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Potential application of hydrogel-based strong anion-exchange membrane for plasmid DNA purification

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ABSTRACT

The potential application of a hydrogel-based strong anion-exchange (Q) membrane to purify plasmid DNAs was evaluated. The maximum binding capacity of plasmid DNA was estimated to be 12.4 mg/ml of membrane volume with a plasmid recovery yield of ~90%. The effect of the inherent properties of plasmid DNA, membrane adsorbent, and the ionic environment on membrane performance was systematically investigated. Plasmid DNAs with smaller tertiary structure tended to have a better recovery than those with larger tertiary structure. Environmental Scanning Electron Microscopy (ESEM) revealed that the hydrogel structure is more porous on one side of membrane than the other. Membrane pre-treatment significantly improved pore distribution and increased membrane porosity resulting in a better adsorption, recovery, and higher flux. The selection of proper operating pH led to further improvement. The relative contribution of these factors to improve membrane chromatography of plasmid DNAs was analyzed using statistical modeling. It was found that the adsorption of plasmid DNA was mainly affected by the available adsorptive area associated with membrane porosity, whereas the recovery of plasmid DNAs was mainly affected by the environmental pH.

1. Introduction

Since the pioneering study of Wolff et al. [1] expressing transgenes that were inserted on a plasmid DNA, there has been a rapid advancement of plasmid-based gene therapy and DNA vaccine development [2–4]. Plasmid DNA therapy is proven to be relatively inexpensive and safe to administer. In addition, plasmid DNAs have higher stability at ambient temperature than conventional viral vaccines and this is considered as an important advantage during long-term storage [4,5]. However, due to its inherently low infection efficacy, a relatively large dose of plasmid DNA is required [6,7] typically in the order of a few milligrams for a full treatment of a patient [5,8]. Currently, there are several plasmid DNA vaccines marketed or under clinical evaluation for the treatment of cancer, infectious and autoimmune diseases. The demand of pharmaceutical-grade plasmid DNAs will be soaring if they prove to be clinically effective [4]. Therefore, an efficient industrial-scale bioprocessing technology for the production and purification of pharmaceutical-grade plasmid DNAs is needed.

Amongst the numerous available methods for plasmid DNA purification, chromatographic techniques are widely adopted because they provide high resolution, use only chemicals that are generally considered safe, and are easily scalable [4,5,7]. The most commonly used method is anion-exchange chromatography which is based on the reversible interaction between negatively charged plasmid DNA and the positively charged chromatographic media [5,9,10]. The application of conventional resin-based anion-exchange chromatography, however, results in low adsorption because the small pores in the resin restrict access to near-micron-sized plasmid DNAs (>0.2 μm). Resins were originally optimized for the purification of nano-sized biomolecules such as proteins (2–10 nm) [7,11–13]. It was shown by confocal microscopy that plasmid DNA adsorption only occurred on the outer surface of the resin and this greatly reduced the binding capacity of the resin beads [14]. Of the 10–100 g of plasmid DNA loaded per litre of resin, only 0.2–2 g could bind [5]. Thus, a substantial amount of chromatographic resin is needed to purify milligrams of plasmid DNA. In addition, it is time-consuming and labour-intensive to pack, clean, and regenerate the chromatographic column [5,15]. Studies have also shown low recovery or irreversible binding of

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plasmid DNA to some chromatographic resins [12,13], either due to the small pore size or strong interaction with the resin material.

Anion exchange membrane chromatography offers a promising alternative to the conventional resin-based column chromatography for large-scale purification. It does not involve packing and cleaning procedures associated with resin-based chromatography. Most importantly, it allows a rapid convective transport of biomolecules through the large pores (~2 μm), in contrast to the diffusive transport through the small pores of the resins (~0.2 μm) [16]. Therefore, a higher rate of mass transfer is possible even at high flow rates [12,16–18]. The large pore size of the membrane also allows better accessibility and greater surface area utilization, thus resulting in a higher binding capacity. Anion-exchange membranes were shown to surpass at least ten fold the counterpart resins in binding capacity on a per volume basis [19]. The scale-up of membrane chromatography is straightforward because the binding capacity is directly proportional to the available membrane surface area, while the scale-up of resin-based chromatography is more challenging.

Several studies reported the use of membranes to produce therapeutic plasmid DNAs with a high purity but at relatively low yield [17,20,21]. For economic reasons, both recovery and yield are important aspects that should be maximized to fully realize the advantage of membrane chromatography. Employing membrane chromatography, a higher yield of plasmid DNA can be achieved with fewer number of processing steps. It is generally unacceptable to have a recovery lower than 70% in any single step of a large-scale production process [9]. Some studies of plasmid DNA purification using anion-exchange membrane addressed these problems. Teeters et al. [19] used different salts and compaction agents to reduce the charge density and the size of plasmid DNA in an attempt to improve recovery, however, optimal recovery was in the range of 63–76% only. Tseng et al. [22] tried to improve plasmid DNA recovery by reducing the binding strength between plasmid DNA and the ion-exchange membrane with various alcohols and chaotropic salts in the washing buffer. They concluded that recovery was hampered by the irreversible binding of plasmid DNA to the membrane support. It is noteworthy that the membrane support in these two studies was polyethersulfone polymer, which makes the membrane partially hydrophobic. Plasmid DNAs are known to adsorb strongly onto hydrophobic chromatography resins [23]. Thus irreversible binding of plasmid DNA to the membrane support was implicated for these polyethersulfone-based membranes. Irreversible binding was also observed in another study employing the polyethersulfone-based ion-exchange membrane [22]. It was therefore suggested that efforts to improve plasmid DNA recovery should focus on the design of membranes employing hydrophilic surfaces [19,22].

The objective of this work is to explore the potential application of a hydrogel-based strong anion-exchange membrane for the purification of plasmid DNA. The membrane used in this study is a composite material incorporating functionalized hydrogel onto the polypropylene membrane support. The hydrophilic hydrogel completely encases the membrane support upon swelling, which provides a hydrophilic surface. It is proposed that the hydrophilic surface has a higher affinity for the highly ionized DNAs than the previously used (polyethersulfone-based) ion-exchange membranes. The combined effects of the inherent properties of plasmid DNA, membrane adsorbent, and the influence of the ionic environment were systematically studied using the strong anion-exchange membrane. Although the application of a weak anion-exchange hydrogel membrane was reported previously [20], to the best of our knowledge, this is the first study of employing a hydrogel-based strong anion-exchange membrane for plasmid DNA purification.

