VERSITA

Biologia **66**/1: 8—17, 2011
Section Cellular and Molecular Biology
DOI: 10.2478/s11756-010-0143-2

Evaluation of zero-length cross-linking procedure for immuno-magnetic separation of *Leptospira*

Stefan Schreier[1,2,3,4], Galayanee Doungchawee[1], Sudarat Chadsuthi[2], Darapond Triampo[2,5] & Wannapong Triampo[2,3,4]*

[1] *Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[2] *R&D Group of Biological and Environmental Physics, Department of Physics, Faculty of Science, Mahidol University, Rama 6 Rd., Ratchatewee, Bangkok 10400, Thailand; e-mail: scwtr@mahidol.ac.th; wtriampo@gmail.com*
[3] *Center of Excellence for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand*
[4] *ThEP Center, CHE, 328 Si Ayutthaya Road, Bangkok 10400, Thailand*
[5] *Department of Chemistry (R3/1), Faculty of Science, Mahidol University, Salaya Campus, Nakhon Pathom 73170, Thailand*

Abstract: Leptospirosis constitutes a major health problem in tropical and subtropical countries and is caused by pathogenic *Leptospira*. Immuno-magnetic separation (IMS) is considered to be an effective pre-enrichment method to isolate *Leptospira* from liquid specimen. We applied an inexpensive and simple IMS protocol using zero-length cross-linkers to immobilize polyclonal anti-leptospiral antibodies onto magnetic particles. The IMS-system has been optimized and evaluated by the assessment of the capture efficiency (CE). Main parameters that influence the conjugation procedure were optimized, including the amount of protein per milligram of magnetic particles, the pH and ionic strength of the conjugation buffer. The bead-bound leptospiral fraction was identified by using acridine orange fluorescence dye. The highest value for CE occurred when using high molar phosphate saline buffer at a pH around the isoelectric point of the antibodies. Finally, up to 3×10^8 leptospiral cells per mL could have been captured with approximately 50 μg of antibody-labelled particles. Strong particle agglutination could be observed during incubation for leptospiral concentrations in the range of 10^7-10^8 cells per mL. Despite covalent binding, we show that the physical adsorption parameters pH and ionic strength of the conjugation buffer greatly affect the entire immobilization process with regard to the CE, thus being able to increase the reactivity of the particles. We therefore conclude that a well-adjusted conjugation buffer for the used chemistry could possibly replace expensive and more complicated antibody immobilization methods.

Key words: *Leptospira*; leptospirosis; immuno-magnetic separation; magnetic particles; antibody immobilization; zero-length cross-linker; carbodiimide.

Abbreviations: BSA, bovine serum albumin; CE, capture efficiency; EDC, (1-3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; EMJH, Ellinghausen and McCullough liquid medium as modified by Johnson and Harris; IEF, isoelectric focusing; IMS, immuno-magnetic separation; NHS, N-hydroxysuccinimide; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; RT, room temperature; TEM, transmission electron microscopy.

Introduction

Leptospirosis is one of the most globally widespread zoonoses occurring especially in tropical and subtropical countries. It is caused by pathogenic spirochete of the genus *Leptospira* (Bharti et al. 2003). The disease is increasingly considered to be a major public health problem in expanding cities as well as in rural areas. Unhygienic conditions lead to the indirect transmission via contact with contaminated water and soil. Less frequently, infection is caused directly by exposure to infected host animals, such as rodents, cows and dogs and their animal waste, mainly urine (Faine et al. 1994). According to the World Health Organization International Leptospirosis Society survey (WHO, Leptospirosis Worldwide) the estimated annual number of leptospirosis cases ranges from 350,000 to 500,000. In humans, highly pathogenic serovars can cause pulmonary haemorrhage and death in more than 10% of infection cases (Smythe et al. 1999; Ko et al. 2009).

Despite the prevalent use of conventional tests, like the microscopic agglutination test, the direct detection of *Leptospira* is presenting large potential for leptospirosis diagnosis. Besides high sensitivity, a great advantage of leptospiral antigen and whole cell detection methods is the use of different types of samples, like urine,

* Corresponding author

Springer

serum or other body fluids (Yan et al. 1998). The pre-enrichment method known as immuno-magnetic separation (IMS) has been effectively used to isolate *Leptospira* prior to its detection by using fluorescence spectroscopy, ELISA or PCR (Taylor et al. 1996; Yan et al. 1998; Fernandes et al. 2008). The highly effective combination of IMS and PCR (IMS-PCR) seems to become an increasingly reliable technique for leptospiral antigen detection in urine and sera (Fernandes et al. 2008). Previous efforts to culture and detect environment-derived leptospiral organisms have been inconsistent, while IMS coupled detection methods provide a great potential to be more successful (Alexander et al. 1975; Henry et al. 1978; Ganoza et al. 2006).

Despite its advantages, IMS is dominated by the much more costly commercial products that may not accommodate poor economies. With this in mind, we have developed a simple and inexpensive IMS-system and have evaluated it for its usefulness in sample collection of *Leptospira*. The system consists of in-house produced, para-magnetic particles and partially purified polyclonal antibodies being immobilized onto the particle surface by zero-length cross-linkers (1-3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) (Grabarek & Gergely 1990; Cabral 1991). This well-known linking procedure is one of the simplest methods for covalent binding of proteins onto carboxyl or amino supports, but has been reported to be less efficient due to random antibody orientation (Danczyk et al. 2003; Jung et al. 2008).

The effectiveness of an IMS-system is mainly defined by its antigen-binding capacity or capture efficiency (CE) (Varshney et al. 2005). The CE is expressed by the percentage of antigen successfully separated from a mixture of other biological compounds present in the sample solution and should be as high as possible in order to increase the sensitivity of subsequent detection methods. However, high CE may be achieved with every IMS-system; it is just a question of how many particles per sample volume are used. We therefore apply the surface area of invested particles per sample volume, defined as cm^2 per mL as another IMS-quality criterion. When calculating the surface area of particles, we assume an ideal smooth spherical surface. In principle, it can be claimed that the higher the quality of an IMS-system, the lower the amount of magnetic particles is necessary for the separation of a given cell amount. This leads to decreased non-specific binding, lower costs, and most important noise reduction that could become an important factor for subsequent electrical or optical detection methods.

The key intention of this study is to develop and evaluate a low-end IMS-system. We therefore investigated effects on CE by modifying the antibody immobilization protocol. Parameters that are known to influence the conjugation behaviour with regard to amount and orientation of immobilized antibodies are the type and concentration of the applied protein and, more importantly, the pH and ionic strength of the buffer (Brash 1996). The antigen-antibody system used herein involved rabbit anti-leptospiral antibodies reactive against pathogenic *Leptospira interrogans* serovar Canicola.

Material and methods

Bacterial strains

Pathogenic *Leptospira interrogans* serovars Canicola, Rachmati and Hebdomadis were used for incubation with immuno-reactive magnetic particles and obtained from the National Leptospirosis Reference Center, the National Institute of Health, Thailand. Stock cultures were weekly subcultured in Ellinghausen and McCullough liquid medium as modified by Johnson and Harris (EMJH) in a 27 °C incubator. Non-target bacterium, *Escherichia coli* was provided from the Bacteria Laboratory Unit at Ramathibodi Hospital in Bangkok, Thailand, and subcultured in Luria-Bertani broth until highly log phase growth.

