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Research Article

Chip electrophoresis of gelatin-based nanoparticles

Recently, biodegradable nanoparticles received increasing attention for pharmaceutical applications as well as applications in the food industry. With the current investigation we demonstrate chip electrophoresis of fluorescently (FL) labeled gelatin nanoparticles (gelatin NPs) on a commercially available instrument. FL labeling included a step for the removal of low molecular mass material (especially excess dye molecules). Nevertheless, for the investigated gelatin NP preparation two analyte peaks, one very homogeneous with an electrophoretic net mobility of $\mu = -24.6 \pm 0.3 \times 10^{-9} \text{ m}^2/\text{Vs}$ at the peak apex ($n = 17$) and another more heterogeneous peak with μ between approximately $-27.2 \pm 0.2 \times 10^{-9} \text{ m}^2/\text{Vs}$ and $-36.6 \pm 0.2 \times 10^{-9} \text{ m}^2/\text{Vs}$ at the peak beginning and end point ($n = 11$, respectively) were recorded. Filtration allowed enrichment of particles in the size range of approximately 35 nm (pore size employed for concentration of gelatin NPs) to 200 nm (pore size employed during FL labeling). This corresponded to the very homogeneous peak linking it to gelatin NPs, whereas the more heterogeneous peak probably corresponds to gelatin not cross-linked to such a high degree (NP building blocks). Several further gelatin NP preparations were analyzed according to the same protocol yielding peaks with electrophoretic net mobilities between $-23.3 \pm 0.3 \times 10^{-9} \text{ m}^2/\text{Vs}$ and $-28.9 \pm 0.2 \times 10^{-9} \text{ m}^2/\text{Vs}$ at peak apexes ($n = 15$ and 6). Chip electrophoresis allows analyte separation in less than two minutes (including electrophoretic sample injection). Together with the high sensitivity of the FL detection – the LOD as derived for the first main peak of the applied dye from the threefold standard deviation of the background noise values 80 pM for determined separation conditions – this leads to a very promising high throughput separation technique especially for the analysis of bionanoparticles. For gelatin NP preparations, chip electrophoresis allows for example the comparison of preparation batches concerning the amount of NPs and gelatin building blocks as well as the indirect assessment of the degree of gelatin cross-linking (from obtained FL signals).

Keywords:

Chip electrophoresis / Fluorescence / Lab-on-a-chip / Gelatin / Nanoparticle

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1 Introduction

Recent years have seen a fast growing interest in nanoparticles (NPs), i.e. particles in the range of up to several hundred nm diameter. Due to their high surface-to-mass ratio, such particles behave substantially different from the corresponding bulk material. These differences concern physicochemical as well as biological properties. For the latter, especially the enhanced interaction of nanosized material with cell mem-

branes (a high particle surface leads to an increase in the contact layer to the environment) and the ability to pass biological barriers (membranes) is remarkable. Furthermore, the capability of NPs to carry other compounds either entrapped within or displayed on their surface leads to interesting applications in various fields like pharmaceutical (for drug delivery and diagnostics) or food industry (e.g. increasing the bioavailability of nutritional components, additives, dietary supplements or functional food ingredients) [1–3]. For many applications biodegradable NPs are advantageous, since they can act as biocompatible substance carriers improving the solubility of the carried compound and allowing sustained substance delivery over a long time period at a given target site. Additionally, the degradation of the carrier leads to NP removal from a biological system. However, interaction of the NPs themselves with cells as well as a load of undesired compounds (contaminations) to NPs (eventually from the particle preparation process itself e.g. cross-linking agents), also leads to toxicological concerns related to nanosized material. Accumulation of NPs may lead to chronic inflammatory reactions.

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Abbreviations: FL, fluorescent; G1–G4, gelatin nanoparticle preparations 1–4; HRV2, human rhinovirus serotype 2; MWCO, molecular weight cutoff; NHS, N-hydroxysuccinimide; NP, nanoparticle; PB, preparation buffer

For recent reviews on NPs refer for example to the following papers [4–8].

In order to develop new analytical methods and adjust existing analytical methods to the fast as well as high throughput detection and characterization of engineered NPs for food applications and drug delivery systems, the European Union initiated the NanoLyse project as part of the seventh Framework Program in 2010 (<http://www.nanolyse.eu>). In this context, we concentrated on method development for the analysis of gelatin-based NPs by on-chip CE. Gelatin as inexpensive and biodegradable matrix was cross-linked via glutaraldehyde forming rather stable globular gelatin NPs according to [9]. Similar NPs are for instance used in pharmaceuticals as drug delivery vehicles (for a recent exemplary research article refer to publication [10]).

CE separates analytes basically according to their charge and size. Cross-linking gelatin via glutaraldehyde leads, besides an increase in analyte stability, most notably, to an increase in particle size after linkage. Additionally, the covalent modification of lysines influences the charge of cross-linked particles. These effects affect the electrophoretic migration behavior of gelatin NPs against the EOF, which in positive polarity analyses is the driving force to move analytes to the detector placed at the cathodic side of the separation channel. We therefore suggest the separation of NPs and less modified gelatin via CE. Although Tigani and colleagues monitored the simultaneous release of not cross-linked gelatin and an antibiotic from a glutaraldehyde cross-linked gelatin matrix via CE [11], to our knowledge CE in the chip format of gelatin NPs has so far not been reported in literature. CE of nanoparticles based on inorganic or polymer material as reviewed for example in [12] or organelles and microorganisms as reviewed for example in [13] was not targeted in the current manuscript.

In a series of previous publications we were able to transfer CE separations to the chip format on a commercially available instrument. In doing so we were able to electrophoretically separate fluorescently (FL) labeled human rhinovirus serotype 2 (HRV2, a bionanoparticle itself), to assess its binding to recombinant receptor fragments or to target its thermal stability [14, 15]. Advantages of electrophoresis in the chip format lay in very short analysis times (electropherograms were recorded typically in one to two min), low consumption of analytes and chemicals, high sensitivity (100 pM LOD for the FL dye Cy5 according to [14]) and good reproducibility of results obtained on single-use chips. Beyond that, chip electrophoresis of FL labeled HRV2 was possible in the absence of detergents which could not be achieved in the capillary format due to non-reproducible spike formation indicating virus aggregation [16]. This allowed electrophoretic analysis of samples not only composed of FL labeled virions but also of liposomes, lipid bilayer enclosed vesicles (as well bionanoparticles) that are not stable in the presence of surfactants [17–20].

Chip electrophoresis of lipid vesicles of up to 400 nm diameter extrusion yielded reasonable results [17] that led us to believe that chip electrophoresis might as well be a suitable method for the analysis and characterization of similar

sized bionanoparticles for example gelatin NPs. In previous analyses we selected FL labeling of analytes via covalent modification of primary amines by application of a dye (NHS ester) detectable with the red laser of the selected instrument ($\lambda_{\text{ex/em}} = 635/685 \text{ nm}$) [14, 15]. It is of note that cross-linking as well as FL modification targets the same reactive groups of the gelatin NP analyte. Nevertheless, depending on the degree of cross-linking, not all reactive groups are covered. Azarmi and colleagues reported about 12% of amino groups still available after glutaraldehyde cross-linking of gelatin employing concentrations of the cross-linker close to the minimum required amount to form stable particles in aqueous solution [21]. Therefore, we selected the FL label Dy649-P1 in its NHS ester form to mark gelatin NPs. The chosen fluorophore additionally exhibits three net negative charges (more information on the dye can be found at <http://www.dyomics.com/dy-649p1.html>) and was therefore suggested to reduce the coating of the employed chip channels via the FL labeled analyte due to electrostatic repulsion from the negative channel wall (electrophoresis was carried out at pH 8.3).

