Gold nanoparticle extraction and reversed electrode polarity stacking mode combined to enhance the capillary electrophoresis sensitivity for conjugated nucleosides and oligonucleotides containing thioether linkers

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Abstract

We present a capillary electrophoretic method for determining two different C8-conjugated deoxyadenosines, and oligonucleotides containing them, in which a psoralen or an acridine molecule is bounded to the base *via* a short alkyl chain containing sulphur ethers at both ends. The sensitivity of the micellar electrokinetic chromatography (MEKC) method was increased by using two preconcentration techniques, micro solid phase extraction (μSPE) followed by reversed electrode polarity stacking mode (REPSM). Parameters that affect the efficiency of the extraction in μSPE and preconcentration by REPSM, such as the kind and volume of the extraction nanoparticle, concentration and injection time were investigated. Under the optimum conditions, enrichment factors were obtained in the range from 360 to 400. The limits of detection (LODs) at a signal-to-noise ratio of 3 ranged from 2 to 5 nM. The relative recoveries of labelled adenosines from water samples were 95–103%. The proposed method provided high enrichment factors, good precision and accuracy with a short analysis time.

On the basis of the advantages of simplicity, high selectivity, high sensitivity, and good reproducibility, this proposed method may have great potential for biochemical applications.

Keywords: Labelled adenosines, Gold nanoparticles, Field amplified sample stacking, Micellar electrokinetic capillary electrophoresis

Introduction

Oligonucleotides are now days widely used for a variety of applications spanning from molecular probes to downregulation of gene expression in cellular studies, and more recently, as molecular scaffolds for the bottom-up construction of ordered aggregates [1], with potential use in devices [2]. In all the above cited applications, extended functionalities can be added to natural oligonucleotides by conjugation with ~~small~~ molecules, still maintaining the basic well known hydrogen bonds schemes [3, 4] that allow the predictable supramolecular assembly of the oligonucleotides. ~~Examples of such "small molecules" include: fluorophores, intercalators and cross-linkers, cleaving groups and others [5, 6].~~ One possible way of preparing conjugated oligonucleotides is that of synthesizing a conjugated nucleoside, and to incorporate it in the chosen position inside the oligonucleotide. The exploitation of the natural ability of the oligonucleotide to bind specifically its predetermined target, make then possible to locate the functional ~~small~~ molecule in a specific predetermined position in the final complex.

As many uses of oligonucleotides are carried on in cellular studies, if not *in vivo*, it is important to be able to analyse them at the lowest possible concentration with minimal interference ~~from the cellular matrices, or more in general~~ from possible interfering components.

Determination of normal and conjugate nucleosides and oligonucleotides in various samples can be performed with different techniques, including immunoassays [7] high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) conjugated with UV detection [8, 9] and mass spectrometry (MS) [10-12]. High-performance capillary electrophoresis (HPCE) has proven to be a rapid and simple technique for separating charged biomolecules and nanoparticles with very high resolution [13]. Its unique advantages, such as a relatively short time of analysis and a high separation efficiency with consumption of a minimal amount of sample and buffer solutions, make HPCE a valuable technique for the determination of biomolecules [14], especially when a reduced amount of sample is available for the analysis. Micellar electrokinetic chromatographic (MEKC) methods with sodium dodecyl sulphate (SDS) and borate phosphate buffers have been applied for the determination of nucleosides by many researchers [15-17]. One of the well-recognized disadvantages of CE is the poor concentration sensitivity, especially when a photometric detector is employed. To solve this problem several techniques have been developed. One of them is the use of high sensitivity detectors [18] another methodology is the sample pre-concentration before (off-line) or after (on-line) sample injection. Among the on-line enrichment methodologies, sample stacking in CE analyses is a particularly useful technique because it is simple to perform, economical, and requires no additional instrumentation [19-21]. In particular, the so called reversed electrode polarity stacking mode (REPSM), a modified technique of the field amplified sample