2. Materials and methods

2.1. Preparation of plasmid DNA

Escherichia coli cells harbouring pUC19 (2.7 kb) [24], pET20b(+) (3.7 kb) [25] and pFlag-PalB (6.4 kb) [26] plasmids, respectively, were grown overnight in Luria–Bertani (LB) media supplemented with 50 μg/ml ampicillin at 37 °C. Cells were harvested by centrifuging the overnight culture at 4000 × *g* for 10 min. MaxiPreps kit (Bio Basic, Inc., Markham, Ontario, Canada) was used to purify plasmid DNA from the harvested cells. The plasmid DNA feed for membrane chromatography experiments was prepared by diluting the purified plasmid DNA to 50 μg/ml in the loading buffer at a desired pH. In each experiment, 2 ml of the diluted plasmid DNA sample was loaded onto the membrane.

2.2. Buffers for plasmid DNA loading, washing, and elution

The loading and washing buffers were 50 mM Tris–HCl, and the elution buffers contained either 0.8 M or 2 M NaCl in addition to 50 mM Tris–HCl. All buffers were prepared in 18 MΩ deionized water and adjusted to pH 7, pH 8, or pH 9.

2.3. Membrane material, pre-treatment, and characterization

The developmental hydrogel-based strong anion-exchange (Q) membranes (25 mm in diameter) were provided by Natrix Separations Inc. (Burlington, Ontario, Canada). The membrane was made by incorporating 3-acrylamidopropyl-trimethylammonium chloride (ATPAC) functionalized hydrogel polymer onto the membrane support. The crosslinking percentage ranged from 9% to 12%. Typical mean dynamic BSA binding capacity is 200 mg/ml of membrane volume at a 10% break through value. The 25 mm disk has a membrane volume of 0.09 ml. The two sides of the membrane differ in their surface texture, with one side being rougher than the other. We thereafter refer to it as the rough side or the smooth side, respectively. The membrane was pre-treated by soaking in a loading buffer for either 0.5 h (as recommended by the manufacturer) or 16 h at room temperature prior to its use. Based on the test results using various soaking times, 16 h was selected to ensure that the hydrogel would swell to its full potential. Both sides of the membrane were characterized by Environmental Scanning Electron Microscope (ESEM, ElectroScan Model E-3, FEI™, Hillsboro, USA) before and after pre-treatment.

2.4. Batch adsorption and desorption

Small membrane pieces with a cross-sectional area of 0.4 cm^2 and the plasmid pFlag-PalB were used in the batch experiments. The membrane sheets were added to the plasmid DNA solutions (up to 180 μg/ml in a loading buffer at pH 8) in 1.5-ml microcentrifuge tubes and the mixtures were allowed to equilibrate for 16 h at ambient temperature in a shaker incubator at 250 rpm. After incubation the plasmid DNA concentration of the liquid phase was measured. The amount of plasmid DNA adsorbed onto the membrane was determined by an overall mass balance, and it was expressed as micrograms per cross-sectional membrane sheet area (cm^2). The batch experiments were conducted in triplicate. Using the Metropolis–Hastings algorithm [29], the adsorption data were analyzed to evaluate the parameters of the Langmuir isotherm (Eq. (1)).

$$q = \frac{q_{max} K c}{1 + kc} \quad (1)$$

where q is the amount of plasmid DNA adsorbed onto the membrane (μg/cm^2), c is aqueous concentration in equilibrium with

the solid phase (μg/ml), q_{max} is the maximum adsorbed plasmid DNA capacity (μg/cm^2), and K is the Langmuir equilibrium constant (ml/μg). The unbound plasmid DNA was washed off by the washing buffer (pH 8), and the elution of the adsorbed plasmid DNAs was carried out by placing the membrane with adsorbed plasmid DNAs in 1-ml elution buffer containing 0.8 M NaCl (pH 8). Desorption was conducted at ambient temperature in a shaker incubator at 250 rpm for 16 h after which no further desorption was observed.

2.5. Membrane chromatography

Membrane chromatography in this study was conducted using a laboratory-scale (10 ml) stirred cell (Millipore, Billerica, USA), which was pressurized by nitrogen to 172 kPa (25 psi) to drive the flow of the plasmid DNA solution across the membrane. For each experiment, a single layer of the pre-treated membrane was overlaid in the stirred cell. The membrane was oriented in two different ways, either with the rough side or the smooth side upward facing the flow. Plasmid DNA solution at 2 ml was loaded onto the membrane and was pressure-driven through the stirred cell. Upon loading onto membrane plasmid DNA is subject to four possible outcomes, i.e. (1) flow through the membrane, (2) adsorb onto the membrane reversibly, (3) adsorb onto the membrane irreversibly, and (4) be rejected by the membrane so that the plasmid DNA remains at the frontal side of the membrane. The amount of plasmid DNA adsorbed onto the membrane was determined by an overall mass balance using Eq. (2)

$$\%Ads = \frac{P_L - P_R - P_{FT}}{P_L} \times 100\% \quad (2)$$

where P_L is the amount of plasmid DNA loaded to the membrane, P_R is the amount of plasmid DNA rejected by the membrane, P_{FT} is the amount of plasmid DNA flowing through un-adsorbed, and $(P_L - P_R - P_{FT})$ is the amount of plasmid DNA adsorbed onto the membrane. After loading the plasmid DNA, the membrane was washed with 2 ml of washing buffer, followed by a two-step elution with elution buffers containing 0.8 M and 2 M NaCl. The percentage recovered from the membrane was calculated using Eq. (3) as follows.

$$\%Rc = \frac{\sum_i P_{Ei}}{P_L} \times 100\% \quad (3)$$

where P_{Ei} is the amount of plasmid DNA eluted in the ith elution fraction, and $\sum_i P_{Ei}$ is the total amount of plasmid DNA recovered from the membrane during the elution. The irreversibly adsorbed plasmid DNA onto the membrane was determined by the difference between the total amount of adsorbed plasmid DNA and the eluted amount using Eq. (4)

$$\%Ads_I = \frac{P_{Ads} - \sum_i P_{Ei}}{P_L} \times 100\% \quad (4)$$

where P_{Ads} $(=P_L - P_R - P_{FT})$ is the total amount of plasmid DNA adsorbed onto the membrane, $P_{Ads} - \sum_i P_{E_i}$ is the amount of plasmid DNA irreversibly adsorbed onto the membrane, and the rest of the variables are the same as those in Eqs. (2) and (3). The experiments were replicated for each plasmid DNA at pH 7, pH 8, and pH 9.