Furthermore, we carried out chip electrophoresis in the presence of electroosmosis and without application of any sieving material as previously described [14, 15]. Our results demonstrate that chip electrophoresis indeed allows analysis of bionanoparticles derived from cross-linked gelatin in a reproducible way. Future research will be directed at the modification of the labeling protocol since in the current setup the removal of low molecular mass material is carried out via self-packed size exclusion chromatography columns employing filters with 200 nm pore size. This setup precludes the analysis of larger particles that are already retarded on the filter. Removal of low molecular mass material from samples as eluate and recovery of gelatin NPs in the retentate in order to allow analysis of gelatin NPs with a larger diameter will be performed in the future.

2 Materials and methods

2.1 Chemicals and reagents

Boric acid (99.99%) as well as SDS (reagent plus grade) was from Sigma Aldrich (Steinheim, Germany), sodium hydroxide (pellets pure) from E. Merck (Darmstadt, Germany), benzoic acid (>99%) from Fluka (Buchs, Switzerland). FL labeling of gelatin NPs was carried out by addition of Dy649-P1 NHS-ester (Dyomics, Jena, Germany, $\lambda_{\text{ex/em}} = 655/676 \text{ nm}$ in ethanol) from a 2.5 mM concentrated stock in DMSO ($\geq 99.9\%$, from Sigma Aldrich). Atto 495 (free acid form of fluorophore, $\lambda_{\text{ex/em}} = 495/527 \text{ nm}$ in water) was from Atto-Tec (Siegen, Germany). Water was of Millipore grade (18.2 M Ω cm resistivity at 25°C). Sephadex G50 (medium grade) was from Pharmacia (Uppsala, Sweden).

Gelatin NPs were purchased from the University of Alberta (Edmonton, Alberta, Canada). Gelatin NPs were

Table 1. Overview of analyzed gelatin NP preparations

Sample number	Nominal gelatin content [mg/mL] before cross-linking	Stock solution
G1	20	H ₂ O (acetone traces from production)
G2	10	18% ethanol in H ₂ O
G3	10	18% ethanol in H ₂ O
G4	40	18% ethanol in H ₂ O

The nominal gelatin content is based on the originally scaled amount of the analyte starting material before cross-linking. It is of note, therefore, that loss of material during gelatin NP production is not accounted for. However, dry mass determination of two representative gelatin NP preparations yielded a deviation of nominal and actual analyte content well within the range of $\pm 10\%$ [w/w] (data not shown) of the nominal gelatin content. Ethanol was added as a biocide.

prepared employing a two-step desolvation method [9] and yielding NPs typically in the size range of 100–400 nm diameter as detected via photon correlation spectroscopy [21]. For gelatin NPs G1, gelatin type B was from bovine skin (Sigma Aldrich), for all other gelatin NP samples gelatin type B from pork/cattle mix (Gelita, Eberbach, Germany) was employed. Gelatin NP preparations are listed in Table 1.

2.2 Instrumentation

Chip electrophoresis was carried out on an Agilent 2100 Bioanalyzer instrument (Waldbronn, Germany) equipped with a red laser ($\lambda_{\text{ex/em}} = 635/685$ nm) and a blue LED ($\lambda_{\text{ex/em}} = 470/525$ nm) for dual detection at indicated wavelengths. Chips from soda lime glass initially manufactured for DNA detection allowed separation of up to 12 samples per chip at 30°C. Chip handling and modifications of the script (part of instrument software usually not accessible to the user, which controls all on-chip analysis steps) were as described [14, 15], to allow for electrophoresis in the presence of electroosmosis and in positive polarity mode. BGE and sample buffer were 100 mM sodium borate, pH 8.3 additionally including SDS at indicated concentrations (each buffer was filtered through 200 nm pore size, sterile, surfactant free cellulose acetate membrane syringe filters from Sartorius, Goettingen, Germany prior to use). Dye concentrations for the setup of the instruments' optics as well as applied voltages for separation were as indicated with individual figures. It is of note that instead of Cy5 as initially reported for the setup of the instruments' optics [14, 15], Dy649-P1 was used.

CE with UV detection was carried out on an Agilent 3D CE instrument employing a fused silica capillary (50 μm id, 375 μm od) from Polymicro Technologies (via Optro-nis, Kehl, Germany) with $L_{\text{tot/eff}} = 59.8/51.3$ cm. Runs were recorded for 9.5 min runtime after 2 min preconditioning with BGE and sample application (25 mbar for 8 s) at positive polarity and 20 kV (33.4 kV/m field strength)

at 30°C (sample and capillary temperature). Runs were followed by 1.5 min postconditioning with 1 M NaOH and water, respectively. Detection was at 200 nm UV absorption. BGE and sample buffer was 100 mM sodium borate, pH 8.3 including 10 mM SDS (filtered as described above).

CE with FL detection was carried out on an Agilent 3D CE instrument equipped with a Picometrics Zetalif FL detector (Ramonville, France) equipped with an HeNe laser with $\lambda_{\text{ex}} = 633$ nm (10 mW from Melles Griot obtained via Merz Brothers, Haid, Austria). A fused silica capillary (50 μm id, 375 μm od) from Polymicro with $L_{\text{tot/eff UV/eff FL}} = 72.0/23.5/57.0$ cm was employed. Runs were recorded for 30 min runtime after 2 min preconditioning with BGE and sample application (15 mbar for 10 s) at positive polarity and 13.8 kV (19.2 kV/m field strength) at 30°C (sample and capillary temperature). Runs were followed by 2 min postconditioning with 1 M NaOH and water, respectively. Detection was at 200 nm UV absorption. BGE and sample buffer was 100 mM sodium borate, pH 8.3 including SDS at indicated concentrations (filtered as above).

Electropherograms from chip CE were aligned to dye peaks, if necessary, electropherograms with UV detection to the peaks of the internal standards (DMSO and benzoic acid) as described [22].

Electrophoretic mobility values from chip CE data were determined after plotting electropherograms (OriginPro 8 SR0 software, v8.0724) and fitting of single Gauss to signal peaks. The corresponding migration time was determined from the peak apex. It is necessary to mention that for well-defined sample components like dye molecules or internal standards Gauss peaks were fitted with Adjusted *R*-Square values typically exceeding 0.990. This was not always the case for analyte-derived peaks indicating particle heterogeneity (see e.g. peak 3 from G2). Also, peak tailing and insufficient resolution between analyte peaks was sometimes a problem (see e.g. peak 5 from G3). In the latter case, the fitting was restricted to the upper part of the corresponding peak to determine its apex. In all cases, obtained migration time values after Gauss peak fitting were taken as good approximation. The EOF was calculated from both major dye peaks (electrophoretic net mobilities derived from CE-LIF measurements) allowing the μ calculation of analyte particles following standard protocols. In the case of CE UV, the μ calculation was based on the peak of the EOF marker (DMSO).