Anti-leptospiral polyclonal antibodies

Leptospira-specific polyclonal antibodies reactive against serovar Canicola were used for immobilization onto magnetic particles and obtained as previously documented (Doungchawee et al. 2005). Immunoglobulins were partially purified by precipitation with 40/50% saturated $(NH_4)_2SO_4$. Before coupling, the purity of the globulin fraction was analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to standard protocols (Laemmli 1970). The separation gel has been stained with Coomassie Brilliant Blue R-250 dye. The electrical properties of the immunoglublins were determined by isoelectric focusing (IEF) following the principle method of two-dimensional electrophoresis (Klose et al. 1995). The focusing strip (Immobiline DryStrip pH 3 – 10NL 12 cm, GE Healthcare) was placed onto a polyacrylamide gel and after SDS-PAGE the separation gel was stained with Coomassie Brilliant Blue R-250 dye.

Immuno-magnetic particles

Iron-oxide para-magnetic particles were synthesized by a co-precipitation method of $FeCl_2$ and $FeCl_3$ in NaOH following the work of An & Chen (2007). $FeCl_2$ and $FeCl_3$ (2:1 mole ratio) were dissolved in HCl and chemically precipitated at room temperature (RT) by adding NaOH solution. Following precipitation, the particles were isolated from the solution by centrifugation and washed with deionized water. Silica surface on the particles was generated with tetraethyl orthosilicate. Subsequently, the silica surface was reacted with 3-aminopropyltriethoxysilane and afterwards, with succinic anhydride under argon gas to generate the carboxylic group on the outer surfaces. Finally, the prepared nanoparticles were washed three-times with deionized water and kept in deionized water at 4 °C until use. The magnetic particles' core consists of multiple magnetite crystals (Fe_3O_4) per particle and are functionalized with approximately 100 nmole/mg carboxyl groups (–COOH).

Particles' size determination and distribution was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments Ltd.). Images of particles were taken using transmission electron microscopy (TEM).

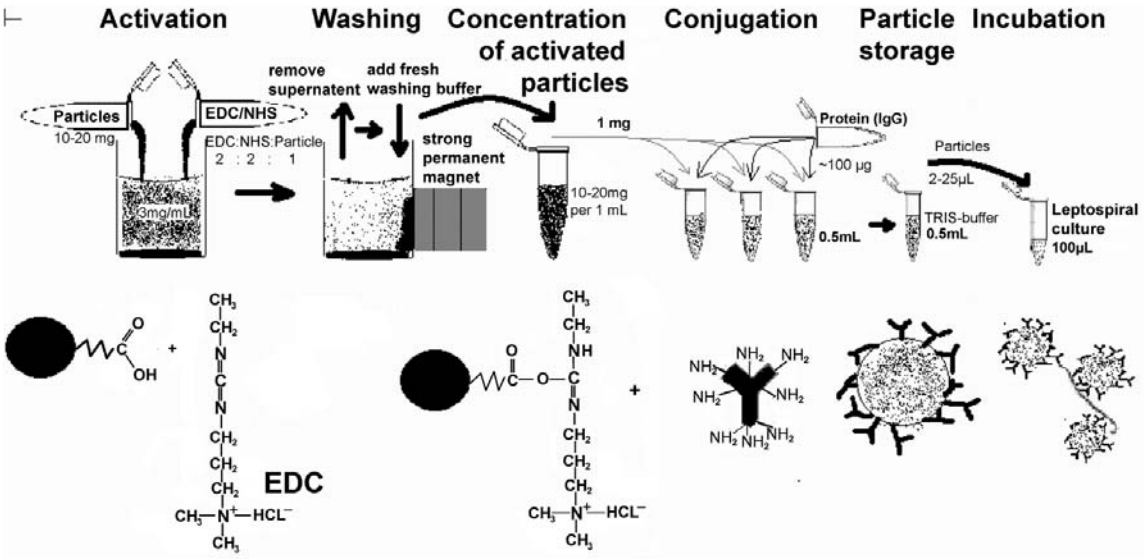

Fig. 1. Illustration of conjugation protocol and application of the IMS-system. The upper part shows the main steps including activation, conjugation and incubation. The lower part shows the underlying chemistry and functionality of each step. Drawings do not fit real dimensions.

Magnetic particle conjugation

For illustration, see Figure 1. Magnetic micro-particles (604 nm in average) were washed once with PBS (pH 7.2, 10 mM) and used for immobilization with immunoglobulins afterwards. For magnetic separation, a strong neodym permanent magnetic (∼1.3 Tesla) was applied to the particle containing solutions for 5 minutes. Various amounts of magnetic beads have been chemically activated by EDC and NHS, 6 mg/mL each. The particle concentration was maintained at 3 mg/mL, as well. The agents were dissolved in 0.5 mL cold PBS (10 mM, pH 7.2) before being added to the magnetic solution. The solution containing the activating agents and the particles was incubated for 45 minutes at RT and occasionally hand-shaken. After washing with PBS buffer (10 mM, pH 7.2), the magnetic solution was concentrated to 1 mL. A standard amount of 1 mg per 0.5 mL was used for immobilization with variable amounts of immunoglobulins and a fixed amount (0.5% concentration in PBS) of bovine serum albumin (BSA). BSA-coated particles were used for an irrelevance test. The experimental procedure consists of various different combinations of PBS conjugation buffer, including variations in pH and ionic strength. The molarities of the buffer depended on the phosphate species, disodium hydrogen phosphate and sodium dihydrogen phosphate. The magnetic particles were washed once and stored at 4 °C in Tris-HCL buffer (pH 7.2) in order to block the remaining active functional groups.

Determination of adsorbed protein

Bradford method was applied using Bio-Rad Protein Assay for the determination of the protein adsorption (Bradford 1976). In brief, protein adsorption was determined indirectly from the protein amount remaining in the supernatant after conjugation process and measured by an automated plate reader (Wallac Victor^{2}V Model 1420 Multilabel HTS counter). 100 µL of Bradford solution (diluted 1:3) was added to 50 µL sample solution. Samples were loaded into 96 well plates with at least 6 repeats. The received data was compared, with bovine γ-globulin (Bio-Rad laboratories: 1.32 mg/mL) serving as protein standard. The optical density was measured at a wavelength of 595 nm. The particles' protein-binding capacity is the difference of initial protein concentration and depleted protein concentration of the supernatant per 1 mg of magnetic particles.

