEI layers and the surface of bare fused silica capillary (15 cm long), (a) 0.6 μM DNA in PB, (b) 2% (v/v in PB) Serum, (c) E. coli in PB, (d) the absolute value of the change of zeta potential for three biomaterials. (PB: pH=7.1, κ=295 μS/cm).

Table 2. S_R of biomaterials adsorbed onto bare fused silica capillary and that with PEI layers.

	Oligonucleotide	Serum	E.coli
pI	4.8	6.6	11.1
S_R(Bare fused silica capillary) /min^{-1}	—	$(2.47 \pm 0.38)\times10^{-3}$	—
S_R(PEI layer) /min^{-1}	$(7.74 \pm 0.22)\times10^{-3}$	$(1.14 \pm 0.64)\times10^{-3}$	0.012

— means no change in surface charge after adsorption.

Conclusions

Herein we proved the applicability of pulsed SP measurement for the characterization of the formation and stability of adsorbed layers inside fused silica capillaries. The assembling process itself could be monitored *in situ*. The results indicated that pulsed SP measurement can be an efficient way for fast evaluation of adsorbed layers inside microchannels. Subtle differences of the charge stability could be measured with this technique due to the unique pulsed flow pattern. The advantages of the method include the better time resolution of the measurement over traditional SP measurement and easier implementation onto microchips or other microfluidic devices.[35] Because capillary may need to be fragmented to reach the inner surface for AFM measurement[54], gold or silver film is necessary for SPR[55], AT-cut quartz crystal is necessary for QCM[56] and the commonly used substrates for ellipsometry is silicon wafer,[57] these traditional surface characterization techniques can hardly be used for microchannels. Pulsed SP measurement can be conducted within micro-channels and the measurement itself was non-destructive, it can be a simple but effective way for fine tuning of the surface modification through physical adsorption.

Acknowledgements

The work is supported by the Natural Science Foundation of China (No. 21175062). F.Y. Li acknowledges the support from the Natural Science Foundation of Gansu Province (No.145RJYA243) and Fundamental Research Funds for the Central Universities (lzujbky-2013-89).

Notes and references

State Key Laboratory of Applied Organic Chemistry, Key Laboratory of Nonferrous Metals Chemistry and Resources Utilization of Gansu Province, Department of Chemistry, Lanzhou University, Lanzhou, Gansu 730000, China. Fax: +86 931 891 2582; Tel: +86 931 8913813; E-mail: puqs@lzu.edu.cn (Q.S. Pu).

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