2.6. Analytical methods

The NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA) was used to measure the plasmid DNA concentration at 260 nm. The absorbance was converted to concentration (ng/μl) using the Beer–Lambert equation, $A = E \times b \times c$, where A is the absorbance, E is the extinction coefficient, b is the path length, and c is the concentration. In addition to the spectroscopic analysis, some plasmid DNA samples were also analyzed by gel electrophoresis for comparison. To perform the analysis, samples were loaded to a 1% agarose gel for electrophoresis at 100 V for 60 min. Then, the gel was stained with ethidium bromide and visualized with a UV transilluminator. The image of the gel was taken by a digital camera, and the intensity of each band was scanned and quantified using an image processor (Image J software from National Institutes of Health, http://rsbweb.nih.gov/ij/). Using these quantified data, the percentages of the rejected and recovered plasmid DNAs were estimated. The molecular size of the tertiary structure of plasmid DNAs was determined based on the electrophoretic mobility.

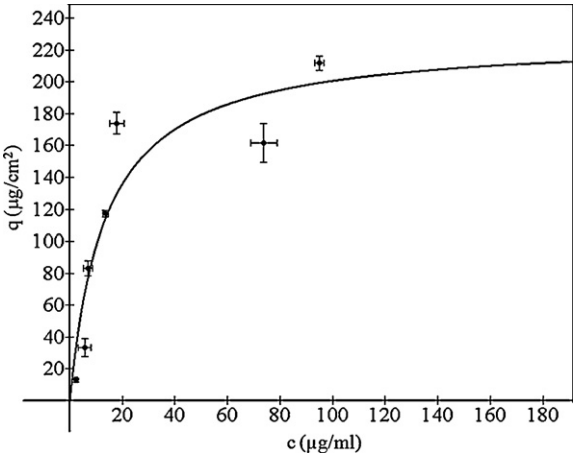

Fig. 1. Batch adsorption isotherm of pFlag-PalB (6.4 kb) on strong anion-exchange membrane in loading buffer of pH 8.

3. Results and discussion

3.1. Batch adsorption and desorption

The selection of the chromatographic material for the economic production of pharmaceutical-grade plasmid DNA is generally based on the adsorption capacity and reversibility. Therefore, the maximum binding capacity and the ability to reversibly bind plasmid DNAs from the hydrogel membrane were investigated in a series of batch experiments. The adsorption isotherm of pFlag-PalB is shown in Fig. 1. The maximum amount of plasmid DNA adsorbed onto the membrane (q_{max}) and the Langmuir equilibrium constant (K) were found to be 227 μg/cm^2 (12.4 mg/ml membrane volume) and 7.4×10^{-2} ml/μg, respectively. It is noteworthy that the maximum uptake capacity per membrane volume is 6 times higher than that of a resin [5]. It is also notable that the maximum uptake capacity per cross-sectional membrane sheet area for plasmid DNA of similar linear size was much higher than that of the membranes used in previous studies [19,27]. Reversible binding of plasmid DNA was assessed by eluting the adsorbed plasmid DNA from the membrane in a batch mode. Results from previous chromatographic experiments performed with a gradient elution using buffer containing 0–2 M NaCl have shown that an elution buffer containing at least 0.6 M NaCl was needed to elute plasmid DNA from the membrane, and a salt concentration of more than 0.8 M NaCl did not improve recovery. Experiments were also performed with a step-wise elution scheme, where an elution buffer containing 0.8 M and 2 M NaCl was used stepwise to see if further elution is possible beyond 0.8 M NaCl. It appears that the majority of the plasmid DNA was eluted with a buffer containing 0.8 M NaCl and further elution with 2 M NaCl did not improve recovery. Therefore, an elution buffer containing 0.8 M NaCl was chosen to desorb plasmid DNA from the membrane in subsequent work. The aver-