Cell capture test

The immuno-reactivity of the prepared particles was tested by mixing a fixed sample size of 0.1 mL bacterial culture with different amounts of magnetic particles (50–500 µg/mL). Serial dilutions of pure culture of the used bacteria were prepared in fresh EMJH medium. For clinical sample tests, leptospiral cells were harvested by centrifugation (13,000×g for 20 min) at RT and washed once in PBS buffer. Leptospirae were concentrated to approximately 10^9 cells/mL and then added to 0.1 mL aliquots of PBS (control), pooled sterile human serum and human urine. Bacterial concentrations were determined before incubation with particles by automated counting as described earlier by Schreier et al. (2009). The incubation was performed at RT in Eppendorf tubes (0.5 mL) under constant rotation (∼48 rotations/min) for 30 minutes. A similar protocol was performed with the BSA-coated and blank particles. After incubation, the tubes were placed nearby a strong permanent magnet for the separation of immuno-magnetic particles from the supernatant. After magnetic separation, bacterial concentrations of the supernatant have been determined by automated counting (Schreier et al. 2009). The CE was calculated by the depletion of bacteria (see below in the *Calculations* part) in the supernatant. For investigations of the bacteria-antibody-particle complex, fluorescence staining was performed during incubation procedure in EMJH medium. Acridine orange (final concentration: 10 µg/mL) was added to the sample solution (0.1 mL) after 20 minutes of particle incubation. Incubation lasted for another 10 minutes with subsequent magnetic separation. The captured fraction was dissolved in 10 µL of PBS (10 mM, pH 7.4) and loaded onto a counting chamber slide. Images of the captured fraction have been taken by an Olympus DP50 CCD camera mounted onto an Olympus BX 50-CU fluorescence microscope and Viewfinder Software at magnifications ×200 and ×400.

Calculations

The capture efficiency, *CE*, reflects the percentage number of captured bacteria for a certain applied amount of particles

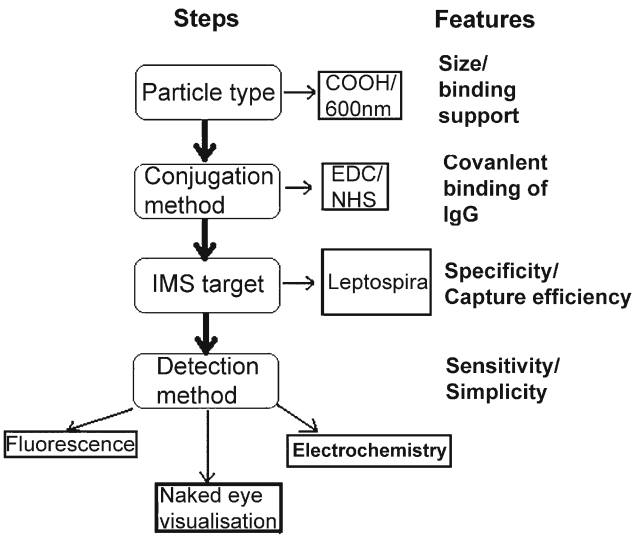

Fig. 2. Scheme of the IMS-system. The scheme illustrates the landmarks of an IMS-system used in this study. It is defined by the type of particle, the conjugation method, the target, and the subsequent detection method. The ultimate goal for our IMS-system is the development of a stand-alone bio-sensing system.

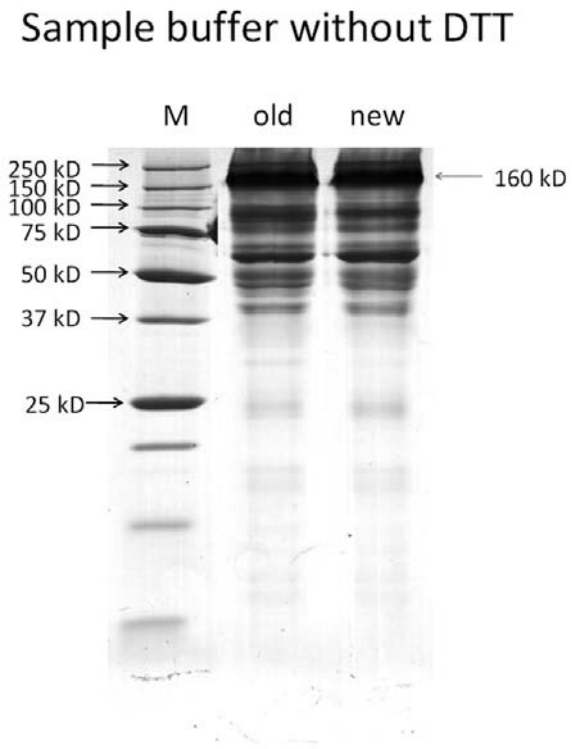

Fig. 3. SDS-PAGE of precipitated rabbit anti-leptospira serum used for conjugation to magnetic particles. Approximately 10 µg of two untreated precipitated globulin fractions that differ in date of production (old: 6 months, new: 1 month) have been loaded onto 12% polyacrylamide separation gel and subsequently stained with Coomassie Brilliant Blue R250.

and is therefore used as a quality criterion of the immuno-magnetic separation system. The CE is calculated indirectly with *Cde* as the depleted amount of leptospiral bacteria and *Co* as the initial amount of leptospiral bacteria before incubation.

$$CE\,(\%) = \left(1 - \frac{Cde}{Co}\right) \times 100 \tag{1}$$

Together with the CE, the total applied active surface of the particles, S_a, served as quality parameter for the comparison of our IMS-system with those of others. The total active surface is the surface of a single particle, S_s, multiplied by the particle amount per mg particle weight, C_p,

$$S_\mathrm{a} = C_\mathrm{p} S_\mathrm{s} = C_\mathrm{p} \pi d^2 \tag{2}$$

where d is a diameter of a single particle.

The particle amount per mg particle weight, C_p, was calculated by dividing the particle volume per mg particle weight, V_mg, by the volume of a single particle, V_p,

$$C_\mathrm{p} = \frac{V_\mathrm{mg}}{V_\mathrm{p}} = \frac{1/j}{\pi d^3/6} \tag{3}$$

where j is the average density of the particles and d is the average particle diameter.

The components of the IMS-system and its possible applications are listed in Figure 2.

Results and discussion

IMS properties

We are convinced that total antibody purification from serum using methods such as affinity chromatography is not necessary for obtaining sufficient reactive magnetic particles as shown in the following sections. We used precipitated anti-leptospiral serum and estimated the composition of the globulin fraction by SDS-PAGE (Fig. 3). The bands show a large fraction of protein with a molecular weight of 150–160 kDa. This band is assumed to be immunoglobulin, due to the particles' positive anti-*Leptospira* reactivity. The blot shows a series of other serum proteins that we define as globular background proteins. The total amount of globular serum protein obtained was about 15 mg/mL. The isoelectric points of the polyclonal antibodies ranged in a first group of lesser quantities from 5.9 to 6.2 and in a second larger group between 6.6 and 8.8. Figure 4a shows a TEM-picture of uncoated particles. The gross particles consist of a multitude of small magnetite crystals. Using dynamic light scattering the magnetic particle size analysis showed a mean peak at 604 nm (± 41 nm) and a size distribution as shown in Figure 4b.

