ION PAIR METHOD TO DETERMINE THE CTAB CONTENT IN GOLD NANORODS SAMPLES

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ABSTRACT

In this work, we report a fast and accessible method for quantification of cetyltrimethylammonium bromide (CTAB) in solutions of gold nanorods (GNRs) by bromophenol blue (BPB) ion-paring formation and spectrophotometric detection. The ion-par method used to quantify CTAB exhibited adequate figures of merit and was applied to the quantification of CTAB present in solutions of GNRs-CTAB and in GNRs-CLPFFD samples obtained by the seed growth method. This type of methodology could be extensive to others surfactants employed for the synthesis of nanoparticles. In addition, this method allows screening CTAB in GNRs samples and consequently would help to know if CTAB concentration is lower than an acceptable cut-off for cell viability analysis.

Keywords: CTAB, gold nanorods, ion-paring, bromophenol blue, quantitation

INTRODUCTION

Nanotechnology offers enormous potential for diagnostics and therapy. Because of their optical and electric properties[1] different types of gold nanoparticles (GNPs) have been studied for biomedical applications[2-7], including imaging[8, 9], biosensing[10, 11]and the delivery of either genes[12, 13]or antitumor drugs for cancer diagnosis and therapy[14].

A type of GNPs are gold nanorods (GNRs) which have a resonance absorption in the near infrared region spectrum. Due to the Plasmon effect at the near infrared region, the GNRs absorb energy and dissipate it locally that could be used in nanomedicine to produce the so-called *molecular surgery*. Thus GNRs can be used for photothermal therapy[15]irradiating at the near infrared region where tissues and cells are essentially transparent (800-1300 nm)[16]favoring the penetration of the electromagnetic irradiation.

GNRs used in therapy must be nontoxic and stable in biological media and should be specific for the target. The complete combination of these three factors has hindered the use of GNRs as carriers in biological and biomedical applications. GNRs synthesis requires the presence of a high concentration (approx. 0.1 M) of the cationic surfactant cetyltrimethylammonium bromide (CTAB) that favors the asymmetric geometry during the synthesis[17]. However, CTAB is known to degrade biomembranes[18-23], raising a significant concern about the cytotoxicity of GNRs-CTAB *in vitro* and *in vivo*. The cytotoxic effects of GNRs-CTAB can be minimized by reducing the CTAB concentration below the critical micellar concentration[24], but this reduction occurs at the expense of GNRs suspension stability, consequently compromising their unique optical properties in biological environments[25]. In order to replace CTAB, GNRs were capped with different molecules such as polyelectrolytes, phospholipids or peptides[26]; however, a residual concentration of the surfactant could remain in the sample. In order to quantify the CTAB present in GNRs solutions, liquid chromatography-mass spectrometry has been used[27]; nevertheless, this method is not always accessible for some laboratories dedicated to nanobiotechnology. Accordingly, a rapid, simple and cheap method would be appropriate for quantifying CTAB.

A typical methodology to quantify cationic surfactants is by ion-pair formation, determining the analyte by titrimetric[28]or spectrophotometric methods[29]. For example, Jia et al. reported a method based on this methodology to quantify carbaryl and its metabolite 1-naphthol in water samples[30]and Gao et al. have been used tetraiodophenolsulfonphthalein as a spectral substitute to characterize the complexation between cationic and anionic surfactants[31]. The dye 4,4'-(3H-2,1-Benzoxathiol-3-ylidene)bis[2,6-dibromophenol] S,S-Dioxide, commonly known as bromophenol blue, (BPB) has been extensively used as an acidic ion-pairing reagent for the determination of cationic and basic drugs through either dye salt formation technique , extraction and spectrophotometric measurement of the complex formed in non-polar solvent[32-35].

Herein, we report a fast and accessible method for the quantification of CTAB in solutions of GNRs by BPB ion-paring formation and spectrophotometric detection. In this work, the developed method was applied to the quantification of CTAB present in solutions of GNRs-CTAB and in GNRs-CLPFFD samples obtained by the seed growth method[36]. In the case of GNRs-CLPFFD the GNRs were functionalized with the amphipatic peptide CLPFFD that recognizes toxic aggregates of β-amyloid involved in Alzheimer's disease for photothermal therapy[26].

