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Research Article

Separation of α -lactalbumin grafted- and non-grafted maghemite core/silica shell nanoparticles by capillary zone electrophoresis

The use of nanoparticles (NPs) in immunodiagnosics is a challenging task for many reasons, including the need for miniaturization. In view of the development of an assay dedicated to an original, miniaturized and fully automated immunodiagnosics which aims to mimic *in vivo* interactions, magnetic zwitterionic bifunctional amino/polyethyleneoxide maghemite core/silica shell NPs functionalized with allergenic α -lactalbumin were characterized by CE. Proper analytical performances were obtained through semi-permanent capillary coating with didodecyldimethylammonium bromide (DDAB) or permanent capillary wall modification by hydroxypropylcellulose. The influence of experimental conditions (*e.g.* buffer component nature, pH, ionic strength, and electric field strength) on sample stability, electrophoretic mobility, and dispersion was investigated using either DDAB- or hydroxypropylcellulose-coated capillaries. Adsorption to the capillary wall and aggregation phenomena were evaluated according to the CE conditions. The proper choice of experimental conditions, *i.e.* separation under -10 kV in a 25 mM ionic strength MES/NaOH (pH 6.0) with a DDAB-coated capillary, allowed the separation of the grafted and the non-grafted NPs.

Keywords:

Core shell silica nanoparticles / Electric field / Electrophoretic mobility / Magnetic nanoparticles / Stability
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1 Introduction

Nanoparticles (NPs) play an important role in today's research in various fields of interests, *e.g.* in nanotechnology [1], medicine [2], and diagnostics assays [3, 4]. Among the NPs of interest under study, superparamagnetic iron oxide NPs are employed for many tasks such as magnetic resonance imaging with NPs as contrast agents [5–7], for hyperthermia treatment [8], drug delivery system [9, 10], separation and purification of stem cells [11], fluorescent cell labeling [12], and DNA purification [13, 14]. For all of these

purposes, modification of NP surface has to be performed. Generally, iron oxide NPs are synthesized by the precipitation process and then an organic (ligands, polymers) or inorganic (silica) shell is formed by using different modes of functionalization [15]. Subsequently, the core/shell NPs can be functionalized using surface anchoring sites by biological entities such as proteins, enzymes, *etc.* [16]. The “biological” moiety then provides the potential of NPs for medicinal and/or biochemical applications.

NPs, both grafted and non-grafted, can be easily characterized by different spectroscopic techniques [17, 18], *i.e.* traditional AFM, transmission electron microscopy [19], and highly effective MS [20, 21], NMR [22], or FTIR [23]. Moreover, CE as a fast and high-throughput alternative to conventional techniques used for NPs' characterization provides information on *e.g.* particles' size distribution and surface charge density and it allows the separation of different population of NPs [24, 25]. Until now, most studies were focused on anionic NPs and only few authors worked with positively charged ones, probably due to some experimental difficulties and unexpected phenomena encountered upon working with cationic NPs [26–28]. VanOrman Huff and McIntire [27] started to use CE for characterization of cationic colloids (amine-modified latex particles) in 1994. However, the particles exhibited negative mobilities probably because they were adsorbed onto the capillary inner

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Abbreviations: APTES, 3-(aminopropyl)triethoxysilane; DDAB, didodecyldimethylammonium bromide; DLS, dynamic light scattering; EDC, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride; HPC, hydroxypropylcellulose; NP, nanoparticle; PEOS, 2-[methoxy (polyethyleneoxy)propyl]-trimethoxysilane; QD, quantum dot; TEOS, tetraethoxyorthosilicate

wall. Similarly, Krivánková *et al.* [28] observed a negative mobility for polyaniline NPs with CTAB-based BGEs, reversed polarity and reversed EOF conditions. These experimental difficulties were overcome by using different strategies for capillary modification [29, 30]. Our group [30] reported on the size-based characterization of different populations of cationic maghemite NPs with different capillary coatings, *i.e.* permanent hydroxypropylcellulose (HPC) and semi-permanent didodecyltrimethylammonium bromide (DDAB) and polybrene. We later studied the CE behavior of zwitterionic bifunctional amino/PEG-modified core/shell NPs, including the evaluation of the acidity constants of the amino and silanol moieties and the amino group density [31]. Ivanov *et al.* [32] published a new approach describing NP–NP interactions during a CE separation. They used functionalized gold NPs (both cationic and anionic) having different absorption maximum for different structures of colloidal NPs. On the basis of the changes in absorption values, they concluded that NPs interacted both together and with the capillary wall.