stacking [22], has good potential for sample enrichment. ~~In line stacking has been applied in monitoring nucleosides reactions by Iqbal et al. to increase the sensitivity of capillary electrophoresis analysis [15].~~ For that which regards off-line enrichment, nanoparticles (NPs) have been used to extract and enrich target analytes from complex matrices thank to their high surface area to volume ratios, which permits higher adsorption capacity for analytes, and allows attachment of biomolecules to the surface of nanomaterials [23, 24]. The binding types of NPs with target analytes include different interaction type mechanisms such as electrostatic or hydrophobic interactions [25]. In addition to these nonspecific interactions, unmodified NPs can also be utilized to capture specific analytes through the formation of chemical bonds. The strong affinity of AuNPs to thiol-containing biomolecules via a gold-thiolate bond has been demonstrated [26], and based on this evidence AuNPs have been shown to be efficient to extract aminothiols in plasma [27] or to adsorb thiol modified nucleosides [28]. However, some reports have also demonstrated the capability of disulfide [29] and thioether [30,31] monolayer encapsulation on gold nanoparticles. While the thiol- or disulfide-based chemistry is often exploited in approaches toward nanoparticle assembly, the exploration of thioether-based coordination chemistry for similar purposes is less employed, and it has not been employed in enrichment phase. In this paper we present the optimization of a capillary electrophoretic method for determining two different C8-conjugated deoxyadenosines, and oligonucleotides containing them [32], in which the functional ~~small~~ molecule is either a psoralen or an acridine derivative. Those molecules are bounded to the base *via* a short alkyl chain containing sulphur ethers at both ends (Fig. 1).

The determination involves the following steps: a) capture of labelled adenosines with AuNPs; (b) centrifuging and washing adenosine-adsorbed AuNPs; (c) release of adenosines from the Au surface through ligand exchange reaction; and (d) separation of released adenosines by CE. We investigated the effect of the number of AuNPs, and the concentration of analytes on the loading and release step. Our goal was the development of an analytical strategy combined with off-line (i.e., SPE) and on-line REPSM MEKC for the separation and sensitive determination conjugated thioether nucleosides and oligonucleotides.

Abbreviations

Acr-dA Acridine-Deoxyadenosine

AuNPs Gold nanoparticle

BGE Background electrolyte

CE Capillary electrophoresis

CZE Capillary zone electrophoresis

dA Deoxyadenosine
MEKC Micellar electrokinetic chromatography
NP Nanoparticle
Pso-dA Psoralen-Deoxyadenosine
P-pso $^{5'}$CGTGCxTCCTAGC$^{3'}$, for x= Pso-dA
P-acr $^{5'}$CGTGCxTCCTAGC$^{3'}$, for x= Acr-dA
REPSM Reversed electrode polarity stacking mode
SDS sodium dodecyl sulphate

Materials and Methods

Apparatus

An Agilent Technologies Capillary Electrophoresis series 7100 system (Santa Clara, CA, USA) was employed for the REPSM and surfactant-modified CE separation of the analytes and of their adducts with AuNPs. The CE system was equipped with diode array detection (DAD). For separations, extended light path (bubble cell) bare fused-silica capillaries (red G1600-61232 I.D.: 50 µm, total length: 60.5 cm, effective length: 52 cm, bubble factor: 3) were obtained from Agilent Technologies (Santa Clara, CA, USA).

Hydrodynamic injection of the Au NP sample was performed at a pressure of 10 mbar applied for 30 seconds. The detection wavelength of the CE system was 520 nm for AuNPs, 260 nm and 268 nm for Pso-dA and Acr-dA respectively, and 260 nm for oligonucleotides. Positive polarity (20 kV) was applied at the capillary inlet for the duration of separation. The pH of the electrolyte was measured using an AMEL pHmeter (Milano, Italy). Before use, the capillary was pretreated through sequential flushing with 1M NaOH for 5 minutes, 0.1M NaOH for 5 minutes and MilliQ (Millipore, Bedford, MA, USA) grade water for 15 minutes. The capillary was also rinsed with water for 3 minutes, 0.1M NaOH for 2 minutes, water for 3 minutes, and running buffer for 5 minutes between each run.

