Analytical Methods

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2013, **5**, 1682

Affinity analysis between immobilized L-arginine and plasmid isoforms provided by surface plasmon resonance†

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A strategy for studying the binding mode of plasmid isoforms to an immobilized amino acid as a model support has been developed using Surface Plasmon Resonance (SPR). The binding of L-arginine to plasmid isoforms of pGL101, pUC19 and pVAX1-*LacZ* (supercoiled, open-circular and linear) is examined by measuring the equilibrium dissociation constants (K_D). L-Arginine was immobilized on a CM5 sensor chip and bound to plasmid isoforms with similar SPR binding profiles (square-shaped) and binding affinities ranging between 10^{-4} and 10^{-9} M. There are significant differences in the apparent affinity that is correlated with the three plasmids (2.39, 2.69 and 6.05 kbp). pGL101 shows the highest binding on the arginine surface followed by pVAX1-*LacZ*, while pUC19 shows the lowest binding. For the three plasmid isoforms, the supercoiled ones have the higher binding affinity to the arginine surface. Different buffer environments affect the interaction strength with an increase in response for Tris–HCl and a marked decrease for high salt concentrations. The knowledge of the affinity parameters is expected to provide further insights into the effect of plasmid topology on the purification by affinity chromatography.

Received 4th February 2013
Accepted 6th February 2013

DOI: 10.1039/c3ay40213d

www.rsc.org/methods

Introduction

The current idea of using nucleic acids as therapeutic agents coupled with the growing demand for DNA purification has led to an interest in understanding the DNA affinity interactions.[1] This demand is particularly strong for plasmid DNA (pDNA), which has applications in the bio-pharmaceutical industry with rigorous demands and requirements, such as larger quantities of pure and active pDNA.[2,3] This has created a need for more effective methods for the separation of plasmid isoforms. For pDNA therapeutics, the supercoiled plasmid is the desired isoform while the linear and open-circular isoforms are regarded as product impurities.[4]

A variety of chromatographic techniques have been developed for the separation of pDNA isoforms, including anion exchange chromatography,[5] thiophilic aromatic chromatography,[6] and affinity chromatography.[7,8] The last technique has been used to specifically capture the desired supercoiled isoform and isolate it based on specific biological functions or individual chemical structure, in a single affinity purification step.[9] Several ligands for affinity purification of pDNA have been investigated.[10,11] The problem of finding a suitable ligand in affinity chromatography is not restricted to specificity, but concerns also the binding strength and the kinetics of the ligand–pDNA interaction.[12] This implies that a ligand with an optimized dissociation constant will facilitate the successful operation of affinity chromatography for pDNA purification.[13] Amino acid-based chromatographic supports have been used in affinity chromatography for pDNA purification, exploiting affinity interactions between amino acids and nucleic acids,[14] offering reduced processing time, less unit operation, and high purity.[15,16] Namely, the L-arginine–bisoxyran–Sepharose support has been used to fully separate supercoiled (sc) and open-circular (oc) pDNA isoforms.[17] The interactions that govern the efficiency of this support for pDNA purification have been explained based on affinity chromatography data[7,17] and analysis of high-resolution protein–DNA structures.[18,19] Here we employ an SPR-biosensor to quantify the binding between L-arginine (acting as an affinity ligand) and plasmid isoforms of pGL101, pUC19 and pVAX1-*LacZ*. L-Arginine was immobilized on the CM5 sensor chip surface to mimic the immobilized ligand affinity chromatographic support. Also the effect of the buffers, HEPES-buffered saline, HBS EP+ (10 mM HEPES, 150 mM NaCl; 0.05% P20 surfactant; 3 mM EDTA, pH 7.4) and buffer C (50 mM $KHPO_4$, 10 mM EDTA, pH 7.5), on the binding of supercoiled pUC19 to the L-arginine surface was analyzed by SPR. The equilibrium dissociation constants were determined for pGL101, pUC19 and pVAX1-*LacZ*.