CE characterization of protein-grafted NPs was performed mainly for quantum dots (QDs) because they can be easily detected by fluorescence techniques. Vicente and Colon [33] evaluated the CE behavior of QDs modified by streptavidin, biotin, and immunoglobulin G using a Tris-borate buffer with poly(ethylene oxide) additive. Some interactions between modified NPs were observed. Feng *et al.* [34] modified QDs by anti-human IgM. The separation between antibody and antibody–antigen complex linked to the QDs was achieved in 25 min in 20 mM sodium tetraborate pH 9.8. In contrast, they noted that antibody without QDs conjugation could not be separated from the complex using the same buffer. QDs modified by horseradish peroxidase and BSA were characterized by Huang *et al.* [35] using borate buffers at pH 8–11. To the best of our knowledge, only one article reports on the CE analysis of iron oxide NPs modified with proteins. Wang *et al.* [36] published CE study of bioconjugation process of iron oxide NPs with BSA, streptavidin, or goat anti-rabbit IgG with 100 mM sodium borate buffer at pH 9.2. They were able to separate conjugated NPs and free proteins mostly in 5 min.

Here, we present the characterization and separation of bifunctional amino/PEG-modified maghemite core/silica shell NPs either non-grafted or grafted with α -lactalbumin. The core/shell NPs were first modified at their surface with 3-(aminopropyl)triethoxysilane (APTES) and 2-[methoxy(polyethyleneoxy)propyl]-trimethoxysilane (PEOS) [31]. α -Lactalbumin was then grafted on this amino/PEG surface [37]. The grafting of these NPs with a biomolecule is a crucial step in the development of a new immunoassay dedicated to allergy diagnosis. The challenge is to perform an immunoassay in a homogeneous liquid phase mimicking *in vivo* immune interactions, instead of in heterogeneous phases as classically performed in ELISAs. These NPs, grafted by one of the partners of the immunological reaction, should be dispersed in a medium allowing the antigen–antibody interaction to take place in a homogeneous liquid phase. In this context, the CE

characterization of α -lactalbumin-grafted NPs and their separation from non-grafted NPs were performed in order to establish the optimal conditions with respect to separation temperature, buffer pH, ionic strength, and electric field strength, for which the colloidal solution remains stable and thus compatible with its use in a subsequent quasi homogeneous immunoassay.

2 Materials and methods

2.1 Chemicals and reagents

Iron (II) chloride tetrahydrate, iron (III) chloride (27% w/w in aqueous solution), iron (III) nitrate, sodium chloride, phosphoric acid (85% w/w), hydrochloric acid, nitric acid, ammonia, acetone, and diethyl ether were purchased from VWR (Strasbourg, France). Tetraethoxyorthosilicate (TEOS), APTES, Tris, MOPS, MES, HEPES, CHES, DDAB, citric acid, DMF, and HPC, α -lactalbumin from bovine milk, *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). 2-(Methoxy(polyethyleneoxy)propyl)-trimethoxysilane (PEOS), containing two to six ethylene oxide groups, was purchased from Gelest (Morrisville, PA, USA). All the chemicals were of analytical grade. Water used throughout was produced by a Direct-Q3 water purification system (Millipore, Molsheim, France).

2.2 Ferrofluid synthesis

Maghemite NPs were prepared by co-precipitation of Fe(II) and Fe(III) salts under alkaline conditions as described by Massart [38]. NPs were then coated by citrate anions and dispersed in water [39]. These NPs were further encapsulated in silica shells. A first shell was prepared in ethanol medium in the presence of ammonia as a catalyst, by condensation of TEOS. The silica shell functionalization was next carried out by simultaneous condensation of a neutral and a cationic aminosilane coupling agent (PEOS and APTES, both 3.4×10^{-4} mol). The concurrent addition of a small amount of TEOS resulted in the formation of a cross-linked silica shell [40]. The silica condensation was carried out overnight and the resulting suspension was then destabilized by addition of diethyl ether. A red precipitate was formed and isolated by magnetic settling. The precipitate was washed twice with a mixture of diethyl ether and ethanol (15:1) and then re-dispersed in MOPS/NaOH buffer (10 mM ionic strength, pH 7.5).