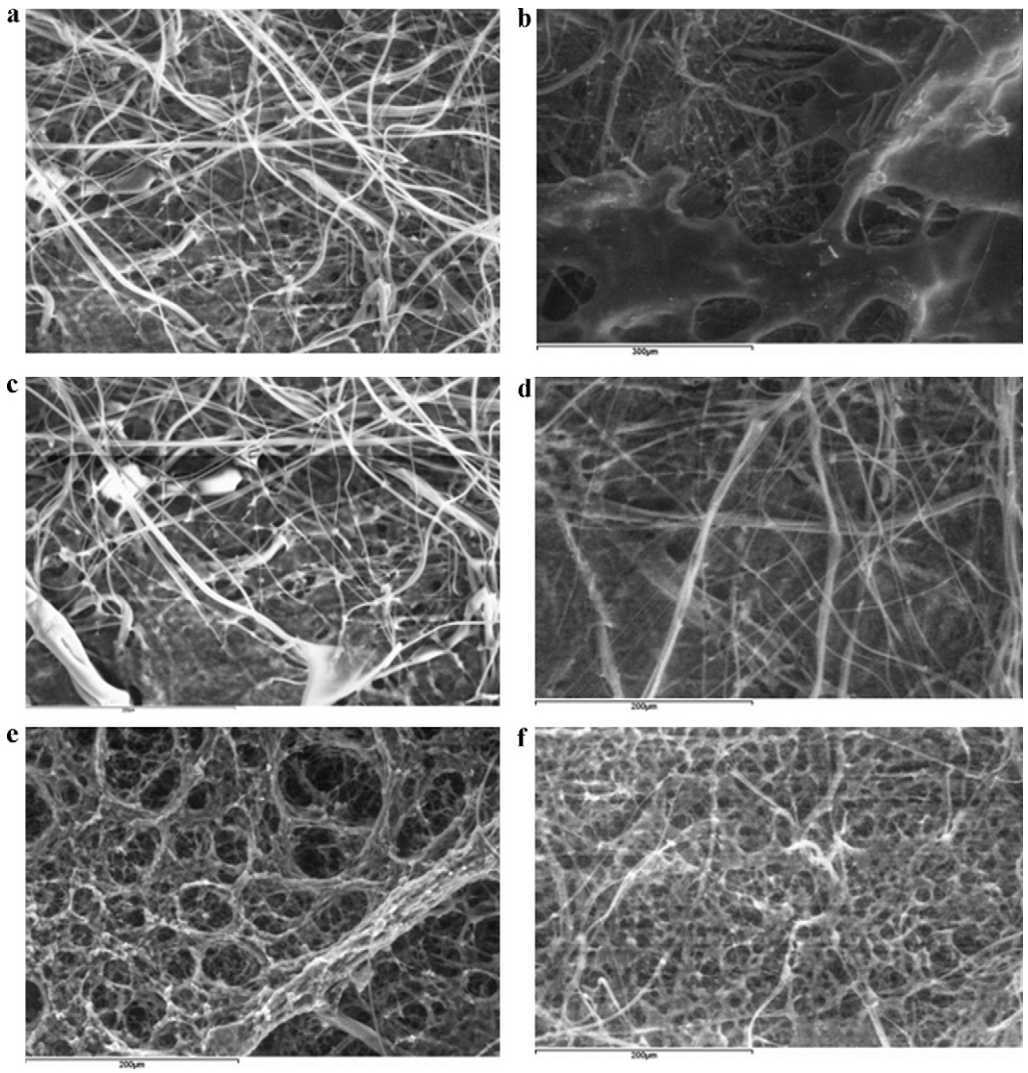

Fig. 2. ESEM images of (a) the rough side of dry membrane, (b) the smooth side of dry membrane, (c) the rough side of 0.5-h soaked membrane, (d) smooth side of 0.5-h soaked membrane, (e) rough side of 16-h soaked membrane and (f) smooth side of 16-h soaked membrane. All at 300× magnification.

age recovery was ~90%. The unrecoverd plasmid DNA might be physically entrapped within the polymeric support as the hydrogel shrinks in solution of high ionic strength that is used to elute plasmid DNAs, or irreversibly adsorbed onto the membrane due to non-specific interaction caused by the high ionic strength of the elution buffer. Despite an incomplete desorption, the average recovery from the hydrogel membrane was substantially higher than the previously reported recoveries at 63–76% using various elution buffers containing salts and compaction agents [19]. It is postulated that the high adsorption and desorption are the inherent property of the present hydrogel-based membrane adsorbent. The batch adsorption experiments were carried out by incubating membrane and plasmid DNA in the loading buffer for 16 h, which was long enough for the hydrogel-based membrane to completely encase the hydrophobic membrane support and develop a more porous structure with a greater accessible adsorptive area (Fig. 2e and f).

3.2. Membrane chromatography

3.2.1. Plasmid tertiary structure

Previous studies [21,28–31] have shown that the recovery of plasmid DNA with a small tertiary structure was often higher than that with a large one. The tertiary structure of plasmid DNA does not depend solely on their primary structure, but also on the degree of supercoiling. The tertiary structures of three plasmid DNAs with different molecular sizes, namely pET20b(+) (3.7 kb), pFlag-palB (6.4 kb) and pUC19 (2.7 kb), were compared by gel electrophoresis. Then, the effect of tertiary structures was related to membrane chromatography performance under identical operating conditions. As shown by the gel electrophoresis results carried out at pH 8 (Fig. 3a), pET20b(+) had the smallest tertiary structure, followed by pFlag-PalB, and pUC19 has the largest tertiary structure. The average recovery of pET20b(+), pFlag-PalB, and pUC19 at pH 8 from the 16-h soaked membrane was 89%, 75%, and 65%, respectively (Fig. 3b), which follows the order of their tertiary structural sizes from the smallest to the largest. Plasmid DNAs with a small tertiary structure have a short hydrodynamic radius so that they will be less restricted by the pores of the membranes. Plasmid DNA with smaller tertiary structure tends to have a lower surface charge density than plasmid DNA with a large tertiary structure, thus forming a fewer number of interactions. The experimental results of this study and other previous studies [21,28–31] are consistent in terms of the observation of higher recovery associated with small plasmid size using anion-exchange membrane chromatography. From a bioprocessing viewpoint, it will be desirable to use plasmid DNA with a small tertiary structure as the vector for gene therapy and DNA vaccines.

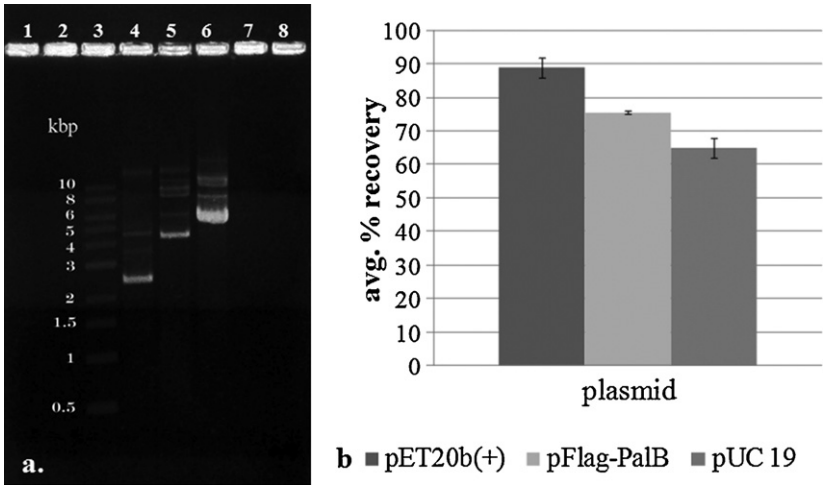

Fig. 3. (a) Gel electrophoresis of pET20b(+) (lane 4), pFlag-PalB (lane 5) and pUC19 (lane 6), and (b) averaged percentage recovery of pET20b(+), pFlag-PalB and pUC 19 at pH 8 with 16-h soaking of the membrane.

3.2.2. Surface texture and membrane orientation

Orientation is another important consideration since the hydrogel membrane used in this study differed in surface texture. To assess the effect of membrane orientation, the membrane was placed with either the "rough" or "smooth" side upward and employing the plasmid pET20b(+). The membrane was pre-equilibrated for 0.5 ht as recommended by the manufacturer. As determined previously, membrane performance is dependent on plasmid size, since small plasmids yield higher recovery. The plasmid pET20b(+), a relatively small molecule in comparison to the other plasmids used in this study, was chosen because it is expected to minimize the effect of plasmid size on membrane performance. Therefore, any difference seen in membrane performance will be more reflective of membrane orientation.