EXPERIMENTAL

Reagents

Cetyltrimethylammonium bromide (CTAB, 99%) was purchased from Sigma and bromophenol blue sodium salt (BPB) was purchased from J. T. Baker. All others reagents were of analytical grade unless indicated otherwise. All solutions were prepared with ultrapure water (ρ= 18 mΩ) from Millipore Milli-Q system.

Apparatus

Spectrophotometric measurements were carried out with an UV–vis spectrophotometer Perkin Elmer Lambda 25, using 1 cm quartz cell and equipped with a computer with Lambda 25 software for acquisition and treatment of data.

Solutions preparation

Stock solutions: 0.1 M CTAB solutions were prepared in Milli-Q water. 1×10^{-3} M BPB solution was prepared in a basic solution of 0.1 M NaOH.

Work solutions: All solutions for the calibration curve were prepared by diluting appropriates volumes of CTAB stock solution in Milli-Q water to obtain solutions in the range of 2×10^{-5} to 1×10^{-4} M.

Synthesis of GNRs-CTAB and GNRs-CLPFFD

GNRs-CTAB were synthesized using the seed-mediated approach[37]. In the first step, a seed solution was prepared to reduce 250 µL of $HAuCl_4$ in a solution of 9.75 mL of 0.1 M CTAB and cold-prepared sodium borohydride (600 µL, 0.01 M). Seeds were kept at 27 °C for 2 h before use. Next, 55 µL of 0.1 M ascorbic acid was added to a growth solution containing 75 µL of 0.01M $AgNO_3$, 9.5 mL of 0.1 M CTAB, and 500 µL of $HAuCl_4$ 0.01 M. Then, 250 µL of HCl 0.1 M and 12 µL of the previously prepared seed solution were added. The solution was allowed to react for 10 min at 27 °C before centrifugation at 7,030g for 15 min. After centrifugation, the supernatant was removed, and the pellet was resuspended in Milli-Q water. Vis-NIR absorption spectra were recorded at room temperature with a 2501PC UV−vis recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan). GNRs were observed using transmission electron microscopy (TEM) with a JEOL JEM-1010 microscope. The specimen was prepared by dropping GNRs on Formvar carbon-coated copper microgrids and letting them dry.

Conjugation of GNRs with CLPFFD.

.For conjugation of CLPFFD to GNRs, GNRs were prepared as described above and after the first centrifugation step, the pellets used to prepare GNRs-

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CLPFFD (pellets that come from 4 mL of original GNRs-CTAB solution) were resuspended in 4 mL of an aqueous peptide solution in a concentration of 0.25 mg/mL, and the final mixture was allowed to react for 72 h at 27 °C. GNRs-CLPFFD was exhaustively characterized as was described previously by Adura et al[26].