This method provides quick and cost-effective means to determine whether an affinity ligand has a suitable strength of binding for an affinity process.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ay40213d

Materials and methods

The 2.39 kbp plasmid pGL101 was provided by DSMZ (Braunschweig, Germany) and 2.69 kbp plasmid pUC19 and 6.05 kbp plasmid pVAX1-*LacZ* were provided by Invitrogen (Carlsband, CA, USA). The restriction enzymes Sma I and Hind III were from Takara (Otsu, Shiga, Japan) and the molecular weight marker HyperLadderTM I was from Bioline (Taunton, MA, USA). SPR analysis was performed using a BIAcore T200 system equipped with a carboxymethylated dextran-coated sensor chip (CM5 research grade). The amine-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide hydrochloride (EDC), 1 M ethanolamine–HCl, HBS, HBS EP+ (10 mM HEPES, 150 mM NaCl; 0.05% P20 surfactant; 3 mM EDTA, pH 7.4) and P20 were purchased from Biacore. Tris–HCl, HEPES, $KHPO_4$, EDTA and borate buffers were purchased from Sigma Aldrich.

Bacterial production of plasmids pGL101, pUC19 and pVAX1-*LacZ*

The pGL101 amplification was accomplished by autonomous replication in *Escherichia coli* (*E. coli*) YMC9. The pUC19 and pVAX1-*LacZ* amplification were performed by autonomous replication in *E. coli* DH5α hosts. The cell growth was carried out using the Terrific Broth medium (20 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 4 mL L^{-1} glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 100 μg ampicillin per mL for the cells transformed with pGL101 and pUC19 and supplemented with 30 μg kanamycin per mL for the cells transformed with pVAX1-*LacZ*. These antibiotics were used as selection markers for the exclusive growth of transformed cells. The fermentation was carried out overnight at 37 °C under 250 rpm shaking. The cells were harvested at the late log phase by centrifugation and were stored at −20 °C.

Alkaline lysis and primary isolation of plasmids pGL101, pUC19 and pVAX1-*LacZ*

The plasmids pGL101, pUC19 and pVAX1-*LacZ* were recovered from *E. coli* bacteria by using the Qiagen (Hilden, Germany) plasmid maxi kit according to the manufacturer's instructions. This protocol is based on a modified alkaline lysis procedure. Briefly, the bacterial pellets, obtained from the centrifugation of 250 mL cell broth at 5445 × *g* (30 min, 4 °C), were thawed and resuspended in 20 mL of 50 mM glucose, 25 mM Tris–HCl, and 10 mM ethylene-diamine tetraacetic acid (EDTA) at pH 8.0. The cell disruption, performed to release the intracellular content, was accomplished by addition of 20 mL of a 200 mM NaOH and 1% (w/v) sodium dodecylsulfate (SDS) solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by adding and mixing 20 mL of a prechilled neutralization solution of 3 M potassium acetate, pH 5.0 (incubation of 20 min on ice). The precipitate was removed by centrifuging twice at 20 000 × *g* (30 min, 4 °C) with a Sigma 3-18K centrifuge. Following lysis, the plasmid DNA is purified by chromatography. The binding of plasmid DNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions. Impurities are removed by a medium-salt wash, and pDNA is eluted in a high-salt buffer, and then concentrated through isopropanol precipitation. The resultant pellet was dissolved in 10 mM Tris–HCl buffer (pH 8.0). The sample obtained at the end of this procedure contained around 90% of sc isoform, while the remaining 10% are due to the presence of oc isoform, as revealed by agarose gel electrophoresis. Pure samples of oc and linear isoforms were prepared from the previous sc-enriched plasmid sample. By this way, the oc plasmid samples were prepared by incubating the sc pDNA samples at room temperature (24 °C). The samples were monitored over time by electrophoretic analysis until the total conversion of sc plasmid to oc isoform was observed (about 3 days). On the other hand, the linear plasmid samples were prepared by enzymatic digestion of pUC19 with the Sma I enzyme and that of pVAX1-*LacZ* with the Hind III enzyme, during 1 h of incubation at 37 °C.