2.3 NP grafting procedure and characterization

The grafting procedure was based on $-\text{NH}_2$ and $-\text{COOH}$ groups linking reaction using EDC as a coupling agent and

sulfo-NHS as a reaction inductor [37]. First, the APTES/PEG maghemite core/silica shell NPs (3.1×10^{14} particles/mL) were mixed with EDC (621.25 nmol/mL) and NHS (962.94 nmol/mL). A solution of α -lactalbumin (24.85 nmol/mL) was next added. This reaction was performed overnight under gentle stirring at 20°C. Then, the grafted NPs were rinsed using a magnetic column with MACS separator magnet (Miltenyi, Paris, France) and re-dispersed in an appropriate buffer for CE. The solid number-mean diameters were obtained by transmission electron microscopy using a JEOL 100CX2 microscope (JEOL Europe, Croissy-sur-Seine, France). Z-average dynamic sizes were measured by dynamic light scattering (DLS) with a Nano ZS Zetasizer instrument (Malvern Instruments, Worcester-shire, UK).

2.4 CE apparatus and mobility measurements

Electrophoretic mobility measurements were performed with a HP ^{3D}CE system equipped with a DAD detector (Agilent Technologies, Waldbronn, Germany). In total, 50 μ m id bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with effective length of 26.5 cm and total length of 35.0 cm were used. Successive hydrodynamic injections were performed in the following order: neutral marker (20 mbar, 2 s), NPs sample (50 mbar, 3 s), and BGE (20 mbar, 2 s). The capillary cassette was thermostated at 18°C if not stated otherwise. The detection wavelengths were 200 and 280 nm for EOF marker and NPs, respectively. Prior to first use, bare fused-silica capillaries were activated by successive rinsing with 1 M NaOH and 0.1 M NaOH for 15 min each and water for 10 min under 925 mbar. Capillary inner walls were either semi-permanently or positively modified with DDAB or permanently and neutrally coated with HPC. The DDAB modification [41] consisted in a 15 min flush with 0.1 mM DDAB solution in BGE followed by 3 min flush with BGE to remove the excess of surfactant and equilibrate the capillary. Between each run, the capillary was rinsed with DDAB solution for 3 min and with BGE for 2 min, still under the aforementioned pressure. For the HPC modification [42], 15 μ L of the polymer solution in water (50 mg/mL) were percolated in the bare fused-silica capillaries at a flow rate of 0.25 μ L/min using a syringe pump (KD Scientific, Holliston, USA). Capillaries were next purged with nitrogen (4 bar) and heated from 60 to 140°C at 5°C/min, held at 140°C for 20 min and then cooled to 25°C at 5°C/min in the oven of ST 200 Stang Instruments gas chromatograph (Perichrom, Saulx-les-Chartreux, France), still under nitrogen flow. Residual EOF was measured using DMF as neutral marker, according to the method by Williams and Vigh [43]. EOF measurements were performed prior and after each repetition series of three to four measurements, in order to perform mobility corrections, if necessary. Capillaries with electroosmotic mobility higher than 2.0×10^{-9} m² V⁻¹ s⁻¹ were discarded.

3 Results and discussion

In view of employing magnetic NPs for immunodiagnostics, amino/PEG functionalized core shell NPs were first synthesized and further modified with α -lactalbumin by a coupling reaction involving the amino groups of NPs and the carboxyl groups of the protein. The core/shell NPs produced with a 1:1 APTES/PEOS ratio presented *ca.* 4000 amino groups per particle with a Z-average hydrodynamic diameter of 60 nm, as determined from DLS [31]. Coupling with α -lactalbumin (molecular mass of *ca.* 14 kDa and *pI* 4.2–4.9 [44, 45]) led to NPs having an increased hydrodynamic diameter (80 nm) and containing about 35 proteins *per* particle [37]. Results obtained from DLS also demonstrate that the α -lactalbumin-grafted NPs were neither aggregated nor bridged together by the protein.