Synthesis of the peptide CLPFFD

Peptide as a C-terminal amide was synthesized using a fluorenylmethyloxycarbonyl (Fmoc) strategy in solid-phase synthesis. The side-chain of cysteine was protected with the trityl group, which is removed during the final cleavage to render the free thiol. Fmoc-protected amino acids were purchased from Iris Biotech (Marktredwitz, Germany). In addition, 1-[bis(dimethylamino) methylene]-1H-benzotriazolium tetrafluoroborate 3-oxide (TBTU), Fmoc-AM handle, and resin MBHA were obtained from Novabiochem. The chemical reagents N,N′-diisopropylcarbodiimide (DIPCI), 1-hydroxybenzotriazole (HOBt), triethylsilane (TES), and N,N'-dimethylaminopyridine (DMAP) were obtained form Fluka (Buchs, Switzerland). Manual synthesis included the following steps: (i) resin washing with DMF (5×30 s), (ii) Fmoc removal with 20 % piperidine/DMF (1×1 min + 2×7 min), (iii) washing with DMF (5×30 s), (iv) washing with DMF (5×30 s) and CH_2Cl_2 (5×30 s), (v) Kaiser's test (with a peptide-resin sample), and (vi) washing with DMF (5×30 s). Peptides were cleaved by acidolysis with trifluoroacetic acid (TFA) using TIS, 2,2´-(ethylenedioxy)-diethanethiol (DOTA) water as scavengers (92.5:2.5:2.5)(v/v/v) for 90 min. TFA was removed with a N_2 stream, and the oily residue was precipitated with dry *tert*-butyl ether. Crude peptides were recovered by centrifugation and decantation of the ethyl ether phase. The solid was redissolved in 10 % acetic acid (HOAc) and lyophilized. The peptide was analyzed using RP-HPLC with a Waters 996 photodiode array detector (λ 443 nm) equipped with a Waters 2695 separation module (Milford, MA), a Symmetry column (C18, 5 µm, 4.6×150 mm), and Millennium software at a flow rate (1 mL/min, gradient) of 5-100 % B over 15 min (A) 0.045 % TFA in H_2O, and B) 0.036% TFA in acetonitrile. The peptide was purified using semipreparative RP-HPLC with a Waters 2487 Dual Absorbance Detector equipped with a Waters 2700 Sample Manager, a Waters 600 Controller, a Waters Fraction Collector, a Symmetry column (C18, 5 µm, 30×100 mm), and Millennium software. The peptide was finally characterized through amino acid analysis with a Beckman 6300 analyzer and through MALDI-TOF with a Bruker model Biflex III. Using MALDI-TOF, the CLPFFD (H-CLPFFD-NH_2) [M^+Na^+]: 762.3 peptide was identified. Peptides were subjected to amino acid analyses, which yielded the following relationships (parentheses denote the theoretical relationship) for CLPFFD: Leu 0.99 (1); Pro 1.06 (1); Phe 2.05 (2); Asp (0.90); Cys not determined. HRMS calculated for $C_{36}H_{50}N_7O_8S$ [M^+H^+], 740.3436; found, 740.3434.

Analytical procedure for determination of CTAB in GNRs samples

Calibration curve preparation for CTAB solutions

Appropriate volumes of CTAB stock solution were diluted with Milli-Q water to obtain working solutions ranging from 2×10^{-5} M to 1×10^{-4} M. Into a series of 15 mL glass stoppered, 5 mL of $CHCl_3$, 1 mL of CTAB and 1 mL of 1×10^{-3} M BPB were added and stirred in a Vortex type stirrer for 30 min. Then the samples were placed upright for 10 minutes and the organic phase was extracted and read in the spectrophotometer at 606 nm against the corresponding blank solution.

Accuracy and precision

The intra-day accuracy and precision were calculated by analyzing three replicates of CTAB solutions at three different concentrations: 4×10^{-5}, 6×10^{-5} and 8×10^{-5} M. The inter-day accuracy and precision were determined by analyzing the three concentrations on three different runs.

Detection (LOD) and quantitation limits (LOQ)

The LOD and LOQ of the method were calculated by using the average (Y_b) and standard deviation (S_b) of the blank estimated response, the calibration curve slopes (m) and the signal noise ratios of 3 and 10, according to the following expressions[38]:

Recovery

$$LOD = \left[\frac{(Yb + 3 \cdot Sb)}{m}\right] \qquad LOQ = \left[\frac{(Yb + 10 \cdot Sb)}{m}\right]$$

Two samples of known concentrations of CTAB (3×10^{-5} M and 8×10^{-5} M) were prepared in the same manner as those of the calibration curve and adding of the reagents used in the GNRs synthesis (ascorbic acid, silver nitrate, CLPFFD) which could interfere with the readings.

Quantification of CTAB in GNRs-CLPFFD and GNRs-CTAB samples

4 mL of both GNRs-CTAB and GNRs-CTAB-peptide sample solutions were centrifuged at 16000×g for 20 min. The supernatant was collected in a glass vial previously washed with *aqua regia*. Then, 1 mL of this sample was taken and assayed as described above. Each sample was assayed in triplicate.

RESULTS AND DISCUSSIONS

To develop an accessible method for the estimation of CTAB concentration in GNRs solutions we analyzed two samples of biological interest: GNRs and GNRs-CLPFFD, that could be useful for photothermal therapy. However, it is necessary to consider that after the synthesis these samples may contain residual amounts of CTAB that can produce cytotoxic effects *in vitro*[24, 26]. Thus, the determination of CTAB in the samples is crucial for biological assays to avoid interference in cell viability studies.