For the REPSM separation the capillary was rinsed with 0.1M NaOH for 3 minutes, water for 3 minutes, and running buffer for 3 minutes between each run. The injection of the Au NP sample was performed throughout at a pressure of 50 mbar applied for a given time followed by an application of voltage at negative polarity (-15 kV) for 24 seconds, switching polarity from negative to positive when the current reached 97–99% of the predetermined current at negative polarity and a waiting time of 5 seconds. All CE experiments were performed at a temperature of 25°C.

Reagents

Sodium dodecyl sulfate (SDS purity 95%) (J. T. Baker, Avantor, Center Valley, PA, USA), sodium hydroxide (Tiolchimica, Rovigo, Italy), hydrochloric acid (Carlo Erba, Milano, Italy), borate buffer ($Na_2B_4O_7$), sodium dihydrogen phosphate (NaH_2PO_4) dimethyl sulfoxide (DMSO) and citrate were obtained from Sigma Aldrich (Steinheim, Germany). The Au NPs used in this study were obtained from commercial sources; their sizes were determined, through TEM analysis. Standard AuNPs having a mean diameter of 10 nm standard deviation (SD): 0.5 nm were obtained from Sigma Aldrich (Steinheim, Germany). MilliQ (Millipore, Bedford, MA, USA) grade water (>18MΩ•cm) was used throughout the experiments.

Psoralen-Deoxyadenosine (Pso-dA) and Acridine-Deoxyadenosine (Acr-dA) and their oligonucleotides P-pso and P-acr (see Fig. 1) were synthetized as described in Ref. [32].

Preparation of BGE

The BGE solutions containing 20 mM $Na_2B_4O_7$ and SDS in the mM concentration range (10, 20, 30, 40 and 50 mM), were prepared with MilliQ water, their pH was adjusted to a given value (~~7, 8,~~ 9.3, 10 and 12) by adding 1.0 M or 0.1 M NaOH in the buffer after diluting it to the final volume. The buffer solutions were filtered through the 0.22 μm PVDF membrane filter Overmolded Millex Millipore (Bedford, MA, USA) before use.

Preparation of Standard Solutions

The 5 mM stock solutions of modified adenosines (Pso-dA, Acr-dA) were prepared in DMSO. All stock solutions were kept at -20 °C. The working solutions of labelled adenosines were prepared by diluting the stock solutions with MilliQ water at the concentrations of 1, 10, 20, 25, and 50 μM for hydrodynamic injection and 0.05, 0.1, 2, 5 and 10 μM, for REPSM injection. The 0.014 mM stock solutions of oligonucleotides were prepared in MilliQ water. All stock solutions were kept at 4°C. The working solutions of oligonucleotides were prepared by diluting the stock solutions with MilliQ water at the concentrations of 0.0035, 0.007, 0.014, 0.035 and 0.07 mM for P-acr and P-pso for hydrodynamic injection and 0.05, 0.1, 2, 5 and 10 μM, for REPSM injection.

Characterization of AuNPs

A double-beam UV–visible spectrophotometer (Jasco V-570 UV-VIS-NIR, Easton, MD, USA) was used to record the absorption spectra of AuNPs.

Transmission electron microscopy (TEM) experiments were performed on Hitachi (Tokyo, Japan) H-800 microscope using an acceleration voltage of 100 kV. The thermionic source was a tungsten filament. Samples for TEM analysis were prepared by placing a drop of the colloidal AuNPs on a clean, dry copper grid coated with a carbon film allowed to dry in air and then used for measurements. The surface charges of gold nanoparticles were characterized by measuring the zeta potential. The zeta potential was measured by using the Zetasizer ZS (Malvern Instruments, Malvern, UK). Zeta potential measurements were performed in borate buffer and 2 nM AuNPs.

Preparation of AuNPs with labelled adenosines and modified oligonucleotides

The maximum absorption wavelength of the AuNPs, which was measured by visible spectrophotometer, was 520 nm. The concentration of 10 nm AuNPs of the used solution, determined using the molar absorptivity of 1.01×10^{8} M^{-1} cm^{-1} @520 nm, was 5.98×10^{12} nanoparticles/mL (9.89 nM). This concentration is denoted as C1.