3.1 Stability of the NPs in different BGEs

Some authors discussed the effect of electrolyte nature on colloidal stability [46–49], *e.g.* rhodamine-doped NPs were more stable in ethanol environment than in MES [46], while thiol-capped QDs were more stable in typical biological buffers such as MES, MOPS, or PBS [47]. In this study, the stability of the α -lactalbumin-grafted NPs in buffers of different composition (anion and cation nature) that could further be employed for the immunodiagnostic test was studied (i) in terms of peak profile, *i.e.* stability during CE separation, (ii) capacity of interaction with specific α -lactalbumin antibody (performed as the immune response [37]), and (iii) storage time. As an example, Fig. 1 shows a typical change in peak profiles according to the stabilization or destabilization of the colloidal solution: (i) Fig. 1A shows a peak-like profile indicating an excellent stability of the NPs

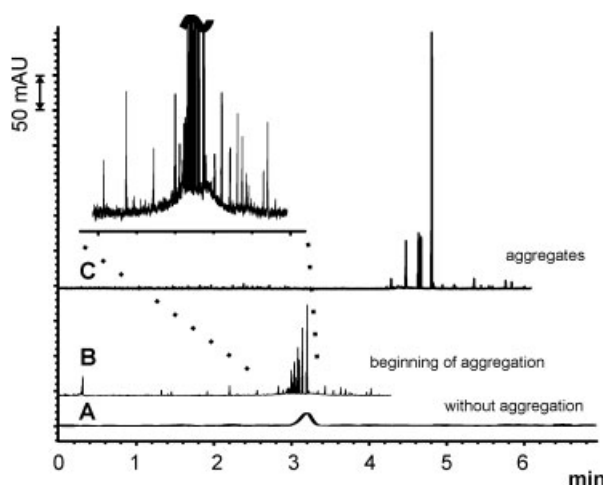


Figure 1. CE profiles of dispersed and aggregated α -lactalbumin-grafted NPs. Bare fused-silica capillary coated with DDAB, 50 μ m id $\times$ 35 cm length (detection, 26.5 cm). BGE: MES/NaOH pH 6.0, ionic strength 10 mM. Applied voltage: –10 kV. Temperature: 25°C. Hydrodynamic injection (50 mbar, 5 s).

suspension (*i.e.* the absence of NPs aggregation) in the electrolyte conditions; (ii) the “spiky” peaks observed in Fig. 1B indicate a beginning of aggregation (destabilization of the suspension); and eventually the spikes observed in Fig. 1C are connected with the formation of aggregates [25]. Thanks to the CE technique, the onset of destabilization was evidenced 1 or 2 days before it could be visualized (by eye) in the stock solution. Table 1 summarizes the results for the buffers studied, all of 10 mM ionic strength. For these experiments, DDAB capillary coating and reversed polarity were employed. In these conditions, NPs migrate in counter-electroosmotic mode. Monovalent organic anions and sodium counter-ion were found to be a good compromise with respect to the stability of the grafted NPs suspension for both CE separation and storage conditions. Moreover, the study of the antigen–antibody recognition proved that only some of these buffers could be used if the biological activity had to be preserved. For example, acetate and formate buffers are known to be

chaotropic and thus not appropriate for antigen–antibody interaction. MOPS, MES, and HEPES buffers proved to be consistent with the present immune interaction studies, as expected.

3.2 Effect of temperature

Temperature is known to affect mobility mainly due to viscosity, but also to zeta potential variations [50, 51]. Moreover, temperature has an effect on physico-chemical equilibria and protein structure. Here, the influence of temperature on the electrophoretic behavior of the NPs, either grafted with α -lactalbumin or non-grafted, was explored between 18 and 60°C in 10 mM MES/NaOH BGE (pH 6.0) and in a capillary modified with DDAB. Figure 2 shows the increase in the mean effective mobility of both grafted and non-grafted NPs with the increase in temperature, together with the variation of the BGE