GNRs with an aspect ratio length/width of approximately four (40 nm/10 nm) were obtained (Fig. 1) by the seed-mediated growth method[26, 37]. GNRs were functionalized with the amphipathic peptide CLPFFD that is derived from the Aβ peptide sequence and recognizes it. The amphipathic peptide CLPFFD has a Cys (C) residue that contains a thiol group, allowing a strong interaction with the gold surface[39]. GNRs-CLPFFD was characterized previously by different techniques X-ray photoelectron spectroscopy as XPS, analysis were characterized instead were characterized of aminoacids, UV-Vis, and Zeta potential[26]. Before determination of CTAB in GNRs samples, the experimental conditions were set.

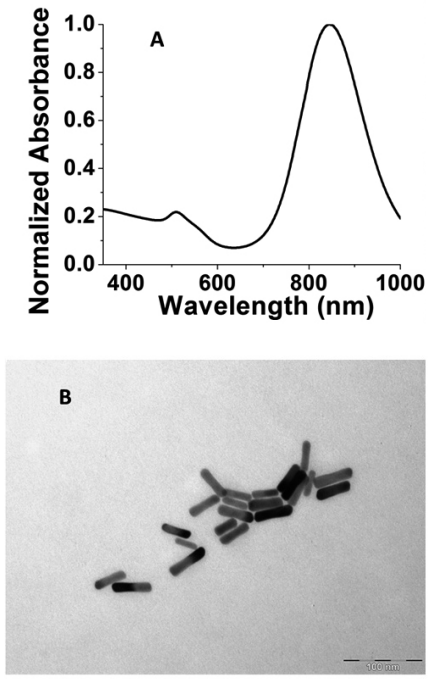

Figure 1. (A) Vis-NIR absorption spectrum of GNRs-CLPFFD and (B) TEM image of GNRs-CLPFFD.

Selection of experimental conditions for the quantification of CTAB in GNRs samples.

Ion-pair complex between cationic surfactants or basic drugs with BPB is widely described in the literature[29, 33-35, 40]. Therefore, based on its ammonium group, CTAB forms an ion-pair complex with BPB. The reaction between CTAB and BPB in 0.1 M NaOH produces a blue colored product that could be extracted in organic solvent. The absorption band of the complexes showed an absorption maximum at a wavelength of 606 nm (Fig. 2). The reagent blanks prepared under similar conditions showed no absorption.

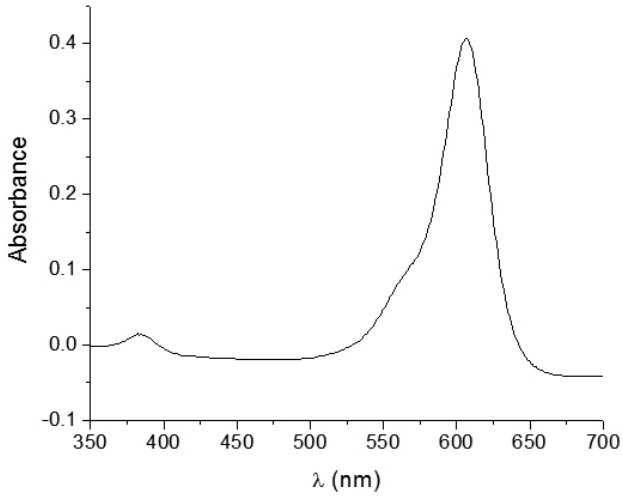

Figure 2. Visible spectrum of CTAB-BPB ion pair complex extracted in chloroform.

In order to verify the stoichiometry of the BPB a titration curve was carried out taking into account the absorbance *vs.* molar ratio CTAB:BPB. As expected, the composition of the ion-pairing complex using the molar ratio method is 1:2 (CTAB:BPB)[41](Fig. 3), due to the fact that at alkaline pH, as in this case, BPB dissociate its -OH and -SO_3H groups and exists as a two-charged anion. The effect of concentration of BPB on the color intensity was studied using different concentrations and the maximum absorption intensity at 606 nm was obtained at 1×10^{-3} M of BPB. In addition, the absorbance of the ion-pair was found to be stable for at least one hour.