Centrifugations were carried out on a tabletop centrifuge, model 1–14 from Sigma (Osterode am Harz, Germany).

2.3 Procedures

For FL labeling of analytes, NP and gelatin-containing stocks were diluted to 7 mg/mL gelatin concentration (based on the nominal gelatin content) in 100 mM sodium borate, pH 8.3 (preparation buffer, PB). Thirty μL of such a solution were mixed with 1.5 μL of labeling dye (Dy649-P1 at 2.5 mM in DMSO), respectively, followed by incubation at ambient temperature under light protection overnight. Removal of low molecular mass material was similar than previously

described for FL labeling of HRV2 [17]. In short, Sephadex G50 was swelled overnight at ambient temperature in PB. Swelled medium was applied twice (two consecutive additions of 480 μL each) to 200 nm pore size spin filters with modified nylon membranes (VWR, Vienna, Austria). Between additions, excess buffer was removed by centrifugation at 1.0×10^3 g for 30 s. Following both Sephadex additions, the material was dried at 1.5×10^3 g for 2 min. Thirty μL of the labeling solution was weighted on top of the dried size exclusion material and was eluted via centrifugation at 1.5×10^3 g for 1 min. Consecutively, the size exclusion column was washed twice with 35 μL each of PB followed by spinning at 1.5×10^3 g for 1 min, respectively. A final centrifugation step was done at 3.0×10^3 g for 1 min. Eluted material was combined and applied to a second column. There, centrifugation was at 1.5×10^3 g for 1 min. The eluted material was weighted and adjusted to an overall 1:5 (v/v) dilution during removal of low molecular mass material by addition of PB (suggesting a buffer density of 1 mg/ μL).

In order to separate gelatin NPs from gelatin of smaller size 300 kDa molecular weight cutoff (MWCO, 35 nm pore size) spin filters from Pall (VWR) were employed. Sixty microliters of FL labeled sample as described above was weighted to 340 μL PB. Subsequently, spin filtration was performed at 1.5×10^3 g for at least 3 min (until the volume retained on the filter was approximately in the range of 60 μL). The eluate was removed and 400 μL PB added on top of the filter. Again spin filtration was done as described above. This procedure was repeated for a third time and the eluates were pooled. The retentate was recovered from the filter, weighted out and adjusted to an overall 1:2.2 (v/v) dilution during gelatin NP enrichment by addition of PB (again suggesting a buffer density of 1 mg/ μL).

2.4 Sample preparation

Samples for determination of Dy649-P1 mobility were in 100 mM sodium borate, pH 8.2 ± 0.1 (ionic strength in the range of 10.3 ± 1 mM) and additionally included SDS at given concentrations. The fluorophore was at 0.5 μM , DMSO at $1:1.0 \times 10^2$ (v/v) dilution.

Bioanalyzer samples were prepared to resemble the composition of the respective BGE. For chip CE in the absence of SDS, samples included approximately 0.32 mg/mL gelatin (based on the initial nominal gelatin content and suggesting complete recovery after FL labeling), for determination of the SDS dependence of obtained signals approximately 0.13 mg/mL gelatin. For all other samples the gelatin content was calculated to be 0.03 mg/mL (again based on the initial nominal gelatin content and suggesting complete recovery after FL labeling). For samples including the retentate of gelatin NP concentration, 0.46 mg/mL gelatin content was calculated based on sample dilutions. However, it is of note, that for the latter case large parts of the proteinaceous sample were depleted during sample preparation by application of 300 kDa MWCO filters. Therefore, a gelatin content of 0.46 mg/mL

only reflects the sample dilution during preparation but not the actual analyte concentration.

UV CE samples were at 0.5 mg/mL gelatin (based on the nominal gelatin content) in BGE and included DMSO at $1:4.0 \times 10^3$ (v/v) and benzoic acid at 0.2 mM.

3 Results and discussion

In a series of papers we demonstrated the applicability of chip electrophoresis on a commercially available instrument for the analysis of bionanoparticles for instance human rhinovirus or liposomes [14, 15, 17, 18]. With the current manuscript we intend to demonstrate the possibility to transfer our initial chip electrophoretic findings to the analysis of other important NPs, especially engineered ones derived from biological material. Therefore, we intended to electrophorese glutaraldehyde cross-linked gelatin (described to form NPs) [21] after modification of accessible primary amines via an FL dye, suitable for detection with the red laser of the 2100 Bioanalyzer instrument ($\lambda_{\text{ex/em}} = 635/685$ nm). We selected for the fluorophore Dy649-P1, which was described with maximal $\lambda_{\text{ex/em}}$ values of 655/676 nm, respectively (in ethanol, according to the manufacturing company). Dy649-P1 contributes three negative charges per covalently attached dye molecule to the analyte/fluorophore complex and thus reduces particle adsorption to the equally negatively charged walls (chip electrophoresis was done at pH 8.3) of the employed analysis chip prepared from soda lime glass.

Although we were aware that cross-linking and FL labeling of gelatin targets the same reactive and surface accessible groups of the analyte, first results already demonstrated the applicability of the employed analysis setup in the characterization of glutaraldehyde-modified gelatin (Fig. 1). Besides peaks related to the employed fluorophore (indicated by asterisk; the LOD for the dye as calculated from the threefold standard deviation of the baseline and the signal height of the first major peak values around 130 pM), two analyte specific peaks were also detected – one sharp peak at around 14.5 s migration time in the given electropherogram (corresponding to an electrophoretic net mobility of $\mu = -18.0 \pm 0.3 \times 10^{-9}$ m²/Vs (number of measurements $n = 4$) in 100 mM sodium borate, pH 8.3 as background electrolyte, BGE) and a very broad, heterogeneous peak between approximately 15.0 and 18.0 s migration time in the given electropherogram (corresponding to an electrophoretic net mobility of between $\mu = -19.6 \pm 0.8 \times 10^{-9}$ m²/Vs and $\mu = -30.3 \pm 0.5 \times 10^{-9}$ m²/Vs ($n = 4$, respectively) in the same BGE). Following the theory of electrophoresis, separation of analytes is according to their charge and size. In the case of an electropherogram recorded for gelatin and gelatin NPs as shown in Fig. 1, this would imply that larger particles migrate faster to the FL detector than smaller analytes. This effect is even more pronounced when one takes into account that larger particles result from gelatin cross-linked to a higher degree and thus are not able to bind dye molecules to such a high extent as compared to smaller particles. The number of bound dye molecules (contributing