Effects of protein concentration on the CE

Different antibody concentrations during the immobilization process are known to greatly affect the adsorption rate and the CE (Lund et al. 1988; Fuentes et al. 2005); we therefore investigated this finding in respect to the IMS-system used within this work. The protein binding capacity of the particles was assumed to be about 20–40 µg/mg of particles. Figure 5 illustrates the protein adsorption for a series of protein total amounts ranging from 35 to 126 µg. First of all, the total adsorbed amounts were rather low. The highest protein adsorption did not exceed 44%. This could be explained by the particles' protein-binding capacity being lower than expected, leading to particles be-

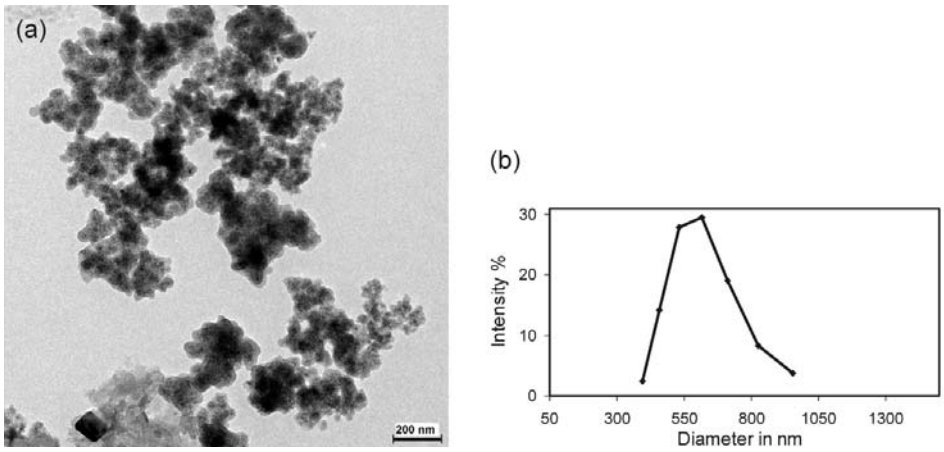

Fig. 4. (a) TEM image of carboxylated para-magnetic particles. Particles have been stored in 20% ethanol solution prior to TEM sample preparation and imaging. (b) Size distribution of magnetic solution was obtained by dynamic light scattering. The distribution curve is the average of six measurement repeats.

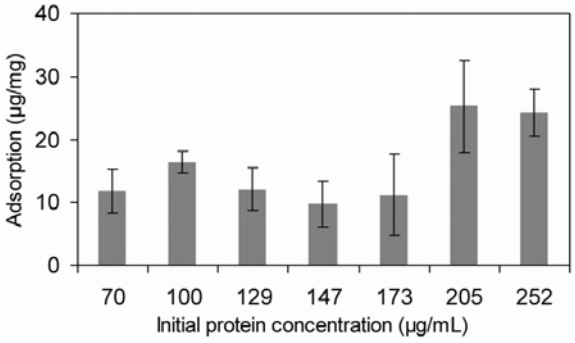

Fig. 5. Total protein adsorption. The total amount of protein is plotted against the amount of adsorbed protein. The adsorption values are calculated from the depletion of protein after the conjugation process. Adsorption is related to 1 mg particle weight and 0.5 mL buffer solution. The error of measurement (automated plate reader) is less than 5%. Error-bars are the result of at least 3 independently repeated experiments.

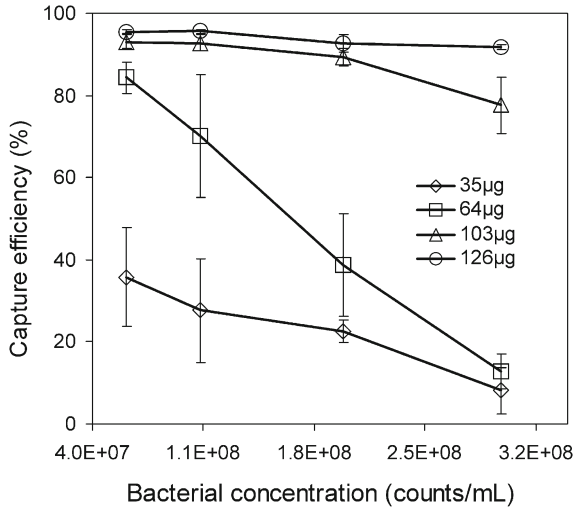

Fig. 6. Capture efficiency – initial protein concentration. The graph shows the percentage of bead-bound *Leptospira* serovar Canicola after incubation. 35 µg, 64 µg, 103 µg and 126 µg of anti-leptospiral globular protein has been conjugated to equal amounts of 1 mg magnetic particles in PBS (100 mM, pH 7.2) and used for incubation (0.5 mg particles per mL) in 0.1 mL EMJH medium at RT.

ing oversaturated with protein. However, the low CE as calculated in Eq. (1) (<40%) of the particles that have been conjugated with low protein concentrations (35 µg/mg) justified the usage of relatively high amounts of protein (Fig. 6). The adsorption chart (Fig. 5) seems to be increased within the rather narrow range of around 100 µg/mg of particles. We explain this adsorption increase by optimal particle-protein interaction. We believe that differences in distance between protein and particle result in different adsorption behaviour so that 100 µg protein per mg particles in 0.5 mL conjugation volume is favourable for adsorption.

Figure 6 shows the CE of the particles that have been conjugated with different protein concentrations over four different cell amounts. The CE values obtained for differently conjugated particles clearly show an increase for higher protein concentrations. Lund et al. (1988) found similar behaviour in the early days of particle coating. They focused on bacteria-bound and -unbound particles for IMS with *E. coli*, and clearly showed a positive correlation between applied protein amount and bead-bound bacteria. Our results, however, demonstrate similar adsorption rates but increased CE. We suggest that both particle-protein and protein-protein interactions occur and influence the system at the site of low energy spots or at the site of covalent binding on the particle surface. Increased protein concentration creates increased competition for binding sites between the different protein types, which is in favour of high molecular weight proteins (Brash 1996). The antibodies account for the group with the highest molecular weight and largest fraction (Fig. 3). For that reason, the percentage of particle-bound antibodies correlates positively with the protein concentration thus increasing the CE. Moreover, antibodies are assumed to adsorb randomly oriented, resulting in moderately sensitive particles, so that larger amounts of particles have to be used for sufficient cell separation.

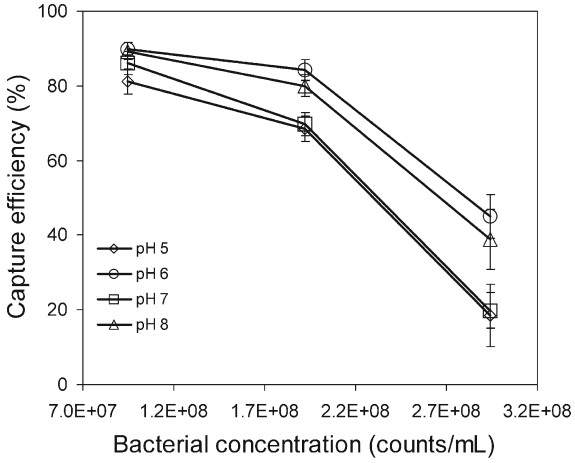

Fig. 7. Capture efficiency – pH. The graph shows the percentage of bead-bound *Leptospira* serovar Canicola after incubation. 103 µg of anti-leptospiral globular protein has been conjugated to equal amounts of 1 mg magnetic particles in PBS (100 mM) at pH 5, 6, 7 and 8 and used for incubation (0.2 mg particles per mL) in 0.1 mL EMJH medium at RT.