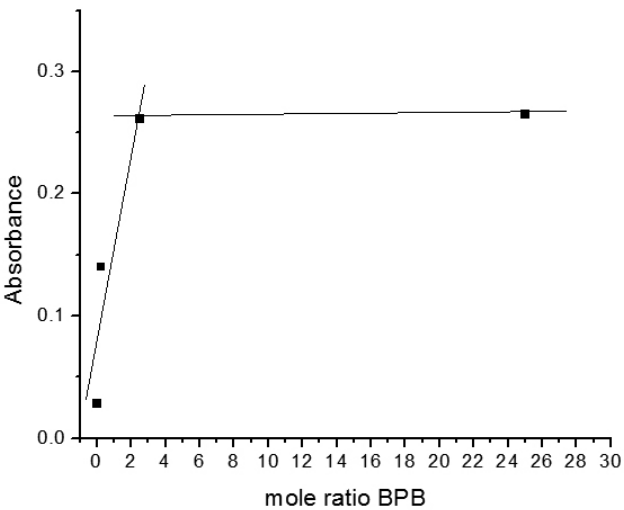

Figure 3. Mole ratio plot of CTAB:BPB

In order to select the optimum experimental conditions, the effects of GNRs, the extraction time on recovery of ion pairing and the dye concentration were evaluated. Chloroform was chosen among other solvents, as well as dichloromethane and ethyl acetate, due to its low cost and quantitative extraction of the ion-pair.

On the other hand, it is important to evaluate the potential GNRs interference in the proposed spectrophotometric method. As can be seen in Fig. 4, GNR exhibits two bands near to 500 and 800 nm and a valley with absorbance values up to 0.1 units, which interfered with the CTAB-BPB ion-pair, requiring the removal of GNRs by centrifugation. The GNRs spectra before and after remotion is shown in Fig. 4.

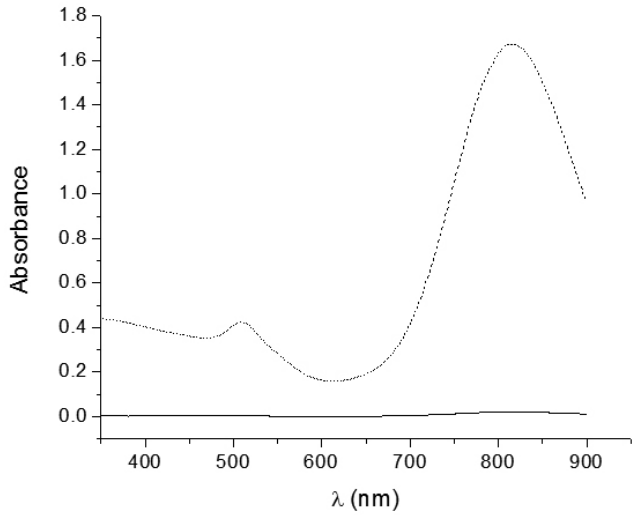

Figure 4. Visible spectra of GNR samples with (—) and without GNRs extraction (---).

Extraction time is an important parameter that influences the recovery of the ion-pair formed. As can be seen in Fig. 5 the absorbance of the ion-pair (at 606 nm) increases up to 30 min of extraction time, and consequently this optimum extraction time was selected.

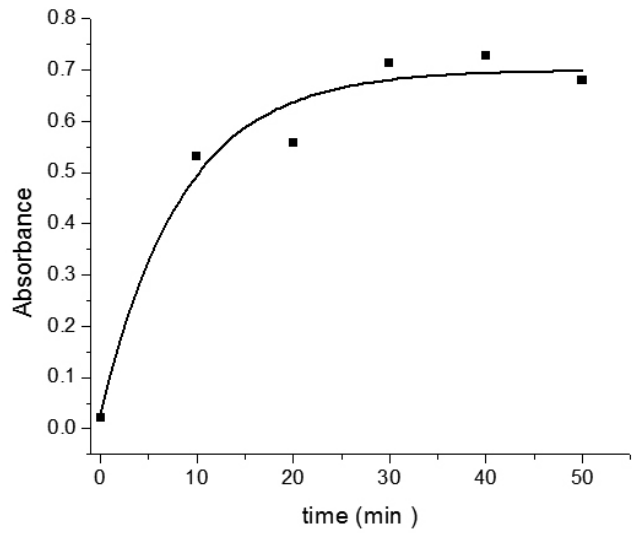

Figure 5. Effect of extraction time on recovery of CTBA-BPB ion pairing.