Table 1 summarizes membrane performance for pET20b(+). There was significantly more plasmid DNAs adsorbed and recovered if the membrane was oriented with the rough side upward in the chromatographic unit. The average rejection of pET20b(+) by the rough and smooth side was 20.5% and 68.0%, respectively. The difference in rejection was also confirmed by gel electrophoresis. As shown in Fig. 4, the band corresponding to rejected plasmid DNA with the rough side up was very faint as compared to the band intensity of the plasmid DNA feed (Fig. 4a); whereas the band corresponding to the rejected DNA with the smooth side up was almost as bright as the band intensity of the plasmid DNA feed (Fig. 4b). The image-processing software, Image J, was used to determine the percentage of rejection and recovery by analyzing the band intensities of the samples loaded onto the gel. The results were consistent with those obtained by spectrophotometric analysis (Table 1). The two sides of the membrane were visualized under ESEM (Fig. 2), and the difference in surface porosity was revealed. The rough side of the membrane was more porous than the smooth side and therefore it provided a larger accessible area for plasmid DNA adsorption. This was consistent with the better plasmid capture when using the rough side up. The flux of the plasmid DNA solution across the membrane was also significantly higher with rough side upward in the chromatographic unit. The larger accessible area on the rough side of the membrane would reduce the extent of rejection, thus less plasmid DNAs would accumulate at the frontal membrane surface which would otherwise restrict the flow of the processing solution.

3.2.3. Membrane pre-treatment and buffer pH

Chromatographic membranes are usually preserved and shipped in a dehydrated state. According to the manufacturer's recommendation, the membrane should be pre-equilibrated in the loading buffer for at least 0.5 h prior to use. However, it was noted that the typical porous structure of hydrogel-based membranes was under-developed after 0.5-h soaking and further swelling and continual pore development were anticipated upon a longer contact with the aqueous environment. Membrane pre-treatment methods involving 0.5 h and 16 h soaking in the loading buffer were compared for their effectiveness.

Environmental Scanning Electron Microscopy (ESEM) was used to characterize both sides of the membrane in a wet state after 0.5-h and 16-h soaking. As shown in Fig. 2, the dry and 0.5-h soaked membranes were similar in structure. However, the porous structure changed significantly if the membrane was soaked for 16 h. The pores were deeper, enlarged and more uniformly distributed on both sides. The porous structure became more complex on the rough side, where interconnected macropores have developed within supermacropores (up to 200 μm). The enlargement in pores would increase the accessible adsorptive area, thus resulting in less intermolecular competition of the plasmid DNA for the membrane binding sites. Also, the electrostatic repulsion of incoming plasmid DNA by the previously captured material on the membrane was expected to be minimized by the well developed porous structure, resulting in a reduction in the amount of rejected plasmid DNA. This could prevent, in turn, the possible formation of a filter cake layer at the frontal side of the membrane, which would otherwise lower the binding capacity and reduce the operational throughput.

The performance of membrane chromatography of various plasmid DNAs using 0.5-h and 16-h treated membranes are summarized in Fig. 5. The average adsorption of pET20b(+), pFlag-PalB, and pUC19 by the 0.5-h soaked membrane was 73%, 68%, and 50%, respectively. The adsorption of pET20b(+) and pFlag-PalB was improved to 96% if the membrane was soaked for 16 h. There was no difference in the adsorption of pUC19 between 0.5-h and 16-h soaking at pH 7, however, the adsorption was improved to 97% after 16-h soaking at pH 8 and pH 9. The application of 16-h soaking resulted in an almost complete adsorption (>96%) for plasmids having different size (Fig. 5), whereas the adsorption onto the 0.5-h soaked membrane was dependent on the plasmid size. It is known that the binding capacity of a membrane for plasmid DNA is strongly dependent on the membrane's available adsorptive area [12,21]. The adsorptive area is, in turn, dependent on the porosity of the membrane. The improvement in membrane porosity and pore distribution with 16-h soaking (see Fig. 2) provided a larger adsorptive area, which negated the size effect on adsorption observed after 0.5-h soaking and improved adsorption significantly.

Table 1
Comparison of averaged percentage adsorption, averaged percentage recovery and flux of loading, washing and elution step between experiments using the rough side and the smooth side of the membrane as the plasmid DNA loading surface for pET20b(+) at pH 8 with 0.5-h soaking of the membrane.

Membrane surface texture	Avg. % adsorption	Avg. % recovery	Loading flux ($ml\,m^{-2}\,s^{-1}$)	Washing flux ($ml\,m^{-2}\,s^{-1}$)	Elution flux ($ml\,m^{-2}\,s^{-1}$)
Rough	79.5 ± 1.3	77.0 ± 1.6	8.4 ± 0.3	5.0 ± 0.2	67.9 ± 0.0
Smooth	32.0 ± 1.4	67.5 ± 0.7	4.1 ± 0.0	4.1 ± 0.0	5.1 ± 1.0

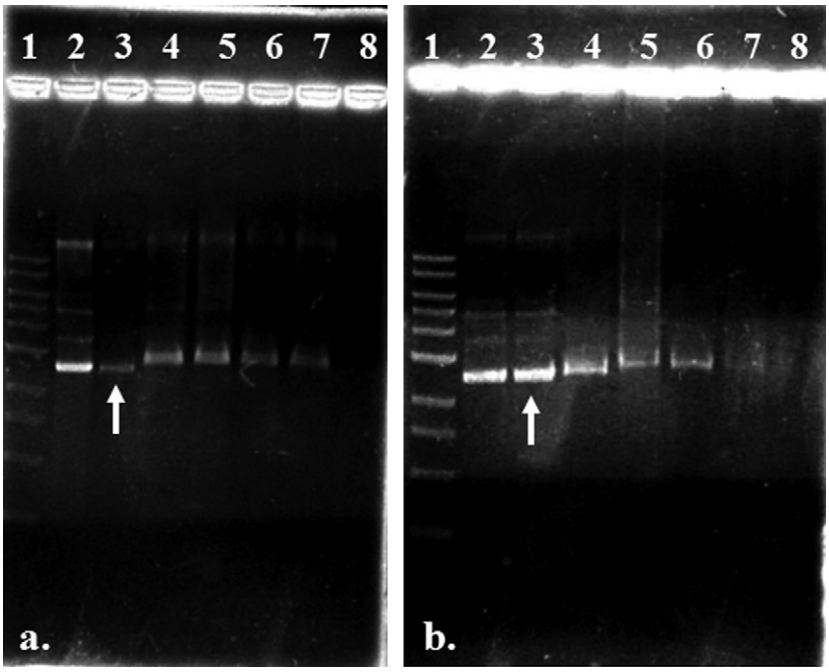

Fig. 4. Gel electrophoresis of samples taken from different steps of (a) experiment using rough side of the membrane as the plasmid DNA loading surface, and (b) experiment using smooth side of the membrane as the plasmid loading surface. In both gel, lane 1 is DNA marker, lane 2 is plasmid DNA feed, lane 3 is the frontal side of membrane after loading and before washing, lane 4 is the frontal side of membrane before first elution with elution buffer containing 0.8 M NaCl, lane 5 is the first elution flowthrough, lane 6 is the frontal side of the membrane before second elution, lane 7 is the second elution flowthrough and lane 8 is the third elution flowthrough. Arrow in both gels is pointed at the band corresponding to rejected plasmid DNA.