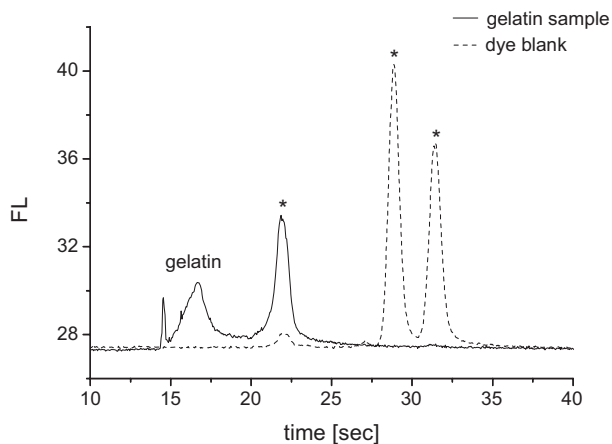


Figure 1. Chip CE of FL labeled, glutaraldehyde cross-linked gelatin NPs; FL labeling and analysis of glutaraldehyde cross-linked gelatin is feasible upon application of chip electrophoresis. Two major and one minor peak originating from the employed dye (Dy649-P1, marked by asterisk) still present in the sample although removal of low molecular weight material carried out prior to analysis can be separated from peaks originating from fluorescing gelatin species. Besides a broad gelatin peak also a second, much more homogeneous analyte peak at 14.5 s migration time in the given electropherogram is detected. The latter peak is considered to represent gelatin NPs. Chip electrophoresis was performed on an Agilent 2100 Bioanalyzer instrument employing chips originally supposed for DNA analysis. Electrophoresis was in positive polarity mode at 800 V applied voltage (approximately 18.9 kV/m field strength). BGE was 100 mM sodium borate, pH 8.3, setup of the instruments optics was via 100 nm Dy649-P1 in BGE. FL was recorded at ($\lambda_{\text{ex/em}}$ = 635/685 nm). FL modified gelatin NP preparation G1 (see Table 1) was analyzed at approximately 0.32 mg/mL gelatin in BGE. Signals for Dy649-P1 were recorded at 20 nM dye concentration in BGE additionally containing approximately 3 μM Atto 495 as additional standard for detection at $\lambda_{\text{ex/em}}$ = 470/525 nm (trace not shown).

three negative charges per bound fluorophore) influences as well the overall charge of analytes and thus their migration against the EOF. From these considerations it follows that the first (sharper) peak probably corresponds to NPs formed from gelatin, whereas the second peak probably results from gelatin that was not cross-linked to such a high degree via glutaraldehyde or did not react at all (for more details see Section 3.3). The heterogeneity of the second analyte peak additionally supports this hypothesis, as incomplete cross-linking (other than a more or less defined number of accessible primary amines of gelatin as suggested for NPs) leads to a variable number of binding sites for FL labeling. A distribution of the number of dye molecules bound to the analyte additionally increases an already given heterogeneity of the nanoparticle starting material. However, before concentrating more on our hypothesis concerning the obtained peak pattern we first attempted to improve the performance of chip electrophoresis for gelatin NP analysis in terms of sensitivity and peak separation.

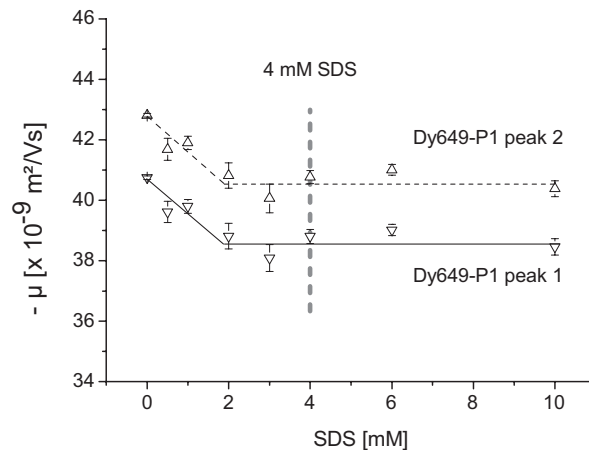


Figure 2. CMC determination by CE-LIF under electrophoresis conditions as employed for chip electrophoresis; the mobility of the two major peaks recorded for the fluorophor Dy649-P1 in dependence on the concentration of the surfactant is determined (average and standard deviation values of three data points per SDS concentration are indicated). Under the given conditions a decline in the electrophoretic mobility of both dye species is observed up to SDS concentrations of 2 mM. For higher surfactant concentrations the electrophoretic mobility of both dye species remains constant at $\mu = -38.59 \pm 0.45 \times 10^{-9} \text{ m}^2/\text{Vs}$ and $\mu = -40.55 \pm 0.46 \times 10^{-9} \text{ m}^2/\text{Vs}$, respectively (average and standard deviation calculated for all data points in the concentration range of 3–10 mM SDS). CE-LIF was in a 50 μm inner diameter fused silica capillary with $L_{\text{tot/eff UV/eff FL}} = 72.0/23.5/57.0 \text{ cm}$ at positive polarity and 19.2 kV/m field strength at 30°C. Detection was at 200 nm UV absorption and via a 10 mW HeNe laser at $\lambda_{\text{ex}} = 633 \text{ nm}$. BGE and sample buffer was 100 mM sodium borate, pH 8.3 including SDS at given concentrations.

3.1 Chip electrophoresis in the presence of SDS

In order to exclude non-covalent interactions between dye and analyte (NPs or gelatin) or analyte and analyte molecules as well as to further reduce analyte attachment to the walls of the separation channel, the addition of SDS to the BGE was assessed. Furthermore, SDS is known to increase the FL response of several fluorophores [23] that would lead to an increased sensitivity of our chip electrophoretic assay. Therefore, we regarded SDS concentrations (i) well below the CMC, (ii) in the pre-micellar region (SDS aggregation, however no micelles) and (iii) above the surfactants CMC. (According to the manufacturer, the CMC of SDS is at 7–10 mM, 20–25°C in water. With increased ionic strength of the employed buffer, the observed CMC value decreases accordingly.) The determination of the SDS CMC in the buffer system employed for chip electrophoresis of gelatin NPs (100 mM sodium borate, pH 8.3 at 30°C) was as described in [24] by calculation of the electrophoretic mobility of an FL dye (Dy649-P1) measured via CE and LIF detection and by relation of the obtained values to the SDS concentration of the BGE. Figure 2 depicts results (average of three measurements each – the standard deviation for each data point is indicated) as obtained for surfactant addition of 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0 and 10.0 mM to the BGE.

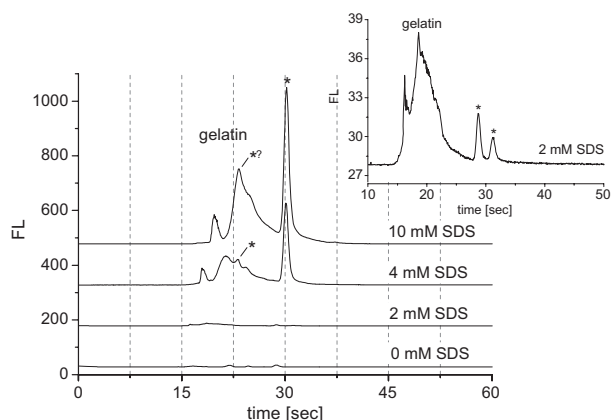


Figure 3. Increasing the SDS content upon chip electrophoresis of gelatin NPs; upon increase of the SDS content of BGE and sample buffer an increase of the FL response for analyte and dye signals is recorded. The inset displays the case of 2 mM SDS addition. The FL increase was most prominent for 10 mM SDS addition. However, in the latter case resolution between peaks was lost. Therefore, further experiments were carried out with BGE and sample buffers containing 4 mM SDS (value above the CMC). Chip electrophoresis was as in Fig. 1 in 100 mM sodium borate, pH 8.3 including SDS at given concentrations. The gelatin content was 0.13 mg/mL. Dy649-P1 peaks are marked with an asterisk.