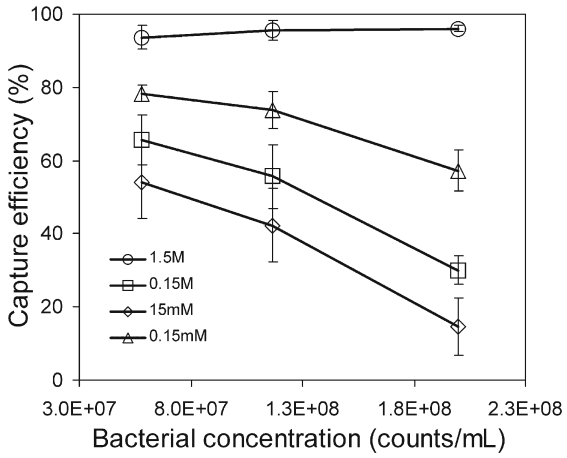

Fig. 8. Capture efficiency – ionic strength. The graph shows the percentage of bead-bound *Leptospira* serovar Canicola after incubation. 103 µg of anti-leptospiral globular protein has been conjugated to equal amounts of 1 mg magnetic particles in PBS (pH 6) at ionic strengths: 0.15 mM to 1.5 M and used for incubation (0.1 mg particles per mL) in 0.1 mL EMJH medium at RT.

In conclusion, the used IMS protocol turned out to be rather inefficient. The antibody adsorption was very low, necessitating high amounts of protein. Furthermore, a large quantity of particles (500 µg/mL) for IMS had to be used in order to achieve 95% CE. In the following CE experiments, 103 µg total amount of protein was used for the conjugation to 1 mg activated magnetic particles in different buffer types at a volume of 0.5 mL.

Effects of buffer pH on the CE

The pH of the conjugation buffer solution has direct effect on the charge of the whole protein and therefore affects the adsorption behaviour of antibodies onto charged surfaces; for that reason it also affects antibodies' activity (Brash 1996). Differences in adsorption and or CE due to pH value variation are clear indicators of electrostatic forces and physical adsorption (Chen et al. 2003). For this experiment (Fig. 7) less than the half of the particle amount (200 µg/mL) was applied when compared to the previous experiment (Fig. 6). The CE sank four fold for the highest analyte concentration, suggesting that changes in pH of the buffer solution did not influence the system to a greater extent. The low influence of pH variations on the CE is expected for our system, as covalent binding is supposed to take place rather than physical adsorption. However, at pH 6 and 8, an increased CE can be observed and explained by also occurring electrostatic and hydrophobic interactions between the differently charged antibodies and the slightly negatively charged particle surface. The lowest pH (pH 5) resulted in the lowest CE. A possible explanation might be the positive charge of the applied antibodies at pH 5 and the assumption that antibodies bind stronger to negatively charged surfaces at lower ionic strength with head-on orientation rather than with end-on orientation, thus resulting in lower reactive particles (Chen et al. 2003). At pH 6, a small percentage of polyclonal antibodies exhibits isoelectric behaviour, so that hydrophobic forces exceed the electrostatic ones. Hydrophobic interaction is believed to increase stability between the antibody's Fc-fragment and the particle surface, which is explained by conformational changes (Buijs et al. 1997). We therefore observe increased CE. At pH 7, a greater number of antibodies are likely to be randomly oriented, so the effect of enhanced CE cannot be observed any more. In this case, electrostatic forces gain influence, as a smaller fraction of antibodies are now negatively charged, which causes repulsion and a greater part of antibodies being still positively charged. At pH 8, the percentage of antibodies at their isoelectric point is increased, so that hydrophobic forces gain influence. A greater conformational instability of the Fc-fragment promotes end-on orientation, as it is believed to be the case at pH 6. However, the CE is slightly decreased in comparison to CE values at pH 6. This might be due to the presence of a certain percentage of negatively charged antibodies, causing repulsion.

Effects of buffer ionic strength on CE

For discussing salt concentration as a parameter that influences the conjugation process, two situations have to be considered. Firstly, buffers with medium and low salt concentration (mM to 0.1 M) that directly influence electrostatic forces and therefore physical adsorption (Chen et al. 2003). In general, it could be stated for liquid-solid interfaces that lower ionic strength results in higher electrostatic attraction or repulsion. Secondly, highest salt concentrations (0.15 M to 2 M) may be discussed differently, as interaction between salt and protein play an important role. Depending on the type of salt and its concentration, protein stabilising or destabilising effects may be observed (Arakawa et al. 1984; Timasheff et al. 2006; Zang et al. 2006). Furthermore, it has been stated that a protein's tertiary structure

has to be considered as rather fragile in solution, and minimal changes in the system may alter conformation (Brash 1996; Batchelor et al. 2004). We investigated the effects of low, medium and high ionic strength on the CE (Fig. 8). The variation of salt concentration revealed notable efficiency improvements. For this experiment, the amount of particles used could be reduced to 100 μg/mL. Figure 8 shows the CE for four different ionic strengths at pH 6 (except 0.15 mM, pH 7.2) over three different leptospiral cell concentrations. The lowest concentration clearly shows enhanced reactivity compared to the higher salt concentration of 15 mM. This is in contrast to the findings of the pH experiment, where we found electrostatic forces being a minor influencing factor of the conjugation system, which is also supported by the fact that both groups, 0.15 mM and 15 mM, did not reveal any differences in protein adsorption (23 μg/mg particles and 23.5 μg/mg particles, respectively). As already mentioned, the protein-protein interactions have to be considered and include electrostatic, steric-repulsion, hydrophobic and van der Waals forces. The equation for free energy as shown below may help to explain how ionic strength influences the adsorption process (Visser 1992):

$$\Delta G = 4\pi\varepsilon R^2\psi^2 \exp\left[\frac{-\kappa\,(h-2R)}{h}\right] \qquad (4)$$

This equation characterizes electrostatic double layer interaction by calculating the free energy between two similarly charged objects in dependence of radius, R, distance, h, surface potential, ψ, and the Debye length, κ. Ionic strength and distance greatly influence the system. If we vary the ionic strength of the conjugation buffer, changes in free energies will occur. Lower ionic strength increases free energy (thus repulsion between molecules) and, *vice versa*, higher ionic strength decreases free energy. We observed higher CE at the lowest ionic strength. Strictly following the theory of the involvement of free energy, larger proteins like antibodies with higher net charge successfully compete with smaller proteins of lower net charge at the binding site, thus resulting in more reactive particles, as the percentage of particle-bound antibodies increases. If we follow this argumentation for the higher salt concentrations (>150 mM), we would expect to find the lowest CE, because electrostatic forces are minimized and antibody orientation occurs more randomly. This was obviously not the case. The higher concentrations (0.15 M and 1.5 M) exhibited increased CE and the highest salt concentration resulted in the highest CE. As mentioned above, high salt concentrations have to be regarded differently. It has been reported for other proteins, such as enzymes, that activity decreases or is enhanced with high salt concentration due to conformational changes (Pugh & Cox 1988). The effects vary depending on the applied type of salt. Destabilising salts are mostly the cause of functional change in the proteins.