Reversibility of the adsorbed plasmids was also assessed for 0.5-h and 16-h soaked membranes. The highest recovery occurred using membranes after 16-h soaking for all three plasmid DNAs (Fig. 5). Upon soaking for 16 h, the hydrophilic hydrogel (shown in Fig. 2) was able to completely encase the hydrophobic membrane support, which reduced irreversible adsorption associated with hydrophobic interaction with the membrane support. There was a much greater improvement in recovery of pUC19 with the use of 16-h soaked membrane as compared to the other two plasmid DNAs, suggesting that the pore size of the membrane impacted the recovery of plasmid DNAs with a larger tertiary structure. The flux through the membrane was significantly increased for all the three plasmid DNAs if 16-h soaked membranes were used (data not shown). For large-scale production, it is important to have a high throughput in addition to a high recovery and yield, and using the rough side of 16-h soaked membrane to purify plasmid DNAs would fulfill these requirements.

Unlike adsorption where the capacity was mainly influenced by the porosity of the membrane, desorption was also affected by the electric interaction between the strong anion-exchange hydrogel and plasmid DNA. It appears from the previous experiments that plasmid DNA with a compact conformation would have a lower overall surface charge density, which would result in less engagement of the surface charges with the membrane and facilitate subsequent elution. However, the ionic environment would also affect the extent of interaction between the charged hydrogel and plasmid DNA [28,29,32]. Therefore, reversibility was also compared at various pH values (i.e. pH 7, pH 8, and pH 9). The highest recovery of pET20b(+), pFlag-PalB, and pUC19 was 89%, 76%, and 77%, respectively. The use of a chromatographic buffer at pH 8 resulted in the highest recovery for pET20b(+) and pFlag-PalB,), whereas the highest recovery of pUC19 was observed using a chromatographic buffer at pH 9 (Fig. 5c). It is proposed that plasmid DNAs may have a smaller tertiary structure (due to supercoiling) and/or a lower overall surface charge under these pH conditions, which would result in less interaction with the membrane, thus in a higher recovery as compared to other (less favourable) pH values. It appears that the most favourable pH for reversibility varies with plasmid DNA, therefore the operating pH should be carefully determined for each individual plasmid. For an economic production of plasmid DNA, irreversible adsorption should be kept at a minimum. It is noteworthy that the pH at which the least amount of irreversible adsorption occurred was the pH where the highest recovery was observed if 16-h soaked membrane was used (Fig. 6). Also, plasmid DNAs with smaller tertiary structure had lower levels of irreversible adsorption. Hence, factors affecting membrane porosity and DNA charge density should be considered to minimize irreversible adsorption for an economic production of pharmaceutical-grade plasmid DNA.

It is important that plasmid DNA retains a supercoiled isoform during the purification process. The biological activity of plasmid DNAs is often associated with their topoisoforms [33,34], and the supercoiled isoform is considered the desired one for therapeutic applications [35]. To assess the isoforms distribution, gel elec-

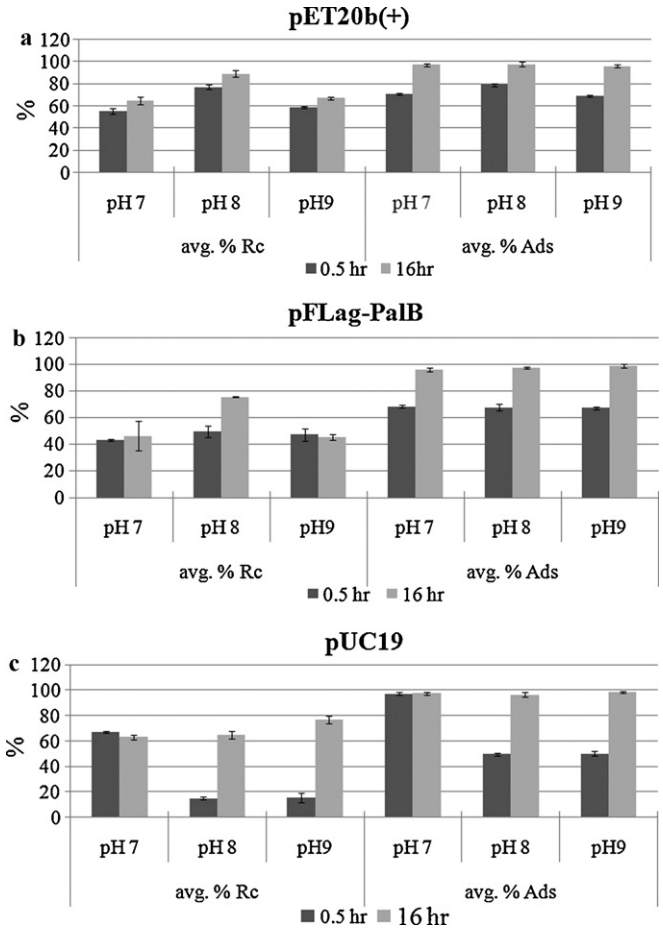

Fig. 5. Comparison of the averaged percentage adsorption and recovery of (a) pET20b(+), (b) pFlag-PalB and (c) pUC19 between experiments using 0.5-h and 16-h soaked membranes at pH 7, pH 8 and pH 9.