As already shown in the electropherogram of Fig. 1, Dy649-P1 yields two main peaks upon chip electrophoresis (28.9 and 31.4 s migration time in Fig. 1, respectively), which were further regarded for mobility calculations. Both dye peaks exhibit a decline in their electrophoretic mobility from $\mu = -42.81 \pm 0.06 \times 10^{-9} \text{ m}^2/\text{Vs}$ and $-40.74 \pm 0.06 \times 10^{-9} \text{ m}^2/\text{Vs}$, respectively, by about $2.2 \times 10^{-9} \text{ m}^2/\text{Vs}$ when SDS is included in the BGE (between 0.5 and 2.0 mM). At SDS concentrations in the range of 3.0–10.0 mM, the electrophoretic mobility of the two dye species remains independent from the amount of added surfactant at $\mu = -40.55 \pm 0.46 \times 10^{-9} \text{ m}^2/\text{Vs}$ and $-38.59 \pm 0.45 \times 10^{-9} \text{ m}^2/\text{Vs}$, respectively (average of all data points between 3.0 and 10.0 mM SDS addition, these values were also employed for future analyte mobility calculations). We therefore reasoned that the CMC of the detergent reached around 2.0–3.0 mM SDS in 100 mM sodium borate, pH 8.3 at 30°C.

We also experienced a clear increase in the FL response upon chip electrophoresis of gelatin NPs in the presence of SDS in the BGE and sample buffer (see Fig. 3). In good accordance with our data determining the CMC of the surfactant in the given BGE, the FL response was most pronounced for electropherograms recorded upon 4.0 and 10.0 mM SDS addition to buffers, i.e. well in the concentration range of the surfactant forming micelles. However, at 10 mM SDS addition resolution between peaks migrating between 21.5 and 28.5 s was lost. Therefore, further experiments were carried out at 4 mM detergent addition. (The LOD for the first major dye peak calculated as previously described improved to approximately 80 pM under these conditions.)

3.2 Variation of the separating electric field and instrument setup parameters

In the next step we assessed the impact of the variation of the separation voltage (Fig. 4A) or the amount of FL dye used for setup of the instruments' optics (Fig. 4B) on obtained electropherograms. As expected, voltage increase led to shorter analysis times, however, at 1200 V (28.4 kV/m field strength), resolution between peaks decreased. As a compromise, further experiments were carried out at 1000 V (23.7 kV/m field strength).

At the beginning of each chip analysis, the optics of the instrument is adjusted to the position of the chip. In order to do so the separation channel of a given chip has to be filled with a fluorophore-containing electrolyte. We therefore questioned whether the variation of the amount of FL dye used for the instrument optics setup would influence the obtained analyte signals. As demonstrated in Fig. 4B no effect of the fluorophore concentration used for setup of the instruments' optics on the analyte signals (for the given concentration range of the FL dye) was observed. As a result we decided to carry out further experiments with the least amount of FL dye tested for setup of the instrument optics (i.e. 25 nM) in order to minimize any carryover effects of the fluorophore between sample wells on the chip.

3.3 Gelatin nanoparticle peak assignment

According to our hypothesis stated above, the first of the two detected analyte peaks (14.5 s migration time in Fig. 1) corresponds to gelatin NPs as larger molecules additionally exhibiting less charge are expected to migrate faster through the separation channel (driven by the EOF) than smaller molecules as for instance gelatin-building blocks. In order to prove this hypothesis, we filtered our samples through 300 kDa MWCO membranes (35 nm nominal pore size as given by the manufacturing company). Gelatin cross-linked to a low degree (building blocks for nanoparticle formation) should pass through the filter, whereas larger particles are retarded. As presented in Fig. 5A, such sample treatment (in combination with less sample dilution) leads to a relative increase of the first, homogeneous analyte peak (14.2 s migration time in the given electropherogram corresponding to an electrophoretic mobility of $\mu = -24.6 \pm 0.3 \times 10^{-9} \text{ m}^2/\text{Vs}$ ($n = 17$) in 100 mM sodium borate, pH 8.3 including 4 mM SDS) in the retentate. Concomitantly, other peaks originating from dye and gelatin-building blocks for nanoparticle formation were greatly reduced. Therefore, experimental results support our hypothesis that relates the more homogeneous analyte peak with a shorter migration time to gelatin NPs.

Figure 5B additionally gives results of a standard CE with UV detection for the comparison of gelatin NP starting material, a corresponding FL modified gelatin sample and a retentate of the same sample after filtration through a 300 kDa MWCO filter (at a comparable analyte concentration). It is of note that CE in the capillary format was carried out in the

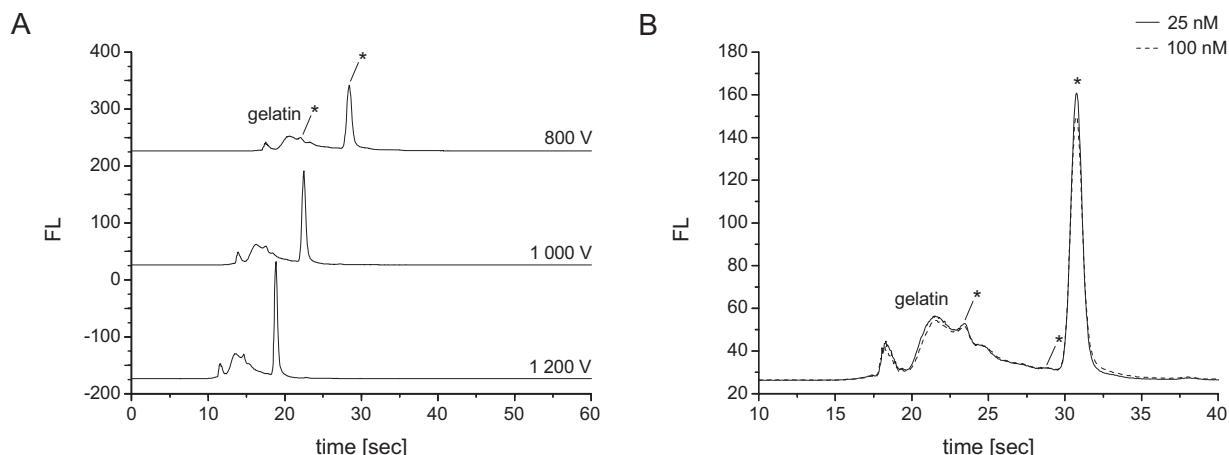


Figure 4. Variation of the applied electric field for analyte separation (A) and the amount of fluorophore used for setup of the instruments' optics (B) during chip electrophoresis; increasing the applied electric field from 18.9 kV/m (800 V applied) to 23.7 kV/m (1000 V) and 28.4 kV/m (1200 V) lead to shorter migration times (A). However, the resolution between the first (homogeneous) and second (broad) analyte peaks worsened at the same time. As a compromise, future electropherograms were recorded with 1000 V separation voltage (23.7 kV/m field strength). Prior to the analysis of each chip, the optics of the Bioanalyzer instrument were adjusted to the position of the chip within the instrument. In order to do so, a fluorophore had to be present in the separation channel of the respective chip. We tested if a variation of the dye amount used would influence the sensitivity of chip electrophoresis (B). Since no such effect was observed, we reduced the dye amount employed for setup of the instruments' optics to the least tested concentration (25 nM) to reduce carryover effects of highly concentrated dye between samples. Chip electrophoresis was in 100 mM sodium borate, pH 8.3 including 4 mM SDS. The gelatin content was at 0.03 mg/mL. Dy649-P1 peaks are marked with an asterisk.