Table 1. Stability of the grafted and non-grafted NPs in different BGEs

BGE ^{a)}	CE profiles	Antibody–antigen interaction ^{b)}	Storage time ^{c)}
MOPS/NaOH pH 6.5–8.0	Stable	Good	~2 days
MES/NaOH pH 5.5–7.5	Stable	Good	~7 days
Acetate/NaOH pH 4.0–5.5	Stable	Fair	> 14 days
Acetate/NH ₃ /LiOH, /KOH pH 4.0	Stable	–	–
HEPES/NaOH pH 7.0–8.0	Irreproducible	Good	~2 days
Formate/NaOH pH 4.0	Irreproducible	Medium	> 14 days
CHES/NaOH pH 8.0–9.5	Aggregates	–	–
Phosphate/NaOH pH 2.5	Aggregates	–	–
Oxalate/NaOH pH 4.0	Aggregates	–	–
Citrate/NaOH pH 3.0, 4.0	Aggregates	–	–
Citrate/Tris pH 3.0, 4.0	Aggregates	–	–

a) BGEs used for CE measurements and storage time studies have a 10 mM ionic strength; BGEs used in antibody–antigen recognition test have a 50 mM ionic strength.

b) Antibody–antigen recognition test is described in detail by Teste *et al.* [37].

c) Approximate possible storage time in a fridge (+4°C) without any change visible to the eye.

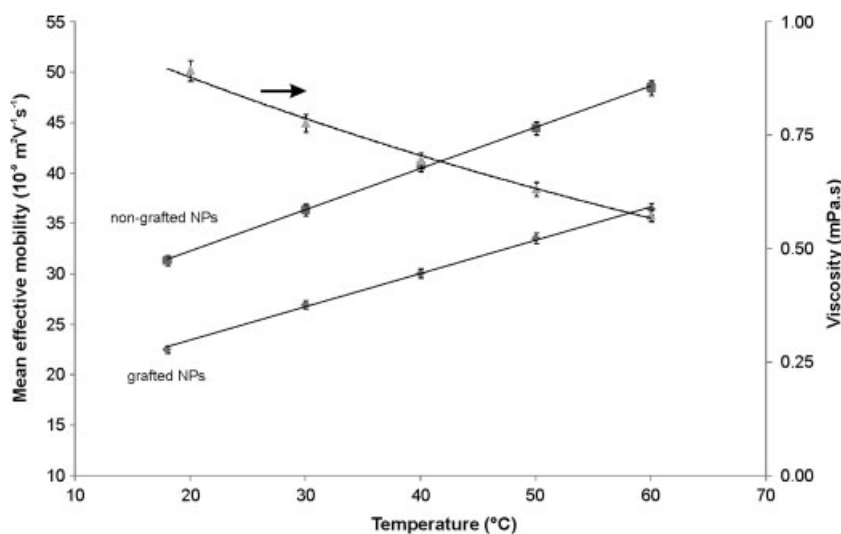


Figure 2. Effect of temperature on BGE viscosity and α -lactalbumin-grafted and non-grafted NPs' mean effective mobility. Experimental conditions as in Fig. 1 except for temperature.