Agarose gel electrophoresis

The structure and purity of the different plasmid isoforms were analyzed by horizontal electrophoresis using 15 cm, 1% agarose (Hoefer, San Francisco, CA, USA) gels, stained with ethidium bromide (0.5 μg mL^{-1}). Electrophoresis was carried out at 100 V, for 40 min, with TAE buffer (40 mM Tris–HCl, 20 mM acetic acid and 1 mM EDTA, pH 8.0).

SPR measurements

Experiments were performed using a BIAcore T200 equipped with a CM5 sensor chip. All binding experiments were carried out at 25 °C in Tris–HCl pH 8 (sterile-filtered and degassed with 0.005% surfactant P20) as running buffer. L-Arginine was covalently attached to the surface of a sensor chip and solutions containing the analytes (isoforms of pGL101, pUC19 and pVAX1-*LacZ*) were passed over the surface.

The immobilization of L-arginine on the CM5 sensor chip was performed at pH 8.5, dissolved in 0.1 M borate solution and using the amine-coupling method,[20] through its α-NH_2 groups, in running buffer of 10 mM HBS-EP+.[21]

The CM5 chip flow cell 1 was left unmodified to act as a reference cell to minimize variations caused by nonspecific binding and bulk refractive index changes. Once the L-arginine-coated sensor chip was stable in the running buffer (Tris–HCl pH 8, flow rate 5 μL min^{-1}), plasmid isoforms pGL101, pUC19 and pVAX1-*LacZ*, in Tris–HCl pH 8, were injected over the surface. The concentrations injected for pUC19 isoforms ranged between 0.7 μM and 0.22 nM, for pGL101 isoforms were between 0.8 μM and 0.27 nM and for pVAX1-*LacZ* were between 1.1 μM and 0.22 nM.

Samples were injected in the order of increasing concentration with each concentration tested for binding in duplicate.

No regeneration solution was required since the plasmid isoforms were removed from the sensor surface.

In addition, blank injections (running buffer without pDNA) were performed prior to each collection of binding data following exactly the same time course as the runs with pDNA. Subtraction of the response of the blank injections from the

data traces (double referencing) removes small but systematic imperfections in the response curve from the experimental data.

The complex was allowed to associate and dissociate rapidly making calculations of kinetics unfeasible.

Hence, all biosensor data were studied by steady-state affinity analysis using BIAevaluation software by averaging the resonance unit values (RU) in the plateau region of the sensorgrams over a 300 to 400 s period. The binding site number and the equilibrium constant were obtained by fitting plots of RU *versus* $C_{analyte}$. Responses were fitted to a single-site bimolecular interaction model, yielding a single equilibrium dissociation constant, K_D. The K_D was estimated when the Chi^2 values were minimized.

Results and discussion

Gel electrophoresis analysis of plasmids pGL101, pUC19 and pVAX1-*LacZ*

The quality of the plasmid stock solutions and the effectiveness of the digestions used to generate the open-circular and linear isoforms were examined by agarose gel electrophoresis. A sample image for the different isoforms of pGL101, pUC19 and pVAX1-*LacZ* plasmids is shown in Fig. 1.

The stock solutions of plasmids pUC19 and pVAX1-*LacZ* were predominantly in the monomeric supercoiled isoform (bright band in lanes 3 and 6, respectively, in Fig. 1), containing a small amount of open-circular pDNA (faint band in lanes 3 and 6). In contrast, the linear samples of pUC19 and pVAX1-*LacZ* (lanes 5 and 8 of Fig. 1) contained only the linear isoform, as well as the monomeric open-circular isoforms presented in lanes 4 and 7 of Fig. 1. For plasmid pGL101, the lanes 1 and 2 contained open-circular and supercoiled isoforms. The structural conformation of the three plasmid isoforms is also reflected in the agarose gel electrophoresis migration. The supercoiled conformation shows a higher migration distance than linear and open-circular isoforms because of its more compact structure, which facilitates the migration through the agarose gel pores.