presence of 10 mM detergent (whereas 4 mM were used for chip electrophoresis) resulting from necessarily higher gelatin NP concentrations upon UV than when employing FL detection. In order to compensate for higher analyte concentrations also the concentration of the surfactant was increased (in order to reduce analyte aggregation). Besides other findings it can be deduced that gelatin NP and gelatin recovery during FL labeling (comparison between starting material and FL particles) was passable, whereas upon application of 300 kDa MWCO filters the gelatin NP recovery was still high, however, the amount of detected gelatin of lower cross-linking degree was greatly reduced. Furthermore, a slight shift in the gelatin NP peak apex (Fig. 5C) of material retentated upon application of 300 kDa MWCO filters to shorter migration times than when compared to the original sample demonstrates that gelatin NP material is also slightly depleted from the corresponding sample. Furthermore, it can be learned that determination of the gelatin NP content of a given sample based on electropherograms with FL detection leads to an overestimation of the amount of less cross-linked gelatin than when compared to gelatin NPs as the latter obviously display less accessible reactive groups for FL modification (compare FL gelatin traces in Fig. 5A and B). Finally, when comparing gelatin NP starting material and FL particles (Fig. 5D) a slight but significant shift in migration of FL labeled gelatin NPs to shorter migration times as a result of covalent sample modification is observed (corresponding to a shift in the electrophoretic net mobility from $\mu = -27.5 \pm 0.1 \times 10^{-9} \text{ m}^2/\text{Vs}$ for the original sample to $\mu = -27.2 \pm 0.1 \times 10^{-9} \text{ m}^2/\text{Vs}$ for the FL gelatin, $n = 4$, electropherograms were normalized according to [22] before calculation, analysis in 100 mM

sodium borate, pH 8.3 including 10 mM SDS). This finding will be discussed in the following paragraph.

Analyzed gelatin NPs are typically in the size range of 100–400 nm (see the Experimental Section); the impact of FL modifications on the size of analytes can therefore be disregarded (in approximation). Thus, it should be possible to relate changes in the migration behavior of analytes to changes of the particle charge instead. Taking these considerations into account and considering that each fluorophore contributes three negative charges to the analyte/fluorophore complex FL gelatin should therefore migrate at a later time position than the original sample. However, in contrast to our expectations the electrophoretic net mobility of FL gelatin is lower than that for the original sample, indicating reduced interaction of FL labeled particles with the applied ionic detergent instead, which in turn leads to a reduced electrophoretic net mobility of the analyte.

3.4 Chip electrophoresis of several gelatin NP preparations

In order to assess the reproducibility of the gelatin NP preparation protocol other gelatin NP preparations were tested for their chip electrophoretic appearance. For a list of tested preparations refer to Table 1. Chip electrophoresis revealed clear differences between gelatin NP preparations (Fig. 6, for a list of observed electrophoretic mobilities of peaks in 100 mM sodium borate, pH 8.3 including 4 mM SDS, refer to Table 2). Interestingly, other gelatin NP preparations failed to initially yield a significant distinct peak for gelatin