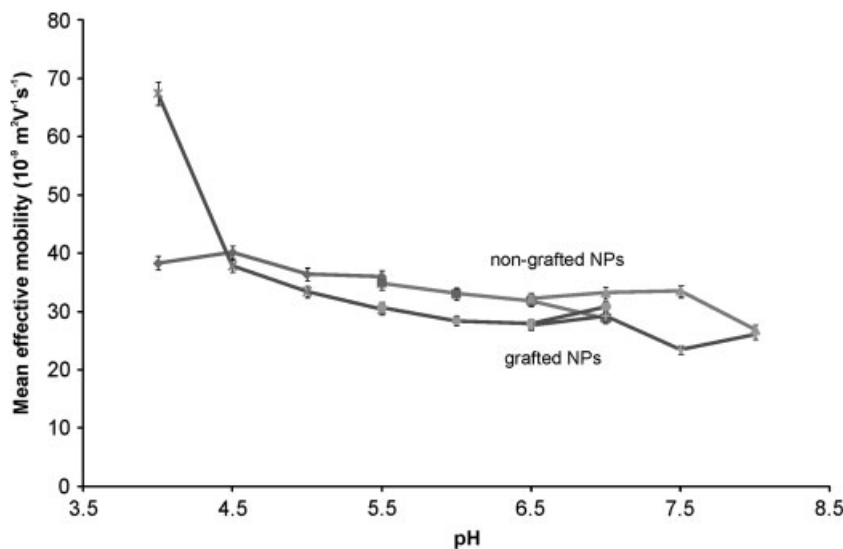


Figure 3. Effect of pH on effective mobilities of α -lactalbumin-grafted and non-grafted NPs. Experimental conditions, see Fig. 1, except for BGEs: acetate/NaOH pH 4.0–5.5, MES/NaOH pH 5.5–7.5, MOPS/NaOH pH 6.5–8.0, all of them having a 10 mM ionic strength; applied voltage: -5 kV.

viscosity, as measured by the protocol by Francois *et al.* [52], using ethyleneglycol for calibration ($\eta = 16.1 \times 10^{-3}$ Pa s at 25°C). No changes in NPs electrophoretic profiles were observed, which indicated that the particles were stable in this temperature range, with no sign of destabilization being noticed. In addition to this, the correction for viscosity led to a constant value of the mean effective mobility over this temperature range, which means that temperature had no measurable effect on the zeta potential of both grafted and non-grafted NPs. In this view, no denaturation of α -lactalbumin that could cause changes in the zeta potential was observed.

3.3 Effect of pH

Subsequently, the influence of BGE pH was studied in the range of pH 4.0–8.0 using acetate (pK_a 4.76 [53]), MES (pK_a 6.10 [53]), and MOPS (pK_a 7.20 [53]) buffers at 10 mM ionic strength with sodium counter-ions, and DDAB-modified capillaries. As expected for particles having *ca.* 4000 amino groups [31] and 35 proteins *per* particle [37], α -lactalbumin-grafted NPs exhibited a positive mobility (Fig. 3) and hence a global cationic surface charge in the whole pH range studied, similarly to the non-grafted ones. Furthermore, in most of the pH range explored, the mobility of α -lactalbumin-grafted particles is slightly lower than that of the non-grafted ones, suggesting a slight decrease of the surface charge density of grafted NPs, as only 35 α -lactalbumin molecules (globally negatively charged at pH values higher than 5) is covalently attached to a surface containing 4000 amino groups. On the contrary, in the special case of acetate/NaOH pH 4.0 buffer, the mean effective mobility of grafted NPs is much higher than the mobility of the non-grafted ones. Since α -lactalbumin has its *pI* value in the range 4.2–4.9 [44, 45], it is globally positively charged in pH 4.0, which increases the surface charge density of NPs in a larger extent than for pH values where

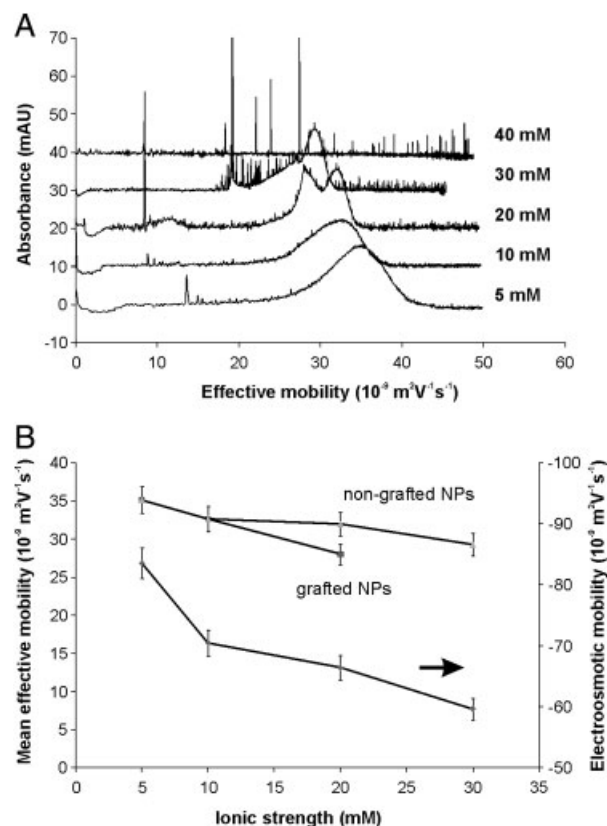


Figure 4. Effect of ionic strength on (A): the mobility-scaled electropherograms of a 1:1 v/v mixture of α -lactalbumin-grafted and non-grafted NPs and (B): their mean effective mobilities and electroosmotic mobility. Experimental conditions as in Fig. 1, except for BGE ionic strengths, as mentioned in the figure.