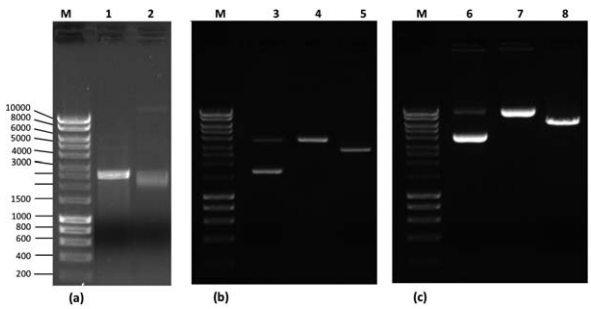

Fig. 1 Agarose gel electrophoretic analysis of the samples prepared with different plasmid isoforms. (a) pGL101 plasmid samples, (b) pUC19 plasmid samples and (c) pVAX1-*LacZ* plasmid samples. Lane M: molecular weight marker; lane 1: pGL101 oc isoform; lane 2: pGL101 sample obtained at the end of alkaline lysis and purification, containing around 90% of sc isoform; lane 3: pUC19 sample obtained at the end of alkaline lysis and purification, containing around 90% of sc isoform; lane 4: pUC19 oc isoform; lane 5: pUC19 linear isoform; lane 6: pVAX1-*LacZ* sample obtained at the end of alkaline lysis and purification, containing around 90% of sc isoform; lane 7: pVAX1-*LacZ* oc isoform; lane 8: pVAX1-*LacZ* linear isoform.

Equilibrium binding experiments by SPR

The equilibrium binding experiments were performed under conditions similar to those for affinity chromatographic assays (temperature, pH and ionic strength).[16] The variation in the amount of bound L-arginine using three CM5 chips was relatively small (less than 3%). The average immobilized density was 215.9 RU.

The experiments showed a low resonance response level, with all the plasmid isoforms saturation of the active surface ranging approximately between 15 and 35 RU (normalized signal) except for the linear pVAX1-*LacZ* isoform for which no signal was detected. Sensorgrams (RU amino acid surface − RU blank surface) for the L-arginine coated surface and the average response data at equilibrium were plotted against each plasmid isoform concentration. Fig. 2(a), (c) and (e) show the sensorgrams of immobilized L-arginine on supercoiled plasmid isoforms pUC19, pGL101 and pVAX1-*LacZ* and Fig. 2(b), (d) and (f) show the equilibrium binding analysis fitted to a steady-state interaction model. The SPR binding profiles were identical for all complexes and are square shaped responses (indicating rapid association and dissociation) and do not have sufficient curvature in both phases (association and dissociation) for kinetic evaluation. Only steady-state binding studies were carried out by averaging the resonance unit values (RU) in the plateau region of the sensorgrams over a 300 to 400 s period.

The K_D values of each plasmid isoforms are presented in Table 1.

There are significant differences in the apparent affinity correlated with the three plasmids (2.39, 2.69 and 6.05 kbp).

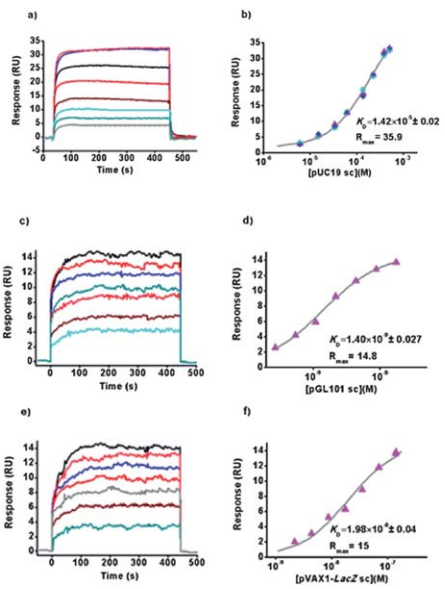

Fig. 2 Sensorgrams (a, c, and e) and equilibrium binding analysis (b, d, and f) of supercoiled plasmids pUC19, pGL101 and pVAX1-*LacZ* on the L-arginine surface. Supercoiled isoforms were injected at various concentrations at 25 °C over the BIAcore chip to which L-arginine was immobilized in 10 mM Tris–HCl pH 8.