Pugh & Cox (1988) used high concentrated cosmotropic salt types (2 M) and found a significant activating effect on recA protein ATPase. They explained their results by assuming an interaction of the high salt buffer with the protein, which changes its conformation thus inducing activation. It is worth mentioning that stabilizing salt types have been used, which are not supposed to change protein conformation. In our system, we also used stabilising salts, when strictly following the Hofmeister series. In a more recent work, Ninham (2006) questioned this rigid point of view of cosmotropic and chaotropic effects, claiming that the Hofmeister series is subject to many more parameters, as defined in former works, therefore the standard sequence has to be set in relation to a far better defined system. Therefore, we may assume different lyotropic behaviour from this system. In particular, hydrogen phosphate and dihydrogen phosphate, which are the main ingredients of PBS, are rather difficult to locate in the lyotropic system. Meyerstein & Treinin (1962) related the spectroscopic shifts of several anions in water with the lyotropic number and failed with hydrogen phosphate. LoNostro et al. (2002) investigated, among other parameters, the effect of salt on polymerisation kinetics and ranked dihydrogen phosphate among the chaotropic salt species ranging between Br^- and HCO_3^-. In contrast, in the Hofmeister series shown in the work of Zhang & Cremer (2006), dihydrogen phosphate is rated as a cosmotropic solute located between $S_2O_3^{2-}$ and flour anion. The standard sequence reported by Ninham (2006) locates dihydrogen phosphate as a cosmotropic salt as well.

Taking these rather confusing and unclear statements into account, we presume salting-in effects for the buffer used in our work, where conformational changes especially at the antibodies Fc-fragment are assumed to occur, thus promoting adsorption with end-on orientation (Buijs et al. 1997). It is also known for low pH values that antibodies exhibit hydrophobic regions by conformational change, specifically at the site of the Fc-region, depending on the time of exposure to the acidic environment (Van Erp at al. 1992; Chang et al. 1995). This behaviour has been subsequently used for proper immobilization of antibodies on hydrophobic dye doped silica (Chang et al. 1995).

The fact of enhanced particle reactivity led to a further reduction of the applied particle amount per separation experiment from 100 to 50 μg/mL. The optimal coated particles were found to be stable over a period of at least three months.

Particle evaluation and capturing pattern

We compared the efficiency of our IMS-system with others by using two parameters. One was the total applied surface area of the particles in cm^2 normalized to 1 mL (see Eq. (3)) of sample solution and the second one was the CE with the highest used amount of cells. Some researchers followed the protocol of bead suppliers, where proposed bead quantities for sufficient IMS exceeding the actual necessary amount. Because of that reason, as well as the great differences between other IMS protocols and the great variation of the average single particle surface, we do not intend to give a

Table 1. Comparison of different IMS-systems.[a]

Target cell	Conjugation method	Bead size (nm)	Applied particles per mL	Applied surface area (cm^2/mL)	Max. No. of cells per mL/ highest CE	Ref/Fig
E. coli O157	Streptavidin-biotin	145	6.4×10^9	4.2	7.2×10^7/95%	Varshney et al. 2005
E. coli O149	SAM anti-sheep precoated (Dynal)	4500	3.2×10^{7b}	20.4	–	Lund et al. 1988
E. coli O157	SAM Protein A (Spherotech)	∼ 4200	3.7×10^7	20.5	10^4/∼50%	Fu et al. 2005
E. coli DSM 498	SiMAG-Polyethyleneimine (Chemicell)	1000	3.5×10^8	11	10^3/99%	Deponte et al. 2004
Leptospira	Carboxylated beads – antibody concentration	604	1.8×10^9	21.6	$\sim 3 \times 10^8$/ > 95%	Fig. 6
Leptospira	Carboxylated beads – buffer pH	604	7×10^8	8.6	$\sim 3 \times 10^8$/ > 95%	Fig. 7
Leptospira	Carboxylated beads – clinical sample	604	1.8×10^8	2.2	$\sim 3 \times 10^8$/ > 95%	Fig. 10

[a] The applied amount of particles in 1 mL sample solution and the applied surface area is calculated following Eq. (2) and (3), respectively.
[b] Assumption: 8×10^7 beads per mg magnetic particles – Data derived from Dynal®.

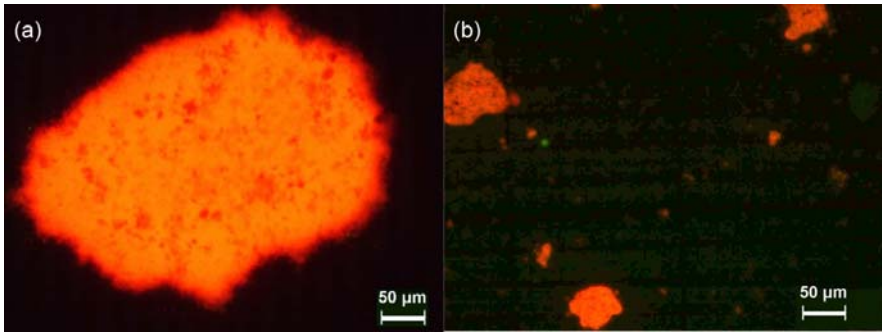

Fig. 9. Fluorescence images of leptospiral bead-bound fraction. Pictures of acridine orange stained agglutinates were taken at magnification ×200 for (a) high leptospiral cell amount (3×10^8 cell/mL) and (b) lower leptospiral cell amount (7×10^6 cells/mL).

statement of quality. But the comparison helps us to locate our system in the great complexity of IMS-systems. Only these works, using pure cultures, have been taken into account.

Table 1 shows our system being efficient using high salt PBS buffer conjugation. The optimized IMS-system required a low surface area per mL of sample solution for sufficient separation. We therefore investigated the bacteria-antibody-particle complex in order to prove the high binding capability of these particles. Figure 9 shows the captured fraction of leptospiral cells that were stained with the fluorescent dye acridine orange. The captured fraction was found to be almost 100% packed into only one to a few agglutinated compounds for highest cell amounts or into several smaller compounds for lower cell concentrations. Macroscopic agglutination was consistent for cell amounts being quantitatively in the range of the applied particle amounts. It should be mentioned that agglutination spontaneously occurred during incubation and was not a product of pellet formation after magnetic separation. Figure 9a shows the packing of both particle and *Leptospira* into one large compound. Lesser packing and more compounds can be observed for lower amounts of *Leptospira* (Fig. 9b).