A known amount of labelled adenosines (0.05–5 µM for Acr-dA; 0.1-7.5 µM for Pso-dA) was added to a measured volume of C1 AuNPs and mixed for 1 h by vortex (VV3, VWR, Milano, Italy). The resulting mixture was centrifuged at 14000 rpm (Eppendorf 5418, Hamburg, Germany) for 30 min and the supernatant was carefully removed up to a residual volume of 20 µL. To remove the excess of labelled adenosines from the pellets, the AuNPs were resuspended in 500 µL of MilliQ water, mixed by vortex 20 min and thus centrifuged, the supernatant was analyzed by CE. The capped AuNPs were thus analyzed by CE and their release was investigated. To remove labelled adenosines from the gold surface, the AuNPs were resuspended in known amount of solutions having different compositions (i.e. pH and SDS concentration), containing 20 mM $Na_2B_4O_7$. After 20 min, the released labelled adenosines were isolated from AuNPs by centrifugation at 14000 rpm for 30 min. The supernatant was directly analyzed by CE. A known amount 1.9 mL of oligonucleotides (obtained by diluting 50 µL of 0.014 mM for P-pso; 0.014 mM for P-acr both prepared in 20 mM $Na_2B_4O_7$ buffer) was added to a measured volume of 10 nm AuNPs solution at concentration C1, a small volume of 500 mM pH 3 citrate HCl buffer was added to the suspension and mixed by vortex, the mixture was incubated at room temperature for 4 h. The solution was centrifuged at 14000 rpm for 30 min to separate the AuNPs from the excess of reagent and the supernatant was analyzed by CE. The AuNPs were then washed 3 times with 400 µl of MilliQ water and thus centrifuged, the supernatant was analyzed by CE. Finally the AuNPs were dispersed in a solution, which contained 20 mM $Na_2B_4O_7$ and 20 mM of SDS. After 48 h, the released oligonucleotides were isolated from AuNPs by centrifugation at 14000 rpm for 30 min. The supernatant was directly analyzed by CE.

Analysis of Pso-dA in urine

Urine samples (8 mL) were collected from healthy adult females. After collection, the samples were frozen and stored at -80 °C immediately. Before the analysis, samples were thawed at room temperature. An aliquot of 3.6 mL was spiked with 40 μL of a solution 20 μM of Pso-dA. Both urine and spiked urine were added with 400 μL of of C1 AuNPs and mixed for 1 h by vortex. After centrifugation at 14000 rpm the supernatant was carefully removed up to a residual volume of 20 μL and the precipitates were suspended in 500 μL of MilliQ water, mixed by vortex 20 min and thus centrifuged. The pellets were resuspended in 400 μL of borate buffer at pH=9.3 with SDS 20 mM. After 20 min, the suspensions were separate by centrifugation at 14000 rpm for 30 min. The supernatants were directly analyzed by MEKC.

Results and discussion

Reverse stacking

To increase the applicability of CE in biomolecules monitoring we developed a stacking REPSM-MEKC method. In fact, REPSM, a CE-based on-line concentration strategy, has been proved increase detection ability for various samples [33, 34], including NPs [35]. In REPSM the sample is introduced into the capillary hydrodynamically, applying a stacking voltage at negative polarity to concentrate the analytes at the interface between the sample zone and the background electrolyte, and then pumping the sample matrix from the capillary under electroosmotic flow (EOF). When the current reaches ca. 97% of its original value, this potential is turned off. A positive potential is then applied to separate the analytes.

The REPSM-MEKC method was optimized with respect to sample loading time, separation buffer concentration, pH of separation buffer, and separation voltage.