the protein is globally slightly negatively charged. Moreover, such acidic conditions could transform the structure of the protein grafted on NPs and modify the hydrodynamic radius and the electrophoretic behavior of the grafted NPs. For the next experiments, 6 was the best pH value, in terms of

providing a good interaction between antigen and antibody, and a good storage stability of the grafted NPs (see Section 3.1 and Table 1).

3.4 Influence of ionic strength

The influence of BGE ionic strength on α -lactalbumin-grafted and non-grafted NPs was studied in MES/NaOH buffers of pH 6.0 over the range 5–100 mM in DDAB-modified capillaries. More and more spiky electrophoregrams, suggesting aggregate formation, were observed for both NPs above a certain threshold of ionic strength (Fig. 4A), this threshold being lower for grafted NPs than for non-grafted ones (30 mM for grafted NPs and 100 mM for non-grafted NPs, when injected separately). Interestingly, the storage stability of NPs was maintained in BGEs of higher ionic strengths (50 mM) for more than 7 days, which means that the stability of such solutions was higher in the absence of an electric field. Within the range of ionic strengths lower than this threshold (5–30 mM), both electroosmotic and NPs effective mobilities decreased upon increasing ionic strength (Fig. 4B), as a result of the zeta potential decrease of both capillary inner wall and particles. A partial separation of the grafted and non-grafted NP populations was obtained at intermediate ionic strengths around 25 mM, showing the existence of a compromise to be met with respect to resolution of the NP populations and their colloidal stability. The aggregate formation above a certain threshold of ionic strength is consistent with the zeta potential of NPs which is decreasing with increasing ionic strength. The electric double layer is narrowing as much at

the threshold thus the energetic aggregation barrier is overcome and aggregates are formed.

3.5 Influence of electric field strength

The effect of electric field on mean effective mobilities and peak widths at 10% peak height was evaluated for applied voltages between 2 and 20 kV (corresponding to electric field strengths of from 57 to 570 V/cm) in all the BGEs described in Section 3.1 using DDAB-coated capillaries. In all the BGEs and when the NPs were injected separately, the effective mobilities of non-grafted NPs were constant over this range of electric field strength, while those of the grafted NPs seemed to be modified with increasing electric field (results not shown). A complex behavior was observed for 1:1 mixture of NPs. For instance at pH 6.0, as shown in Fig. 5A, a slight decrease in effective mobility was observed upon increasing voltage, which could not be explained by Joule heating, which would produce the reverse effect. Alternatively, this suggested an interaction with the capillary wall at low voltage, as more time is allowed to the interaction and as the particles migrated counter-electroosmotically in this case. Concurrently, the partial separation obtained at low voltage was lost on increasing voltage, because of increasing peak width (Fig. 5B, in mobility unit). This behavior did not appear to be consistent with the preceding assumption of wall interaction/adsorption, suggesting that other effects on the colloidal suspension may have taken place, such as those of electric field strength and/or liquid phase composition. At this point, additional experiments were performed at pH 6.0 using a neutrally coated capillary,