To complete the evaluation step, the IMS-system has been tested with human serum and urine, respectively. Figure 10 depicts the CE values for a total particle amount of 5µg in 0.1 mL PBS, serum and urine over four different bacterial concentrations. The particles showed high efficiency for all solution types. However, a drop in CE can be noticed for urine. This may be due to high concentrations of urea, which is known to destabilize proteins and could negatively affect the antibodies reactivity. Furthermore, at lower leptospiral concentrations, the partilces' CE seems to decrease for serum and urine when compared to PBS. This can be explained by non-specific binding, which is a reported problem of IMS (Skjerve et al. 1990; Goodridge et al. 1999).

Specificity

Non-specific binding was tested for non-target bacteria: *E. coli* and *Leptospira* serovars Hebdomadis and Rachmati. For our experiments optimal coated particles (50 µg/mL) were used for incubation with 0.1 mL *E. coli* pure cultures and mixed cultures (*E. coli* and *Leptospira* serovar Canicola). The CE of the mixed cultures (approximately 3×10^7 *E. coli* and 2×10^6 leptospiral cells) was found to be 98%, suggesting that the particles are capable of effectively sorting out a minority of leptospiral cells with less than 5% loss within a sample containing other biological compounds. Particles showed no reactivity against serovars Rachmati and Hebdomadis. However, the magnetic particles showed some affinity for *E. coli* bacteria (Fig. 11). We observed an uptake in *E. coli* cells of 14–17% depending on the bacterial concentration. The captured fraction of *E. coli* was investigated by using the fluorescent dye acridine orange (Fig. 12). The image shows single bacterial cells

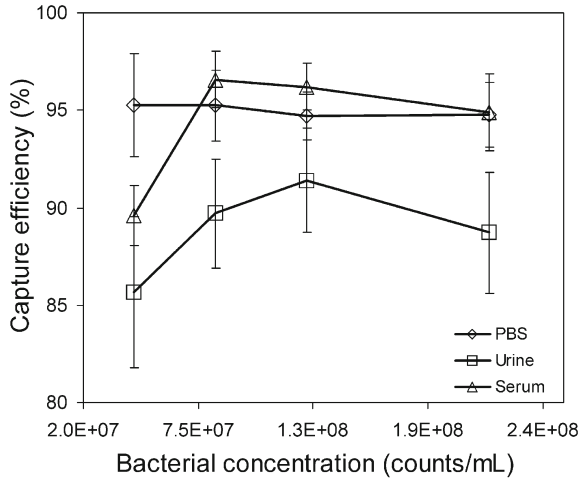

Fig. 10. Capture efficiency – clinical sample. The graph shows the percentage of bead-bound *Leptospira* serovar Canicola after incubation. 126 μg of anti-leptospiral globular protein has been conjugated to equal amounts of 1 mg magnetic particles in PBS (1.5 M, pH 6) and used for incubation (50 μg particles per mL) in 0.1 mL PBS (100 mM, pH 7.2), pooled human serum and urine at RT. The values obtained are the average of three independently repeated experiments.

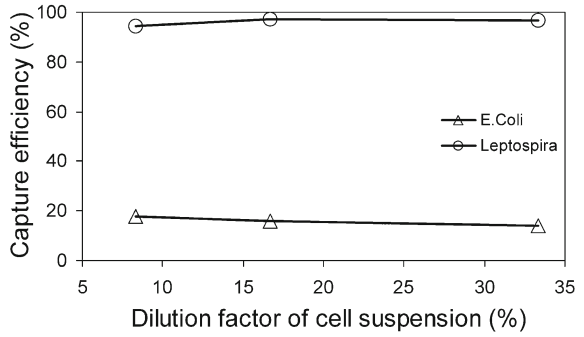

Fig. 11. Capture efficiency – specificity. Initial leptospiral pure culture (2.5×10^8 cells/mL) serves as control. *E. coli* cells were grown until high concentrated (10^9 per mL) and a pure culture (0.1 mL) was incubated subsequently with optimal prepared particles (50 μg/mL). Due to different cell amounts of both types of bacteria, the x-axis provides the information of the dilution factor. Control and sample underwent equal treatment.

without any agglutination behaviour. This capturing behaviour leads us to conclude that cross-reaction is present with binding forces far weaker than for *Leptospira*. Additionally, an irrelevance test was performed with *Leptospira* serovar Canicola in EMJH medium using BSA-coated and blank (non-treated) particles, which are expected to be not reactive. The testing was performed under the same conditions as with reactive particles. As a result, the CE was 0% on average for both BSA- and non-coated particles.

To conclude, we showed that despite using partly purified anti-leptospiral IgG and zero-length cross-linkers for protein immobilization, our IMS-system turned out to be efficient for separation and concentration of *Leptospira* in various solutions. Moreover, we demonstrated that properties of physical adsorption

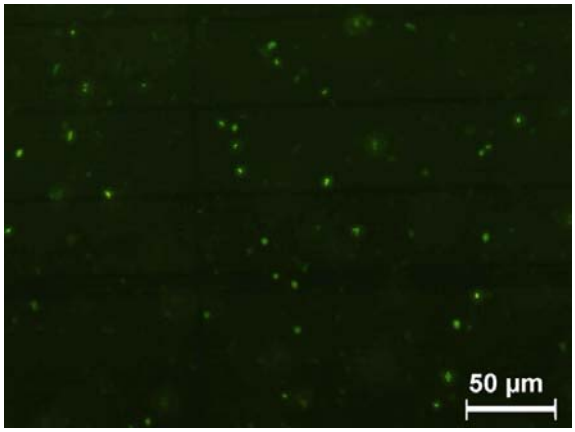

Fig. 12. Fluorescence image of *E. coli* bead-bound fraction. The picture of the acridine orange stained captured fraction was taken at magnification ×400.

have effect on the conjugation process by varying pH and ionic strength of the conjugation buffer. High salt buffer (1.5 M PBS, pH 6) revealed highly reactive particles assuming optimal antibody immobilization.

Acknowledgements

The authors thank Mr. David Blyler for his editorial suggestions and providing comments. The authors also thank Dr. Palangpon Kongsaeree and Khomsan Tiensomjitr of Mahidol University for providing magnetic particles and know-how. This work was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), the Thailand Research Fund (TRF), the Commission on Higher Education (CHE), and the Thailand Center of Excellence in Physics.

References

Alexander A.D., Evans L.B., Baker M.F., Baker H.J., Ellison D. & Marriapan M. 1975. Pathogenic leptospiras isolated from Malaysian surface waters. Appl. Microbiol. **29:** 30–33.

An Y. & Chen M. 2007. Preparation and self-assembly of carboxylic acid-functionalized silica. J. Colloid Interface Sci. **311:** 507–513.

Arakawa T. & Timasheff S.N. 1984. Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding. Biochemistry **23:** 5912–5923.

Batchelor J.D., Olteanu A., Tripathy A. & Pielak G.J. 2004. Impact of protein denaturants and stabilizers on water structure. J. Am. Chem. Soc. **126:** 1958–1961.