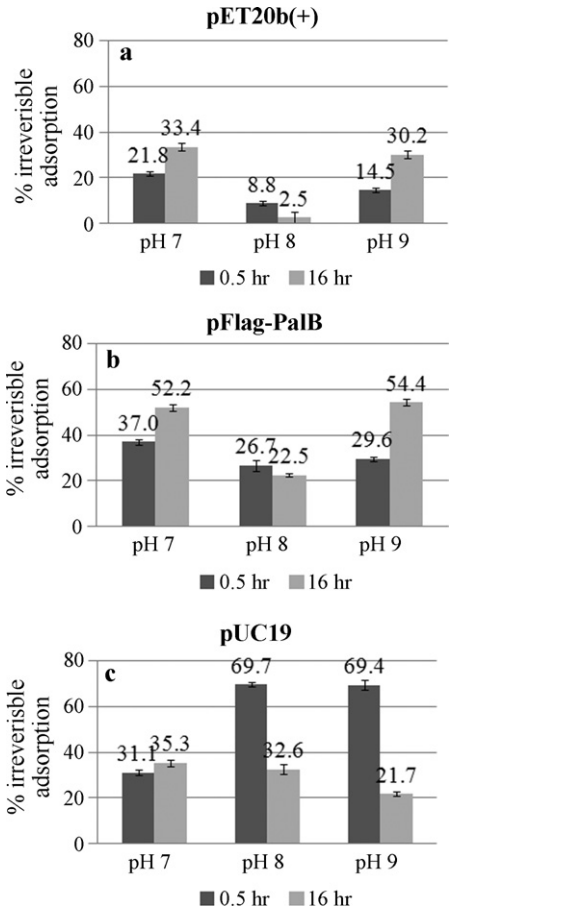

Fig. 6. Percentage irreversible adsorption of (a) pET20b(+), (b) pFlag-PalB and (c) pUC19 to the membranes that were either soaked for 0.5 h or 16 h at pH 7, pH 8 and pH 9.

trophoresis of the plasmid DNA samples was performed before and after membrane chromatography. As shown in Fig. 4, the majority of the loaded plasmid DNAs was in the supercoiled isoform, and this was maintained during the chromatographic procedure.

3.3. Factorial contribution to membrane performance

Since all factors investigated herein appeared to be relevant to membrane performance, it would be valuable to obtain the

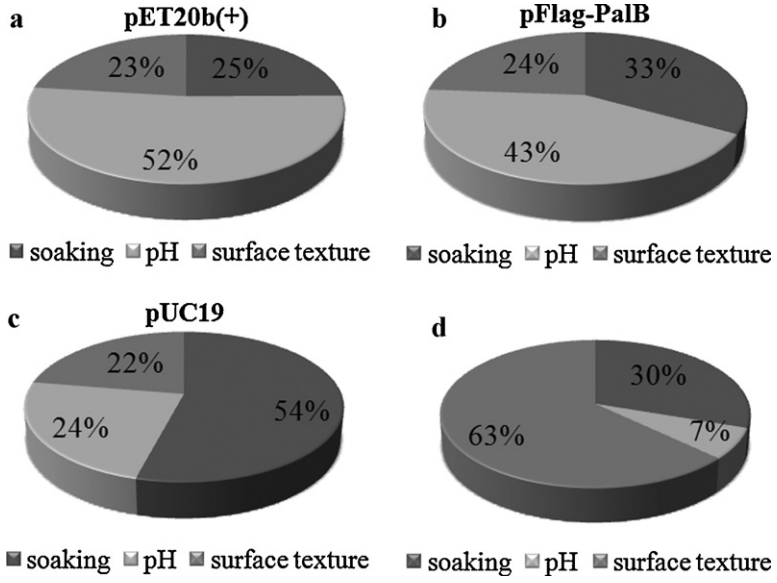

Fig. 7. The relative contribution of membrane soaking time, pH of the buffer and the orientation of the membrane to plasmid recovery of (a) pET20b(+), (b) pFlag-PalB, (c) pUC19, and (d) the relative contribution of the above mentioned factors to plasmid adsorption of pET20b(+) to the membrane.

contributing level of each factor for any future improvement of membrane design. Therefore, the relative contribution of various factors, including membrane orientation, membrane soaking time, and chromatographic buffer pH, to the performance of membrane chromatography was determined using fractional factorial analysis. The results are summarized in Fig. 7. Note that the contribution of each factor to the recovery was evaluated for all three plasmid DNAs (Fig. 7a–c). However, a similar evaluation associated with the adsorption behaviour was evaluated for pET20b(+) only (Fig. 7d) since the experiments were performed at more levels of the membrane orientation factor only for this plasmid DNA. Factorial design allows the determination of the effect of a given factor at several levels of the other factors so that the conclusions are valid over a range of experimental conditions [36]. The fractional factorial experiments comprised a $2 \times 2 \times 3$ level (orientation $\times$ soaking time $\times$ buffer pH) design. The three-factor interactions were confounded; consequently the main effects and the two-factor interactions were evaluated. Based on the analysis, the interactions between the factors were insignificant. The main effects "explained" 80% or more of the total variability. Evidently, the response at the "best" buffer pH was not dependent on either the orientation of the membrane or the length of soaking. In a similar vein, membrane orientation and soaking time appeared to be independent variables with minimal interaction. For both pET20b(+) and pFlag-PalB, the effect of the buffer pH on the recovery was significantly greater than that of the membrane soaking time and membrane orientation. However, the effect of the buffer pH and membrane orientation on the recovery of pUC19 was minor, as compared to the effect of the membrane soaking time. One can conclude that the recovery of plasmid DNAs with a small tertiary structure is mostly influenced by the buffer pH, whereas the recovery of plasmid DNAs with a large tertiary structure is mainly dependent on the membrane pore size. For the adsorption of plasmid DNAs (Fig. 7d), the effect of membrane orientation was the greatest, followed by the membrane soaking time, while the buffer pH has the smallest effect. It is noteworthy that the combination of membrane orientation and soaking time determines the available adsorptive area of the membrane; therefore, the adsorption of plasmid DNAs was primarily dependent on the available adsorptive area of the membrane. The result is consistent to previous observations [12,21].