Analytical development

After experimental variables were optimized calibration curves were constructed by plotting absorbance at 606 nm *vs.* concentration. In Table 1, the figures of merit of the calibration graphs obtained are shown. Linear regression in the concentration range of 2.0×10^{-5} to 1.0×10^{-4} M was obtained, with adequate values of repeatability and reproducibility for the studied concentrations.

Table 1. Analytical parameters for the developed method.

Parameter	
Repeatability, CV (%)	2.8 (4.0×10^{-5} M) - 2.1 (6.0×10^{-5} M) - 2.2 (8.0×10^{-5} M)
Reproducibility, CV (%)	2.9 (4.0×10^{-5} M) - 4.2 (6.0×10^{-5} M) - 3.1 (8.0×10^{-5} M)
Concentration range (M)	2.0×10^{-5} - 1.0×10^{-4}
Calibration curve	A = 10844 C [M] – 0.155 (r = 0.9993; n = 9)
Detection limit (M)	7.48×10^{-6}
Quantitation limit (M)	1.73×10^{-5}

CTAB has been determined by using different spectroscopic methods based on colorant ion pairing formation, with typical ranges of LODs from 0.12 to 0.38 μM by using Resonance Rayleigh scattering with BPB[42] and Eosin Y[43], Resonance light scattering with azoviolet[44] and Fluorescence quenching with eosin Y[45]. Meanwhile, the lower LODs have been obtained by flow injection analysis using Cu(II)-chromazurol S–surfactants complexes[46] (0.05 μM) and by complexes formation of $Al^{3+}$and Be^{2+}withChromeAzurol S[47] (0.025 μM). Finally, LODs of 0.8 μM and 3 μM have been obtained by using a spectrophotometric method with sequential injection[48] and non-extraction flow injection using eriochromeblack-T, respectively[49].In this sense, our method presents a LOD and LOQ that allow establishing whether a solution of GNRs contains cytotoxic concentration. Different values of IC_{50} *in vitro* cytotoxicity of CTAB have been reported depending on the cell lines assayed[50]; for example, Takahashi et al. determined that IC_{50} of CTAB for HeLa cells is around 10 μM, which is in the same order of our LOD[51].

To check the selectivity of the proposed method, the peptide used in the GNRs-CLPFFD synthesis was added, demonstrating that no interference with CTAB-BPB ion-pair is produced, obtaining recove values over 90 % with CV values lower than 3%.

In GNRs-CTAB and GNRs-CLPFFD samples we determined the CTAB content. To avoid the interference of GNRs a centrifugation of the sample was carried out and in the supernatant the CTAB concentration was determined. In the case of GNR-CTAB the concentration was $8.19\times10^{-4} \pm 1.08\times10^{-5}$ M while in GNR-CLPFFD the concentration was $5.48\times10^{-5} \pm 1.22\times10^{-5}$ M. The presence of CTAB in the solutions could be attributed to a desorption from the surface to the solution. Remarkably, the presence of lower concentration of CTAB in GNR-CLPFFD sample with respect to GNR-CTAB could be attributed to the chemisorption of the peptide CLPFFD (which contains a thiol) on the gold surface with a subsequent replacement of the CTAB which leads to a reduction of the surfactant final concentration.

Concluding remarks

The ion-par method to quantify CTAB exhibited adequate figures of merit and was applied to GNRs samples. This type of methodology could be extensive to others surfactants employed for the synthesis of nanoparticles. In addition, this method allows screening CTAB in GNRs samples and consequently would help to know if CTAB concentration is lower than an acceptable cut-off for biological analysis.

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