Table 1 Equilibrium analysis data at 25 °C

	K_D (M)
pUC19 sc	$1.42 \times 10^{-5} \pm 0.02$
pUC19 oc	$1.92 \times 10^{-4} \pm 0.06$
pUC19 ln	$1.48 \times 10^{-4} \pm 0.04$
pGL101 sc	$1.40 \times 10^{-9} \pm 0.03$
pGL101 oc	$3.30 \times 10^{-9} \pm 0.01$
pVAX1-*LacZ* sc	$1.98 \times 10^{-8} \pm 0.02$
pVAX1-*LacZ* oc	$3.92 \times 10^{-8} \pm 0.03$

Plasmids pGL101 and pVAX1-*LacZ* were chosen due to their different size and base composition. Plasmids pUC19 and pGL101 present an equal distribution of 25% of each base, while the sequence of pVAX1-*LacZ* revealed the presence of 22% and 28% of adenine and guanine, respectively.

pGL101 showed the highest binding on the arginine surface followed by pVAX1-*LacZ*, while pUC19 showed the lowest binding. These results suggest a non-linear tendency between the plasmid size and binding, since the smaller (pGL101) and larger (pVAX1-*LacZ*) plasmids have the highest affinity. The strongest interaction obtained with the smaller plasmids could be attributed to their higher compactness degree that allow for more interactions per surface area, inducing cooperative effects and increasing the stability of the interaction, thus leading to higher affinity to the L-arginine surface. However, the larger plasmid pVAX1-*LacZ* showed higher affinity for the arginine surface than pUC19 (see Table 1). This can be explained by the fact that the plasmid pVAX1-*LacZ* has a higher guanine content when compared with pUC19, which could account for this favoured interaction. This can be attested by amino acid–nucleotide studies that recognized arginine to interact preferentially with guanine.[19]

Regarding the equilibrium dissociation constants of the three plasmid isoforms, the supercoiled isoforms have the higher binding affinity to the arginine surface. The major difference between isoforms was found for pUC19, as the affinity increased approximately by 10-fold for the supercoiled pUC19 relatively to the open-circular and linear isoforms. Concerning open-circular and linear plasmid isoforms, the affinity increased slightly (1.3-fold). The increase in binding is negligible for pGL101 and pVAX1-*LacZ* supercoiled and open-circular isoforms (0.4- and 0.5-fold, respectively). For linear pVAX1-*LacZ*, no signal was detected.

The fact that the supercoiled isoform binds strongly to the L-arginine surface could be attributed to the ability of the supercoiled isoform to move in space very rapidly due to its flexible and dynamic conformation in solution, bringing distant sites into proximity. This effect is more evident in the supercoiled isoform than in the relaxed form and thus favours the interaction with the L-arginine surface. Also, the tighter interwounding due to the shielding of the phosphate backbone usually tends to compact more the supercoiled pDNA and to expose the bases.[22]

Previous chromatographic experiments using the L-arginine–agarose support to purify pDNA revealed that the supercoiled isoform presented a stronger interaction with the support than the open-circular form.[17] In this case, the chromatographic interaction was promoted by using a low ionic strength buffer and the selective elution of the bound species was achieved by increasing the NaCl concentration. It was verified that the supercoiled plasmid was eluted at a higher ionic strength, indicating a stronger interaction.[17]

To compare the salt dependence of the binding between pUC19 isoforms of pDNA and the L-arginine surface, three buffers were analyzed HEPES-buffered saline, HBS EP+ (10 mM HEPES, 150 mM NaCl; 0.05% P20 surfactant; 3 mM EDTA, pH 7.4) and buffer C (50 mM $KHPO_4$, 10 mM EDTA, pH 7.5). The relative response (RU) of pUC19 supercoiled at a concentration of 0.105 μM using these buffers is presented in Fig. 3.