Bharti A.R., Nally J.E., Ricaldi J.N., Matthias M.A., Diaz M.M., Lovett M.A., Levett P.N., Gilman R.H., Willig M.R. & Gotuzzo E. 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. **3:** 757–771.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

Brash J.L. & Wojciechowski P.W. 1996. Interfacial Phenomena and Bioproducts. Marcel Dekker, New York, 510 pp.

Buijs J., White D.D. & Norde W. 1997. The effect of adsorption on the antigen binding by IgG and its F(ab')$_2$ fragments. Colloids Surfaces B: Biointerfaces **8:** 239–249.

Cabral J.M.S., Kennedy J.F. & Taylor R.F. 1991. Protein Immobilization: Fundamentals and Applications. Marcel Dekker, New York, 377 pp.

Chang I.N., Lin J.N., Andrade J.D. & Herron J.N. 1995. Adsorption mechanism of acid pretreated antibodies on dichlorodimethylsilane-treated silica surfaces. J. Colloid Interface Sci. **174:** 10–23.

Chen S., Liu L., Zhou J. & Jiang S. 2003. Controlling antibody orientation on charged self-assembled monolayers. Langmuir **19:** 2859–2864.

Danczyk R., Krieder B., North A., Webster T., HogenEsch H. & Rundell A. 2003. Comparison of antibody functionality using different immobilization methods. Biotechnol. Bioeng. **84:** 215–223.

Deponte S., Steingroewer J., Löser C., Boschke E. & Bley T. 2004. Biomagnetic separation of *Escherichia coli* by use of anion-exchange beads: measurement and modeling of the kinetics of cell–bead interactions. Anal. Bioanal. Chem. **379:** 419–426.

Doungchawee G., Phulsuksombat D., Naigowit P., Khoaprasert Y., Sangjun N., Kongtim S. & Smythe L. 2005. Survey of leptospirosis of small mammals in Thailand. Southeast Asian J. Trop. Med. Public Health **36:** 1516–1522.

Faine S., Adler B., Bolin C. & Perolat P. 1994. Leptospira and leptospirosis. Medisci, Melbourne, 386 pp.

Fernandes C.P.H., Seixas F.K., Coutinho M.L., Vasconcellos F.A., Moreira Â.N., Conceição F.R., Dellagostin O.A. & Aleixo J.A.G. 2008. An immuno-magnetic separation-PCR method for detection of pathogenic *Leptospira* in biological fluids. Hybridoma **27:** 381–386.

Fu Z., Rogelj S. & Kieft T.L. 2005. Rapid detection of *Escherichia coli* O157: H7 by immunomagnetic separation and real-time PCR. Food Microbiol. **99:** 47–57.

Fuentes M., Mateo C., Guisan J.M. & Fernández-Lafuente R. 2005. Preparation of inert magnetic nano-particles for the directed immobilization of antibodies. Bionsens. Bioelectron. **20:** 1380–1387.

Ganoza C.A., Matthias M.A., Collins-Richards D., Brouwer K.C., Cunningham C.B., Segura E.R., Gilman R.H., Gotuzzo E. & Vinetz J.M. 2006. Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic *Leptospira*. PLoS Med. **3:** e308.

Goodridge L., Chen J. & Griffiths M. 1999. Development and characterization of a fluorescent-bacteriophage assay for detection of *Escherichia coli* O157: H7. Appl. Environ. Microbiol. **65:** 1397–1404.

Grabarek Z., & Gergely J. 1990. Zero-length crosslinking procedure with the use of active esters. Anal. Biochem. **185:** 131–135.

Henry R.A. & Johnson R.C. 1978. Distribution of the genus *Leptospira* in soil and water. Appl. Environ. Microbiol. **35:** 492–499.

Jönsson U., Malmqvist M. & Rönnberg I., 1985. Immobilization of immunoglobulins on silica surfaces. J. Biochem. **227:** 373–378.

Jung Y., Jeong J.Y. & Chung B.H. 2008. Recent advances in immobilization methods of antibodies on solid supports. Analyst **133:** 697–701.

Klose J. & Kobalz U. 1995. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis **16:** 1034–1059.

Ko A.I., Goarant C. & Picardeau M. 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nature Rev. Microbiol. **7:** 736–747.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

LoNostro P., Lopes J.R., Ninham B.W. & Baglioni P. 2002. Effects of cations and anions on the formation of polypseudorotaxanes. J. Phys. Chem. **106:** 2166–2174.

Lund A., Hellemann A.L. & Vartdal F. 1988. Rapid isolation of K88+ *Escherichia coli* by using immunomagnetic particles. J. Clin. Microbiol. **26:** 2572–2575.

Meyerstein D. & Treinin A. 1962. The relation between lyotropic and spectroscopic properties of anions in solution. J. Chem. Soc. **66:** 446–450.

Ninham B.W. 2006. The present state of molecular forces. Progr. Colloid Polym. Sci. **133:** 65–73.

Pugh B.F. & Cox M.M. 1988. High salt activation of recA protein ATPase in the absence of DNA. J. Biol. Chem. **263:** 76–83.

Schreier S., Triampo W., Doungchawee G., Triampo D. & Chadsuthi S. 2009. Leptospirosis research: fast, easy and reliable enumeration of mobile leptospires. Biol. Res. **42:** 5–12.

Skjerve E., Rorvik L.M. & Olsvik O. 1990. Detection of *Listeria monocytogenes* in foods by immunomagnetic separation. Appl. Environ. Microbiol. **56:** 3478–3481.

Smythe L.D. 1999. Leptospirosis worldwide. Wkly Epidemiol. Rec. **74:** 237–242.

Taylor M.J., Ellis W.A., Montgomery J.M., Yan K.T., McDowell S.W.J. & Mackie D.P. 1996. Magnetic immuno capture PCR assay (MIPA): detection of *Leptospira borgpetersenii* serovar hardjo. Vet. Microbiol. **56:** 135–145.

Timasheff S.N., Lee J.C., Pittz E.P. & Tweedy N. 1976. The interaction of tubulin and other proteins with structure-stabilizing solvents. J. Colloid Interface Sci. **55:** 658–663.

Van Erp R., Linders Y.E., van Sommeren A.P. & Gribnau T.C. 1992. Characterization of monoclonal antibodies physically adsorbed onto polystyrene latex particles. J. Immunol. Methods **152:** 191–199.

Varshney M., Yang L., Su X.L. & Li Y. 2005. Magnetic nanoparticle-antibody conjugates for the separation of *Escherichia coli* O157: H7 in ground beef. J. Food Prot. **68:** 1804–1811.

Visser H. 1992. Protein Interactions. VCH Verlag, Weinheim.

Yan K.T., Ellis W.A., Montgomery J.M., Taylor M.J., Mackie D.P. & McDowell W.J. 1998. Development of an immunomagnetic antigen capture system for detecting leptospires in bovine urine. Res. Vet. Sci. **64:** 119–124.

Zhang Y. & Cremer P.S. 2006. Interactions between macromolecules and ions: the Hofmeister series. Curr. Opin. Chem. Biol. **10:** 658–663.

Received April 11, 2010
Accepted November 4, 2010