Different concentrations of borate buffer, 15, 20, 40 and 60 mM, and different pH values, ~~7.10, 8.00,~~ 9.3, 10.04 and 12.10 were investigated. As the concentration of borate was increased, a longer migration time was observed. In addition, the retention time of the labelled adenosines increases with the pH. It was also found that by increasing the SDS concentration in the BGE, the separation improves however for concentrations larger than 50 mM long retention times lead to peak broadening. The best resolution of the analytes was obtained using a 20 mM sodium tetraborate buffer at pH 9.30 containing 20 mM SDS, with an applied voltage of 20 kV. Fig~~s~~ 2 ~~2a and b~~ show the elegropherogram of mixture of Pso-dA and Acr-dA under optimal condition.~~versus SDS concentration and pH respectively.~~

Different sample plugs were tested in order to determine the highest sample plug length to be injected without worsening the separation profile by REPSM. To improve the sensitivity of the method, the injection time was varied between 25 s and 100 s using a 50 mbar hydrodynamic injection. The peak area of the three analytes increased with the injection time, but injection time longer than 50 s gave lower area reproducibility therefore 50 s were chosen.
To examine the quantitative REPSM method, the results in terms of limit of detection were compared with the conventional MEKC method (see Table 1). It can be observed that the REPSM-MEKC method gave around twenty-fold lower limits of detection.

Loading of conjugate adenosines on AuNPs

To verify whether or not citrate-AuNPs can interact with labelled adenosines, the extinction spectra of the citrate-AuNPs were examined ~~after the addition of labelled adenosines~~. The Surface Plasmon Resonance (SPR) peak of citrate-AuNPs was located at 520.0±0.5 nm (*n*=3) in (spectrum A in Fig. 3). As shown from TEM data in the inset of Fig. 3, AuNPs have an average diameter of about 10 nm in agreement with the data from the supplier. Upon the addition of Pso-dA, the SPR peak of the AuNPs was shifted to 543 ±0.6 nm (spectrum B in Fig. 3) and its intensity decreased. Similar trends were observed in the spectra of Acr-dA, and of P-pso and P-acr (spectrum C in Fig.3). It has been reported that the adsorption of uncharged nucleosides (e.g. adenosine and inosine) could cause AuNPs aggregation, in such a case the colloidal solution undergoes an instant red-to-blue color change [36]. In the studied concentration range, the spectra of the colloid after addition of labelled adenosines do not turn to blue, the shift in the spectra is possibly due to the modification of the dielectric environment close to the NP surface which, in turn, is related to the interaction of the surface with a molecule [37]. The interaction between nucleosides and AuNPs is quite complicated and has been the subject of extensive debate. Briefly, binding of nucleosides to AuNPs through the nitrogen has been reported, [37, 38], in other work hydrophobic interactions were indicated as the adsorption drive force [39]. These differences in the interaction responsible for adsorption partially derive from different colloidal composition for what concerns both the adsorbate solution, the NP surface and the media. Moreover, it should also be considered that the compounds investigated in the present study were conjugate nucleosides containing thioether groups, and the adsorption of organosulfur compounds on the AuNP surface, has also already been reported [30]. In fact, it is well known that alkanethiols are the most popular ligand for AuNPs [40], additionally, the use of dialkyl sulphides [41, 42] and protected thiols has also been reported [43]. The thioether–gold coordination is much weaker than the covalent thiolate–gold interaction, and it increases for multidentate ligands comprising more than one thioether unit [44, 45]. In general, monothioethers need longer chain

lengths or costabilization in order to give stable, redispersible gold colloids [46]. The detailed study of binding mechanisms lies beyond the scope of the present work. However, we observed that for the studied oligonucleotides, the binding was found to be strong enough to allow their capture on the surface of the AuNPs. In order to qualitatively understand if this interaction is mainly due to functional molecules and the tioether linker or to the nucleoside, the adsorption of dA (deoxyadenosine) was investigated. In Fig. 4 the electropherograms before and after the contact with AuNPs are reported. It can be seen that the adsorption of dA on AuNPs is negligible. To quantify the adsorption of labelled adenosines on AuNPs, their concentrations obtained by REPSM-MEKC in the solution before and after the contact with AuNPs were measured. The effect of equilibration time on the adsorption was evaluated by measuring the concentration of the labelled adenosines in contact with AuNPs during time. The concentration observed for the modified adenosines reached a constant value after 30 min of incubation.Consequently, to assure a contact time longer than the equilibration time, 1 h was chosen for the experiments. In addition, the influence of the concentration of the analytes was considered. Fig. 5 shows the adsorbed quantity as a function of the labelled adenosines in the solution after equilibration. Extraction with 400 µL of AuNP at C1 concentration with 400 µL of solution was almost complete at concentrations lower of 2 µM for Pso-dA and 2.5 µM for Acr-dA, at higher concentrations the particles were saturated. On the basis of these results, in order to obtain a quantitative adsorption of the two adenosines the quantity were chosen lower than the saturation coverage and the equilibration time was set to 1 h. These adsorption results confirmed that AuNPs could be used for extracting the labelled adenosines from an aqueous solution.