The binding profiles (square wave) using these buffers were similar to those for Tris–HCl. It was found that the maximum binding between supercoiled pUC19 and immobilized L-arginine was observed in 10 mM Tris–HCl, followed by 10 mM HEPES-buffered saline and buffer C, whereas the lowest binding was observed in 10 mM HBS EP+. These results show that the interactions between supercoiled pUC19 and the L-arginine surface are mainly due to electrostatic forces. The widely employed buffer Tris–HCl pH 8 enhanced the electrostatic interactions between the supercoiled form and L-arginine surface. In contrast, under salt concentration (150 mM NaCl) phosphate groups of the supercoiled plasmid backbone are effectively screened by the salt counterions resulting in a more compact and less regular structure.[23] The electrostatic potential of the surface repels cations condensed on the plasmid. In this case the interactions between the guanidinium cations of arginine and the anionic phosphate groups in the supercoiled plasmid backbone are less favourable. This result also agrees with the conditions already established in the chromatographic experiments for the purification of pDNA using the L-arginine support.[7] Usually, an increased salt gradient (NaCl) is used in chromatography to elute the plasmid isoforms in the order of increasing charge density.[7]

In the case of buffer C, the HPO_4^- increases the repulsions on the supercoiled plasmid backbone and some interactions could be favoured by the guanidinium cation surface. In the presence of HEPES buffer, the electrostatic interaction between the L-arginine surface and the supercoiled plasmid backbone could be more favoured, since HEPES at neutral pH is a

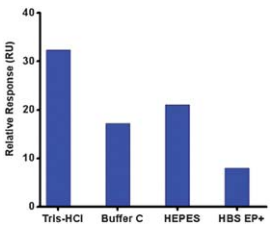

Fig. 3 Relative response (RU) of pUC19 supercoiled at 450.5 s from the beginning of injection at a concentration of 0.105 μM over a L-arginine surface using 10 mM Tris–HCl (pH 8), HEPES-buffered saline, HBS-EP+ (10 mM HEPES, 150 mM NaCl; 0.05% P20 surfactant; 3 mM EDTA, pH 7.4) and buffer C (10 mM EDTA, 50 mM $KHPO_4$, pH 7.5).

zwitterionic molecule with a positive charge on one of the protonated nitrogens. On one hand this can reduce the repulsive electrostatic interaction between charged phosphate groups of supercoiled chains. On the other hand the electrostatic potential of the surface attracts the negative charge of the sulfonic group.

Conclusions

The binding affinity of plasmid isoforms pUC19, pGL101 and pVAX1-*LacZ* to the L-arginine surface was examined by surface plasmon resonance. L-Arginine was immobilized on the CM5 sensor chip surface to mimic the immobilized ligand affinity chromatographic support. With the presented results, it is only possible to carry out steady-state binding studies. Kinetic calculations were unfeasible since the complexes associate and dissociate rapidly from the surface. pGL101 showed the highest binding on the arginine surface, followed by pVAX1-*LacZ*, while pUC19 showed the lowest binding. The highest affinity was found for the supercoiled isoforms of the three plasmids, while the lowest was found for the relaxed isoforms.

Previous chromatographic experiments using the L-arginine–agarose support to purify pDNA revealed that the supercoiled isoform presented a stronger interaction with the support than the open-circular form.

Distinct buffers affected the interaction strength with an increase in response for Tris–HCl and a marked decrease for HBS EP+. Likewise, an increased salt gradient (NaCl) is used in chromatography to elute the plasmid isoforms by increasing the charge density.

Therefore, the separation of plasmid isoforms by affinity chromatography can benefit from the affinity analysis provided by the SPR biosensor.