4. Conclusion

Membrane chromatography is promising to overcome several major challenges associated with the large-scale production of plasmid DNAs. In this study, a hydrogel-based strong anion-exchange membrane with a high binding capacity was used to demonstrate its potential applicability for plasmid DNA purification. While the desired property in membrane chromatography is reversible adsorption, rejection and irreversible adsorption of plasmid DNAs can be frequently observed. Using the hydrogel-based membrane, the extent to which these undesirable events occurred was found to be dependent on various factors, including the membrane porosity, the soaking time, the buffer pH and the size of plasmid DNA tertiary structure. Porosity appears to have a major impact on performance. The more porous side of the membrane would provide a larger accessible area for plasmid DNA adsorption, thus the rejection due to the restrictive membrane pore size and the repulsion by the previously bound plasmid DNA could be minimized. In this study it was shown that the porosity can be greatly increased by properly pre-treating the hydrogel membrane. With the pre-treatment, pores were enlarged, well-structured, and evenly distributed so that the binding capacity for plasmid DNAs could be substantially enhanced. In addition, the hydrophilic supermacroporous hydrogel could completely encase the membrane support and the irreversible adsorption of plasmid DNAs to the hydrophobic membrane support was reduced. The approach greatly reduced plasmid DNA loss associated with the irreversible adsorption to the membrane support that is commonly observed for many commercially available anion-exchange membranes. The tertiary structure of plasmid DNA, as determined by the degree of supercoiling also had an effect. While the size effect on the adsorption was negated by the enlargement of pores by proper pre-treatment, but the size effect persisted for desorption. In general, a higher desorption was observed for small plasmid DNAs. Buffer pH had a more pronounced impact on desorption behaviour of plasmid DNA than other factors because it could potentially affect the size of the tertiary structure of plasmid DNA and/or surface charge density. Through a careful selection of the operating pH, the recovery can be further improved. The optimal pH appeared to be plasmid-dependent. The high convective flow through the supermacroporous structure of the hydrogel membrane, as compared to the diffusive transport through the interior of the resin beads, would be an important processing benefit.

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References

[1] J. Wolff, R. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P. Felgner, Science 247 (1990) 1465.
[2] J.W. Shiver, E.A. Emini, Annu. Rev. Med. 55 (2004) 355.
[3] M.M. Gottesman, Cancer Gene Ther. 10 (2003) 501.
[4] Y. Han, S. Liu, J. Ho, M.K. Danquah, G.M. Forde, Chem. Eng. Res. Des. 87 (2009) 343.
[5] K.J. Prather, S. Sagar, J. Murphy, M. Chartrain, Enzyme Microb. Technol 33 (2003) 865.
[6] P. Tiainen, I. Galaev, P.O. Larsson, Biotechnol. J. 2 (2007) 726.
[7] G.N.M. Ferreira, G.A. Monteiro, D.M.F. Prazeres, J.M.S. Cabral, Trends Biotechnol. 18 (2000) 380.
[8] W.W. Leitner, H. Ying, N.P. Restifo, Vaccine 18 (1999) 765.
[9] J. Stadler, R. Lemmens, T. Nyhammar, J. Gene Med. 6 (2004) S54.
[10] A. Eon-Duval, G. Burke, J. Chromatogr. B 804 (2004) 327.
[11] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, J. Chromatogr. A 1069 (2005) 3.
[12] P. Tiainen, P.E. Gustavsson, A. Ljunglöf, P.O. Larsson, J. Chromatogr. A 1138 (2007) 84.
[13] E. Unsal, T. Bahar, M. Tuncel, A. Tuncel, J. Chromatogr. A 898 (2000) 167.
[14] A. Ljunglof, P. Bergvall, R. Bhikhabhai, R. Hjorth, J. Chromatogr. A 844 (1999) 129.
[15] D.M.F. Prazeres, G.N.M. Ferreira, G.A. Monteiro, C.L. Cooney, J.M.S. Cabral, Trends Biotechnol. 17 (1999) 169.
[16] C. Haber, J. Skupsky, A. Lee, R. Lander, Biotechnol. Bioeng. 88 (2004) 26.
[17] M.A. Teeters, S.E. Conrardy, B.L. Thomas, T.W. Root, E.N. Lightfoot, J. Chromatogr. A 989 (2003) 165.
[18] P. Guerrero-German, D.M.F. Prazeres, R. Guzman, R.M. Montesinos-Cisneros, A. Tejeda-Mansir, Bioprocess Biosyst. Eng. 32 (2009) 615.
[19] M.A. Teeters, T.W. Root, E.N. Lightfoot, J. Chromatogr. A 1036 (2004) 73.
[20] G.M. Miladys Limonta, M. Pupo, O. Ruiz, BioPharm 23 (2010).
[21] A.G. Grunwald, M.S. Shields, Anal. Biochem. 296 (2001) 138.
[22] W.C. Tseng, F.L. Ho, T.Y. Fang, S.Y. Suen, J. Membr. Sci. 233 (2004) 161.
[23] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, S.A.M. Martins, G.N.M. Ferreira, D.M.F. Prazeres, Biotechnol. Bioeng. 68 (2000) 576.
[24] C. Yanisch-Perron, J. Vieira, J. Messing, Gene 33 (1985) 103.
[25] D. Gunasekera, R.G. Kemp, Protein Exp. Purif. 16 (1999) 448.
[26] Y. Xu, A. Yasin, T. Wucherpfennig, C. Chou, World J. Microbiol. Biotechnol. 24 (2008) 2827.
[27] C.S. Chang, H.S. Ni, S.Y. Suen, W.C. Tseng, H.C. Chiu, C.P. Chou, J. Membr. Sci. 311 (2008) 336.
[28] D.R. Latulippe, A.L. Zydney, Biotechnol. Bioeng. 99 (2008) 390.

[29] E. Arkhangelsky, B. Steubing, E. Ben-Dov, A. Kushmaro, V. Gitis, Desalination 227 (2008) 111.
[30] J.C. Murphy, G.E. Fox, R.C. Willson, J. Chromatogr. A 984 (2003) 215.
[31] A. Eon-Duval, R.H. MacDuff, C.A. Fisher, M.J. Harris, C. Brook, Anal. Biochem. 316 (2003) 66.
[32] Y.L. Lyubchenko, L.S. Shlyakhtenko, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 496.
[33] M.J. Molloy, V.S. Hall, S.I. Bailey, K.J. Griffin, J. Faulkner, M. Uden, Nucleic Acids Res. 32 (2004) e129.
[34] L. Cupillard, V. Juillard, S. Latour, G. Colombet, N. Cachet, S. Richard, S. Blanchard, L. Fischer, Vaccine 23 (2005) 1910.
[35] J. Urthaler, W. Buchinger, R. Necina, Chem. Eng. Technol. 28 (2005) 1408.
[36] D.C. Montgomery, E.A. Peck, G.G. Vining, Introduction to linear regression analysis, 3rd ed., John Wiley & sons, Inc., USA (2001).