The particles obtained after incubation and centrifugation, were washed by suspension in water and separated by centrifuge, the supernatant was injected in the CE system to determine the concentration of the labelled adenosines eventually released and the procedure was repeated twice. We found that, for both the analytes considered (i.e. Pso-dA and Acr-dA), the concentration in the two aqueous solutions was below the detection limit, and no peaks were revealed in the electropherograms. This finding indicates that the concentration of released adducts in water (i.e. AuNP-Pso-dA and AuNP-Acr-dA) were lower than the detection limit of the method.

Release of labelled adenosines from their adducts with AuNPs

To evaluate the release of labelled adenosines from their AuNP adducts, the labelled nucleosides adsorbed on the NP surface were suspended in 100 µL of buffered solutions at different pH values (the conjugated particles were prepared as described in section 3.2). It can be seen in Fig. 6a, that the percentage of released Pso-dA is negligible in the 3-9 pH range and increases at pH higher than

10 or lower than 2. To increase the release of the compounds in the solution the effect of SDS was investigated at pH=9 (see Fig. 6b). When SDS concentration was increased from 0.1 to 10 mM an increase of the area of the analyte in the electropherograms recorded at 260 nm was found, at SDS concentration higher than 10 mM the area of the compounds reached a plateau. The maximum release is thus obtained for SDS concentrations higher than the critical micelle concentration for SDS (8.1 mM).This finding indicates that the labelled adenosines are physisorbed on AuNP, and they are displaced by the surfactant. SDS can indeed be adsorbed onto gold surface [47, 48].
Based on the previous results, for the extraction of the labelled nucleosides a buffered solution at pH= 9 with 20 mM SDS was chosen. The electrophoretic mobilities of the released labelled adenosines were equal to those obtained for the compounds before the capture process. Therefore, the concentration step does not affect the labelled adenosines.
Under the conditions above mentioned, the peak areas of the compounds in the electropherograms obtained by MEKC increased significantly after the extraction. In particular, the extraction with the AuNPs resulted in a 4.2, and 3.8-fold increase in the response of the method (peak area recorded at 260 and 268 nm for Pso-dA and Acr-dA, respectively). This result is in agreement with a four-fold improvement in the sensitivity due to a nearly complete adsorption from the initial 400 µL volume (before extraction) and desorption to a final sample volumes of 100 µL (after extraction), confirming that thioether labelled nucleosides can be completely extracted from aqueous solution by using AuNPs. The effect of the sample volume on the enrichment of the labelled adenosines was also investigated, generally by increasing the sample volume, the peak area in the electropherograms of the analytes after enrichment increases, until the particle saturation is reached. For a given compound, the sample volume to achieve particles saturation depends on the concentration of both the analyte solution and the AuNPs. At the concentration of 0.05 µM (C_i) and by using 100 µL of C1 AuNP solution using and 1.9 mL (V_i) of sample solution, the recovery obtained from three repetition was 101±8.3%. The recovery was calculated as:

$$R(\%) = 100\,\frac{C_f V_f}{C_i V_i} \qquad (1)$$

where C_f corresponds to the concentrations of labelled adenosines in the final volume of supernatant and 100 µL (V_f). In turn C_f values were calculated from calibration curves built with standard solutions.
The enrichment factor after the extraction procedure (i.e. V_i/ V_f), was 19.
The LOD for Pso-dA and Acr-dA at a signal to noise ratio of three, in the normal hydrodynamic injection technique MEKC (without extraction) were 1.62 and 0.71 µM respectively, and the LOD

in the normal hydrodynamic injection technique MEKC (with extraction) were 0.092 and 0.052 μM for Pso-dA and Acr-dA respectively.