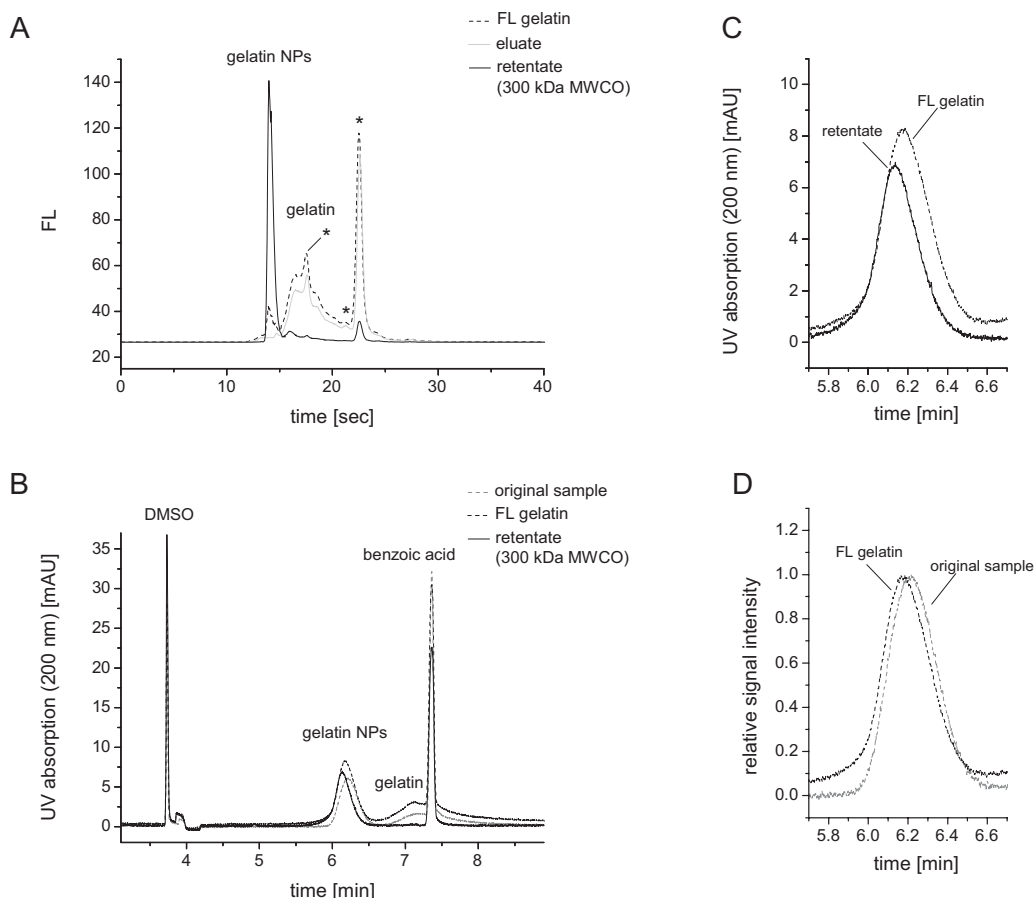


Figure 5. Separation of FL gelatin by filtration (35 nm pore size) into two differently sized species; application of a 35 nm pore sized filters allowed us to differentiate between gelatin NPs and smaller analyte particles (A). We found the first (homogeneous) peak (14.2 s migration time in the given electropherogram corresponding to an electrophoretic mobility of $\mu = -24.6 \pm 0.3 \times 10^{-9} \text{ m}^2/\text{Vs}$ ($n = 17$) in 100 mM sodium borate, pH 8.3 including 4 mM SDS as BGE, see Table 2) to correspond to the larger analyte species (retentate of filtration), whereas the broader, heterogeneous peak corresponds to analyte molecules below the filter size cutoff (eluate of filtration, i.e. gelatin cross-linked to a lower degree). CE employing UV detection allowed us to compare samples according to their concentration as well as peak mobilities in order to assess the sample recovery during labeling (B) and gelatin NP concentration (C) as well as to compare FL modified particles with unmodified analytes (D). Chip electrophoresis was in a positive polarity mode at 1000 V applied voltage (approximately 23.7 kV/m field strength). The setup of the instruments' optics was via 25 nM Dy649-P1 in BGE. FL was recorded at ($\lambda_{\text{ex/em}} = 635/685 \text{ nm}$). FL modified gelatin NP preparation G1 (see Table 1) was analyzed at approximately 0.03 mg/mL gelatin in BGE for FL gelatin and eluate samples. The retentate was approximately factor 18 less diluted. Dy649-P1 peaks are marked with an asterisk. CE UV was in a 50 μm inner diameter fused silica capillary with $L_{\text{tot/eff}} = 59.8 / 51.3 \text{ cm}$ at positive polarity and 33.4 kV/m field strength at 30°C. Detection was at 200 nm UV absorption. BGE and sample buffer was 100 mM sodium borate, pH 8.3 including 10 mM SDS. Samples were at approximately 0.5 mg/mL gelatin and included DMSO and benzoic acid as internal standards.

NPs as found for the first tested gelatin NP preparation from 20 mg/mL gelatin (as seen e.g. in Fig. 5A). However, filtration of samples through 300 kDa MWCO filters again reveals some similarities between samples (Fig. 7A) as peaks between 13 and 16.5 s migration time in given electropherograms (electrophoretic net mobility μ between $-23.3 \pm 0.3 \times 10^{-9} \text{ m}^2/\text{Vs}$ and $-28.9 \pm 0.2 \times 10^{-9} \text{ m}^2/\text{Vs}$ – $n = 15$ and 6, respectively – in 100 mM sodium borate, pH 8.3 including 4 mM SDS) increased relative to peaks of gelatin not cross-linked to a high degree. Interestingly, some preparations showed two peaks in the retentate of filtration, corresponding to gelatin NPs as well as gelatin, not cross-linked to such a high degree. Since filtration through 300 kDa MWCO filter does not yield a sharp

cutoff, such behavior can be explained. Additionally, our data indicate great differences between gelatin NP preparations. Therefore, it is not surprising, that also the size of gelatin not cross-linked to such a high degree is strongly varying.

It is of note that especially for the 40 mg/mL gelatin (G4) NP preparation an approximate size of NPs well above 200 nm was expected. If this is indeed the case, the current FL labeling protocol (including removal of unreacted dye via Sephadex G50 columns and filters with 200 nm pore size) would interfere with analyte recovery, as larger NPs are retarded by the employed filter membrane. As demonstrated when CE UV results for gelatin NP starting material and FL particles for G4 are compared (Fig. 7B), this is indeed the

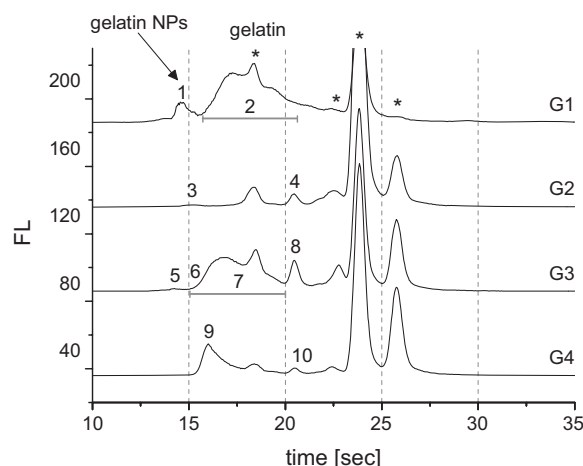


Figure 6. Comparison of several gelatin NP preparations via chip electrophoresis under previously determined conditions; several gelatin NP preparations (see Table 1 for sample parameters) were subjected to FL labeling and chip electrophoresis. As can be seen from electropherograms all tested gelatin NP preparations appeared slightly different (for a list of electrophoretic mobilities of indicated peaks (peaks 1 to 10) refer to Table 2). Chip electrophoresis as in Fig. 5 for approximately 0.03 mg/mL gelatin samples.

Table 2. Electrophoretic net mobilities μ of FL gelatin or gelatin NP peaks

Sample ID	Peak	$\mu^a)$	$SD^a)$	n
G1	1 ^{b)}	−24.6	0.3	17
	2	−27.2 to −36.6	0.2/0.2	11
	2'	−28.9	0.2	6
G2	3 ^{b)}	−25.5	0.2	14
G3	4	−34.9	0.1	14
	5	−23.3	0.3	15
	6	−26.6	0.3	6
G4	7	−25.8 ^{c)} to −34.1	0.6/0.2	10
	8	−34.9	0.1	15
	9	−27.8	0.3	8
	9'	−27.3	0.2	5
	10	−34.9	0.1	8

BGE contains 4 mM SDS. Peak numbering is demonstrated in Figs. 6 and 7, respectively. Average values as well as standard deviations for n replicates are given. a) [10^{-9} m²/Vs] b) It is of note that the indicated peaks are not homogeneous but consist obviously of several species (compare e.g. to Fig. 7, peak 3 from gelatin preparation G2). c) For samples not subjected to additional gelatin NP concentration, sometimes peak 6 can be separated from peak 7. However, separation was not sufficient in all cases to allow for exact determination of the μ value of peak 6, instead peak broadening of peak 7 is observed, which is reflected in the higher SD value of peak 7.

case – the peak corresponding to gelatin NPs is missing after FL labeling.