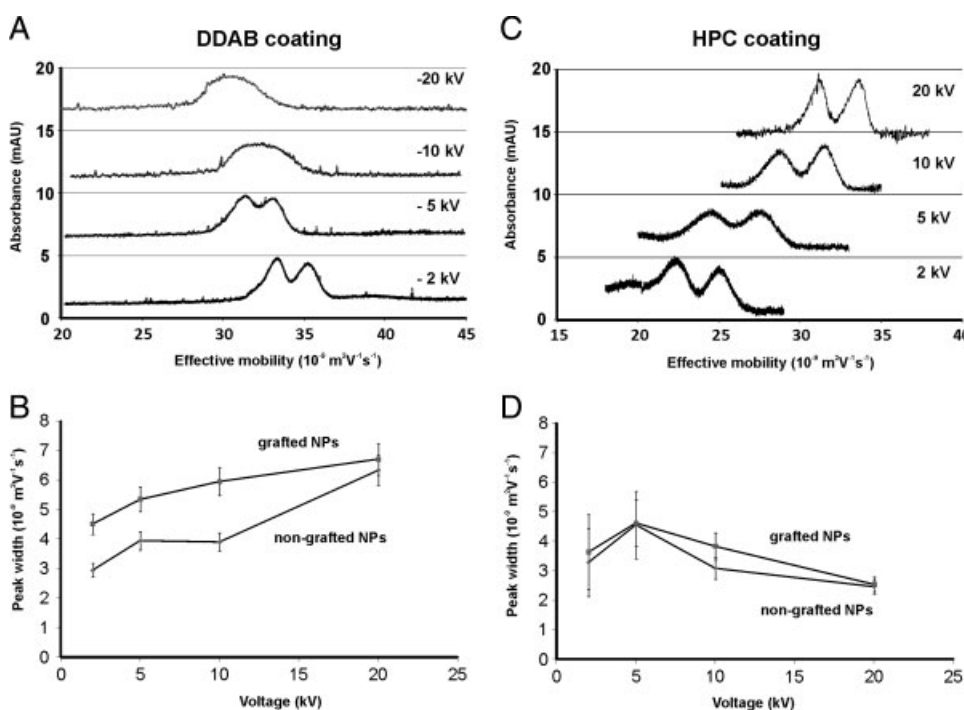


Figure 5. Influence of applied voltage on the separation of a 1:1 v/v mixture of α -lactalbumin-grafted and non-grafted NPs using DDAB- or HPC-coated capillaries. Experimental conditions: see Fig. 1, except for applied voltages, as mentioned in the figures. In mobility-scaled electrophoregrams, the first peak belongs to the grafted NPs and the second one to the non-grafted NPs. Peak widths were measured at 10% peak heights. Sequence of migration depended on the capillary modification, in DDAB, grafted NPs migrated first, in HPC, non-grafted NPs migrated first.

and a baseline shift was observed at low voltage behind the peaks (in time-scaled electrophoregrams) with effective mobilities increasing upon voltage (Fig. 5C). This may be consistent with adsorption to the capillary wall at low voltage, since there is no EOF in this case. Figure 5D shows the corresponding non-monotonous variation of peak width with voltage, including an increase in peak width with voltage in the range of low voltages (below 5 kV), for which adsorption was suspected. For higher voltages, a decrease in peak width was observed, leading to a higher resolution between the two NP populations at high voltages. This behavior was thus different from those observed using positively charged capillaries. In conclusion, the use of lower electric field strengths with the DDAB coating is favorable for the separation of NPs while higher electric field strengths are more suitable for the HPC coating. With respect to the previous results, the best conditions for the separation of both grafted and non-grafted NPs are 25 mM ionic strength MES/NaOH buffer, pH 6.0, with a DDAB-coated capillary and electric field strength of 285 V/cm (10 kV) with reversed polarity.

4 Concluding remarks

CE was shown to be an effective method for characterizing the functionalization of cationic maghemite core/silica shell NPs with α -lactalbumin. The best separation of grafted from non-grafted NPs was achieved using a 25 mM ionic strength MES/NaOH buffer, pH 6.0, and a DDAB-coated capillary, which enables to better characterize batches of NPs and improve functionalization process. From this study, two important points can be highlighted: (i) it is possible to detect the onset of NPs aggregation according to the composition of the medium or to time, whereas it cannot still be visualized, for instance, 1 or 2 days in advance. (ii) A marked effect of the electric field strength on NPs electrophoretic behavior was noticed, which confirms its detrimental influence on colloidal stability. Finally, CE appeared as an easy, fast, and reliable alternative to the common characterization techniques implemented for NPs. CE characterization of antigen-grafted NPs will help us to establish appropriate conditions for the antigen–antibody interaction in different steps of the development of an immunoassay mimicking *in vivo* conditions.

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