The particles after the release of analytes were washed twice with milliQ water and 10 mM citrate solution and the enrichment of Pso-dA was repeated on the AuNPs recovered by using the procedure above described. The extraction-release procedure was repeated thrice on the same AuNPs particles. The extraction was carried out with 400 μL of AuNP at C1 concentration with 400 μL of 2 μM Pso-dA solution, and a contact time of 1 h. The loading percentage of Pso-dA on AuNPs was calculated from its concentration measured by REPSM-MEKC before and after the contact with AuNPs. Loading percentages were 97%, 100%, 96%, respectively in the first, second and third trial. The release was obtained with 400 μL of the buffered borate solution at pH= 9.3 and 20 mM SDS. Recovery percentages in the first, second and third reuse cycle were 100%, 98%, 105% respectively. This finding indicates that the solid phase of the off-line enrichment procedure can be easily regenerated and can be reused, thus decreasing the waste of materials.

Finally when the REPSM-MEKC method was applied to the extracted solution of labelled nucleosides which was obtained from the release of the analytes from their adducts with AuNPs, an enrichment factor of about 380 was obtained, which is in good agreement with the total enrichment factor given by the product of the enrichment in the extraction procedure (about 19) and that due to REPSM injection (about 20). To the best of our knowledge, this is the first example of the use of citrate-AuNPs for the successful extraction of labelled adenosines combined with stacking injection to increase the sensitivity of CE analysis. The LOD, at a signal to noise ratio of three, in the REPSM injection technique MEKC (with extraction) were 5.5 and 2.3 nM for Pso-dA and Acr-dA, respectively.

It has been reported that REPSM-MEKC method can also be directly applied to AuNPs. For instance, the surfactant CE separation coupled with RESPM injection increases the detection sensitivities of a factor of about 5 for AuNPs with diameter of 5 nm as reported in Ref. [35]. Starting from this finding AuNP-labelled adenosines adducts were analyzed by CE. The zeta potential of employed AuNPs in the running buffer was negative -41.3 ± 0.9 mV, indicating the stability of the suspension. On the other hand, their stability in buffer containing SDS inside capillaries commonly employed for electrophoretic separation has been already demonstrated [35]. In Fig. 7 it can be seen the separation of the two components: Pso-dA and its AuNP adduct obtained at different concentration of SDS. The adducts were partially dissociated during the electrophoretic separation in SDS buffers and both the free Pso-dA and its AuNP conjugate were detected, thus indicating that adduct suspensions were not stable in the running buffer. It can be also noticed that the separation increases with decreasing the surfactant concentration in BGE. It is out of the scope

of the present study to investigate the kinetic of the Pso-dA release in SDS buffer. However, from this experiment it can be assessed that CE-diode array detection technique could allow also for separation and identification of surface labelled nanoparticles, if performed under stable conditions. The extraction procedure was applied to urine sample in order to explore the feasibility of the methodology in practical cases. When the supernatant obtained from the extraction with AuNPs of urine was analized by REPSM-MEKC, no peak corresponding to Pso-dA was detected (see Fig. 8a). Instead in the urine sample spiked with Pso-dA, a peak corresponding to the analyte was identified (see Fig. 8b). The identification was carried out by comparing the eletropherogram of the urine sample to that of a water spiked solution prepared with the same procedure of the urine sample (Fig. 8c).