Acknowledgements

Carla Cruz and Ângela Sousa acknowledge the post-doctoral grants from FCT (SFRH/BPD/46934/2008 and SFRH/BPD/79106/2011, respectively). This work was supported by FCT, the Portuguese Foundation for Science and Technology (PTDC/EBB-BIO/114320/2009) and PEst-C/SAU/UI0709/2011 COMPETE.

Notes and references

1 Y. Han, G. You, L. K. Pattenden and G. M. Forde, *Process Biochem.*, 2010, **45**, 203–209.
2 M. M. Diogo, S. C. Ribeiro, J. A. Queiroz, G. A. Monteiro, N. Tordo, P. Perrin and D. M. F. Prazeres, *J. Gene Med.*, 2001, **3**, 577–584.
3 FDA Fada, Guidance for industry (draft guidance): consideration for plasmid DNA vaccines for infectious disease indications, FDA office of communication. Training and manufacturers assistance (HFM-40), Rockville Pike, MD 20852-1448, USA, http://www.fda.gov/cber/gdlns/plasdnavac.htm, 2006, 1401.
4 K. Yoshinaga and Y. Suzuki, *Agric. Biol. Chem.*, 1983, **47**, 919–920.
5 D. L. Varley, A. G. Hitchkock, A. M. E. Weiss, W. A. Horler, R. Cowell, L. Peddie, G. S. Sharpe, D. R. Thatcher and J. A. J. Hanak, *Bioseparation*, 1999, **8**, 209–217.
6 R. Lemmens, U. Olsson, T. Nyhammar and J. Stadler, *J. Chromatogr., B*, 2003, **784**, 291–300.
7 F. Sousa, T. Matos, D. M. F. Prazeres and J. A. Queiroz, *Anal. Biochem.*, 2008, **374**, 432–434.
8 F. Sousa, D. M. F. Prazeres and J. A. Queiroz, *Trends Biotechnol.*, 2008, **26**, 518–525.
9 M. M. Diogo, J. A. Queiroz and D. M. F. Prazeres, *J. Chromatogr., A*, 2005, **1069**, 3–22.
10 A. Sousa, F. Sousa and J. A. Queiroz, *J. Sep. Sci.*, 2010, **33**, 2610–2618.
11 N. E. Labrou, *J. Chromatogr., B*, 2003, **790**, 67–78.
12 T. H. Nguyen and M. Elimelech, *Biomacromolecules*, 2007, **8**, 24–32.
13 Y. Han, S. Gras and G. A. Forde, *AIChE J.*, 2009, **55**, 505–515.
14 N. M. Luscombe, R. A. Laskowski and J. M. Thornton, *Nucleic Acids Res.*, 2001, **29**, 2860–2874.
15 J. Urthaler, R. Schlegl, A. Podgornik, A. Strancar, A. Jungbauer and R. Necina, *J. Chromatogr., A*, 2005, **1065**, 93–106.
16 F. Sousa and J. A. Queiroz, *J. Chromatogr., A*, 2011, **1218**, 124–129.
17 F. Sousa, D. M. F. Prazeres and J. A. Queiroz, *J. Gene Med.*, 2009, **11**, 79–88.
18 M. M. Hoffman, M. A. Khrapov, J. C. Cox, J. Yao, L. Tong and A. D. Ellington, *Nucleic Acids Res.*, 2004, **32**, D174–D181.
19 F. Sousa, C. Cruz and J. A. Queiroz, *J. Mol. Recognit.*, 2010, **22**, 2–14.
20 D. G. Myszka, M. D. Jonsen and B. J. Graves, *Anal. Biochem.*, 1998, **265**, 326–330.
21 C. Cruz, E. J. Cabrita and J. A. Queiroz, *Anal. Bioanal. Chem.*, 2011, **401**, 983–993.
22 F. Sousa, D. M. F. Prazeres and J. A. Queiroz, *Arch. Biochem. Biophys.*, 2007, **467**, 154–162.
23 T. Schlick, B. Li and W. K. Olson, *Biophys. J.*, 1994, **67**, 2146–2166.