It is of further note that especially G2 does not yield such a high FL response as G1 that might result from differing numbers of surface accessible primary amines in given gelatin NP preparations. Alternatively, these preparations might include gelatin NPs to a lesser content. Therefore, future research

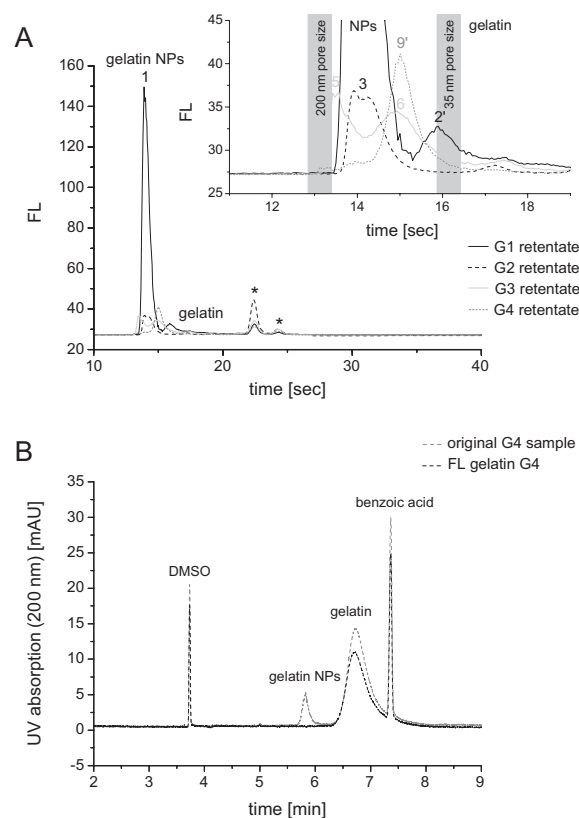


Figure 7. Chip CE demonstrates that other gelatin NP preparations also include particle species retarded upon filtration (35 nm pore size filters), i.e. gelatin NPs; application of 35 nm pore sized filters also leads to retardation of FL labeled analytes for other gelatin NP preparations indicating the presence of NPs with electrophoretic net mobilities between $\mu = -23.3 \pm 0.3 \times 10^{-9}$ m²/Vs and $-28.9 \pm 0.2 \times 10^{-9}$ m²/Vs ($n = 15$ and 6 , respectively) in 100 mM sodium borate, pH 8.3 including 4 mM SDS (see Table 2 for details (peak number 1 to 10)) (A). However, it is of note that for no other tested gelatin NP preparation the FL response for gelatin NPs was as high as for the first tested preparation G1, which might either indicate differences in gelatin NP concentration or in the number of surface accessible primary amines needed for FL labeling. It is of further note that with the current FL labeling protocol gelatin NPs larger than 200 nm diameter are lost during purification as can be demonstrated by CE with UV detection (B). Electrophoresis conditions as with Fig. 5.

will also concentrate on CE UV measurements of gelatin NP preparations. The analyte species with the electrophoretic net mobility $\mu = -34.9 \pm 0.1 \times 10^{-9}$ m²/Vs ($n = 37$) in 100 mM sodium borate, pH 8.3 including 4 mM SDS as found for several gelatin NP preparations (see Table 2) could not be identified, so far.

4 Concluding remarks

With the current manuscript we were able to demonstrate chip electrophoresis of FL samples for the characterization of gelatin NPs. By application of filters of 35 nm pore size for removal of smaller particles and 200 nm pore size

during FL labeling we were able to enrich gelatin NPs in this given range relative to gelatin cross-linked to a lower degree. It was shown that all investigated gelatin NP preparations differed concerning the amount of gelatin NPs and gelatin cross-linked to a lower degree as well as in the amount of surface accessible primary amines. The correlation between the size of NPs and their respective electrophoretic mobility was not possible due to at the moment not available exact size data of gelatin NP preparations. Therefore, future research will be aimed at the determination of the particle size of gelatin NPs. Additionally, we will concentrate on the protocol for FL labeling of analytes in order to reduce loss of larger NPs due to employed filters as well as on CE UV measurements to assess particle recoveries. As a final goal we intend to set up a method for gelatin nanoparticle detection in food matrices via chip electrophoresis.

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5 References

- [1] Kuan, C. Y., Yee-Fung, W., Yuen, K. H., Liong, M. T., *Crit. Rev. Food Sci. Nutr.* 2012, 52, 55–71.
- [2] Chaudhry, Q., Scotter, M., Blackburn, J., Ross, B., Boxall, A., Castle, L., Aitken, R., Watkins, R., *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 2008, 25, 241–258.
- [3] Lopes, C. M., Martins-Lopes, P., Souto, E. B., *Pharmazie* 2010, 65, 75–82.
- [4] De Jong, W. H., Borm, P. J., *Int. J. Nanomedicine* 2008, 3, 133–149.
- [5] Kumari, A., Yadav, S. K., Yadav, S. C., *Colloids Surf. B Biointerfaces* 2010, 75, 1–18.
- [6] Mahapatro, A., Singh, D. K., *J. Nanobiotechnology* 2011, 9, 55.
- [7] Jian, F., Zhang, Y., Wang, J., Ba, K., Mao, R., Lai, W., Lin, Y., *Curr. Drug Metab.* 2012, 13, 440–446.
- [8] Xu, T., Zhang, N., Nichols, H. L., Shi, D., Wen, X., *Mater. Sci. Eng. C* 2007, 27, 579–594.
- [9] Coester, C. J., Langer, K., van Briesen, H., Kreuter, J., *J. Microencapsul.* 2000, 17, 187–193.
- [10] Kuntworbe, N., Al-Kassas, R., *AAPS PharmSciTech* 2012, 13, 568–581.
- [11] Tigani, D., Zolezzi, C., Trentani, F., Ragaini, A., Iafisco, M., Manara, S., Palazzo, B., Roveri, N., *J. Mater. Sci. Mater. Med.* 2008, 19, 1325–1334.
- [12] Surugau, N., Urban, P. L., *J. Sep. Sci.* 2009, 32, 1889–1906.
- [13] Subirats, X., Blaas, D., Kenndler, E., *Electrophoresis* 2011, 32, 1579–1590.
- [14] Kolivoska, V., Weiss, V. U., Kremser, L., Gas, B., Blaas, D., Kenndler, E., *Electrophoresis* 2007, 28, 4734–4740.
- [15] Weiss, V. U., Kolivoska, V., Kremser, L., Gas, B., Blaas, D., Kenndler, E., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2007, 860, 173–179.
- [16] Kremser, L., Petsch, M., Blaas, D., Kenndler, E., *Electrophoresis* 2006, 27, 1112–1121.
- [17] Weiss, V. U., Bilek, G., Pickl-Herk, A., Blaas, D., Kenndler, E., *Electrophoresis* 2009, 30, 2123–2128.
- [18] Bilek, G., Weiss, V. U., Pickl-Herk, A., Blaas, D., Kenndler, E., *Electrophoresis* 2009, 30, 4292–4299.
- [19] Weiss, V. U., Bilek, G., Pickl-Herk, A., Subirats, X., Niespodziana, K., Valenta, R., Blaas, D., Kenndler, E., *Anal. Chem.* 2010, 82, 8146–8152.
- [20] Bilek, G., Matscheko, N. M., Pickl-Herk, A., Weiss, V. U., Subirats, X., Kenndler, E., Blaas, D., *J. Virol.* 2011, 85, 8368–8375.
- [21] Azarmi, S., Huang, Y., Chen, H., McQuarrie, S., Abrams, D., Roa, W., Finlay, W. H., Miller, G. G., Lobenberg, R., *J. Pharm. Pharm. Sci.* 2006, 9, 124–132.
- [22] Reijenga, J. C., Martens, J. H., Giuliani, A., Chiari, M., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2002, 770, 45–51.
- [23] De, D., Datta, A., *J. Phys. Chem. B* 2011, 115, 1032–1037.
- [24] Tellez, A., Weiss, V. U., Kenndler, E., *Electrophoresis* 2008, 29, 3916–3923.