Capture and Release of oligonucleotides by using AuNPs

Oligonucleotide probe-linked AuNPs have found wide application in biosensors, in general they were obtained as alkanethiol-capped oligonucleotides self-assembled on Au-NPs by Au-thiol bond. One of the method to prepare those probes was that proposed by Mirkin [49] based on the chemisorption step for linking thiol oligo probes to citrate-protected AuNPs. It should be noted that, in addition to terminal linkage of DNA and AuNP through Au—S linkage, nonspecific binding of nucleotides to AuNP through the nitrogen-containing bases, or not specific interactions were also described [37, 50]. Nonthiolated DNA was found to protect AuNPs against salt-induced aggregation as long as the DNA ~~is~~ was single stranded, short, and unfolded [36]. In this case, the adduct ~~is~~ resulted from an adsorption process. In general, the DNA loading capacity in adsorption is much lower than that that can be achieved by thiolated DNA. However, it has been found that at low pH, the loading capacity of AuNP for nonthiolated DNA containing polyadenine fragment, can be increased and their conjugates maintain the full functions of DNA, allowing for molecular recognition [52]. In the present work, the adsorption of labelled oligonucleotides (see Fig. 1) was carried out at acid pH. The increase in loading capacity at low pH is due to the decrease of electrostatic repulsion among negatively charged AuNPs and oligonucleotides as reported in Ref. [52]. A small volume (i.e., 50 μL) of stock solution (14 μM in 20 mM NaH_2PO_4 buffer pH 7.10) was diluted in 1.9 mL of buffer (spiked solution), at this diluted solution 100 μL of C1 AuNPs was added, and the suspension was mixed via a brief vortex mixing. A small volume of 500 mM pH 3.00 citrate-HCl buffer was added to the suspension. After brief vortex mixing, the sample was allowed to incubate at room temperature for 4 h, the mixture was centrifuged at 14000 rpm, and the supernatant was removed.

The pellets obtained after incubation and centrifugation, were washed by suspension in water and separated by centrifuge. The conjugates were resuspended in the phosphate buffer and the suspensions of the AuNPs superficially labelled with oligonucleotides were analysed by CE. As shown in Table II, the AuNPs adducts migrated at different rates than AuNPs and in particular the conjugates migrated more slowly.

This finding confirms that the proposed method is able to separate efficiently AuNPs on the basis of their size and surface properties. It should also be noticed that in the present study, the oligonucleotides employed, have a thioether linker which is more easily displaced than a terminal thiol group linker [54].

The release of adsorbed oligonucleotides was carried out at neutral pH by adding NaCl 0.1 M [50] in 100 μL of SDS borate buffer, and the recovery was 88±11% (see Eq. 1) with an enrichment due to the extraction equal to 17. Release of nonthiolated DNA from their AuNP adducts in neutral pH solution has also been reported in Ref. [50]. The solutions of the oligonucleotides P-pso and P-acr ~~solutions,~~ before extraction were analyzed by REPSM-MEKC method, carried out in the same condition above described for the labelled adenosines. An increase in the sensitivity of the method was obtained (see Table I), with an ~~the~~ enrichment factor of 21±8 ~~with~~ respect to hydrodynamic injections. Finally, by coupling the extraction procedure with REPSM method an enrichment factor of about 360 was obtained ~~(see Fig. 8)~~. The LOD, at a signal to noise ratio of three, in the REPSM injection technique MEKC (with extraction) were 3.3 and 4.3 nM for P-Pso and P-Acr respectively.

Conclusions

In this paper we present the optimization of a capillary electrophoretic method for determining two different C8-conjugated deoxyadenosines, and oligonucleotides containing them, in which either a psoralen or an acridine moiety are linked to the adenosine through an alkyl linker containing two thioether functions. The choice of the linker allows a reversible capture of the compounds on gold nanoparticles, ensuring at the same time enrichment and separation from possible interfering components (at least in aqueous solutions).

The combined two step enrichment method based on off -line micro solid phase extraction (μSPE) on gold nanoparticles followed by REPSM-MEKC can be applied for the separation and analysis of labelled nucleosides and oligonucleotides. The enrichment procedure is simple and does not require additional instrumentation. The method does not employ solvent and the gold nanoparticles employed as solid phase can be reused, therefore, it satisfies the criteria of green analytical

chemistry procedure. The proposed enrichment method considerably increases the sensitivity of capillary electrophoresis. In addition, the separation strategy developed can also be employed to investigate surface modified nanoparticles.

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