Talanta 99 (2012) 538–543

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

# Gold nanoparticle-biotinylated liposome hybrids as analytical reagents for biotin determination using a competitive assay and resonance light scattering detection

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ARTICLE INFO

*Article history:*
Received 9 March 2012
Received in revised form
8 June 2012
Accepted 15 June 2012
Available online 20 June 2012

*Keywords:*
Gold nanoparticle-biotinylated liposome hybrids
Competitive avidin–biotin affinity assay
Flow injection system
Resonance light-scattering detection
Food analysis

ABSTRACT

The preparation of hybrid nanostructures formed by gold nanoparticles (AuNPs) into biotinylated liposomes and their analytical application are presented. The surface of negatively charged AuNPs was modified with 1-dodecanethiol and the NPs were encapsulated into biotinylated liposomes using the rapid solvent evaporation method. Liposomes were resized by both mechanical shaking and ultrasound treatments and filled liposomes were separated from empty liposomes using sucrose density gradient centrifugation. The analytical usefulness of AuNP-liposome hybrids as amplification probes for biotin determination was checked using the competitive affinity reaction based on the avidin–biotin interaction and biotilynated phospholipids for the synthesis of the liposome hybrids. The method was automatized using a flow system and measuring the resonance light scattering signal. The dynamic range of the calibration graph was 0.001–20 $\mu g\,mL^{-1}$, ($r^2=0.9998$, $n=14$), with a detection limit of 0.3 $ng\,mL^{-1}$. The precision, expressed as relative standard deviation (RSD%), was lower than 5% and the sampling frequency was 9 $h^{-1}$. The approach has been applied to the determination of biotin in food samples, with recovery values ranging between 88.2 and 105.2%.



## 1. Introduction

The special vesicular structure of liposomes, which consists of a lipid bilayer enveloping an internal aqueous compartment, and the easy functionalization of their surface have given rise to their use in a wide range of analytical methods involving chromatography, capillary electrophoresis, immunoassay, sensors and microfluidic systems [1]. Liposomes have shown their usefulness as signal-amplification reagents using their capability to entrap or attach different hydrophobic, amphipathic or hydrophilic molecules. Fluorescent liposomes, obtained by entrapping a fluorescent organic dye or a lanthanide chelate have been widely described for this purpose.

Liposomes containing AuNPs have been proposed for delivery AuNPs in biomedical applications, in a similar way to the use of liposomes as effective carriers for drugs and other therapeutic agents in clinical treatments [2]. These NPs have been shown to provide therapeutic enhancement in radiation therapy [3], but they have short circulation lifetimes and limited accumulation into tumor cells when they are directly delivered. However, the incorporation of AuNPs into or the surface of liposomes allows the improvement of their stability, circulation lifetime and cellular uptake [4]. Three types of AuNP-liposome hybrids have been described, according to the position of the NPs in the liposomes: liposomes containing AuNPs in the inner phase, liposomes with AuNPs at the lipid membrane, and liposomes modified with AuNPs on the surface [5]. The two first hybrids involve the formation of liposomes in the presence of AuNPs, while the third type consists on the physical adsorption of the AuNPs on the surface of previously formed liposomes.

This article shows for the first time the usefulness of AuNP-liposome hybrids as signal-amplification reagents alternative to fluorescent liposomes. The study describes the synthesis and purification of biotinylated-hybrid liposomes containing AuNPs and their application in a competitive assay for biotin determination in food samples using a flow system and resonance light scattering (RLS) as detection system. The reaction of avidin with the liposome hybrids causes a high radiation scattering, which decreases in the presence of the analyte. Although the aggregation of biotinylated AuNPs with streptavidin has been previously studied using spectrophotometry [6,7], it has not used for analytical purposes.

Biotin is a water soluble vitamin of the B-complex, also known as vitamin H or vitamin $B_8$, which acts as coenzyme in different carboxylase-mediated metabolic reactions, such as gluconeogenesis,

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http://dx.doi.org/10.1016/j.talanta.2012.06.029

biosynthesis of fatty acids and metabolism of amino acids [8]. This vitamin is present in a wide variety of foodstuffs at low concentration levels, in the free form or covalently bound to proteins of the food matrix. The first methods developed for biotin determination were microbiological assays, which attain a high sensitivity but also show low precision, selectivity and accuracy [9,10]. Several liquid chromatographic methods have been described for the determination of biotin using, mainly, fluorimetry or mass spectrometry as detection system [9,11,12], which allow the separation of biotin and biotin derivatives. Fluorimetric detection requires the use of a post-column derivatization reaction. A third group of methods for biotin determination are binding assays based on the high affinity constant of the reaction of avidin or streptavidin with this vitamin [9]. These methods, which are very selective, involve the use of a competitive format and a biotinylated reagent with a measurable property, such as luminescence or enzymatic activity. The simultaneous use of liquid chromatography with an avidin silica gel column and a biotinylated fluorescent reagent has given rise to a very sensitive and selective method for biotin determination [12].

The method described here shows the potential advantages of biotinylated liposome hybrids containing AuNPs as an alternative amplification reagent, using the capability of these NPs to scatter the radiation. The use of biotinylated liposomes, together with the biotin–avidin or biotin–streptavidin system, has found some analytical applications. A microfluidic preconcentrator has been recently described using biotinylated liposomes containing a fluorescent dye, which did not show fluorescence owing to the quenching effect of the high concentration of dye molecules into the liposomes [13]. Streptavidin-conjugated magnetic beads, previously immobilized in the microfluidic channels using a permanent magnet, were used to capture the liposomes, which were then lysed by flowing a detergent that caused the release of the dye, obtaining a significant fluorescence enhancement. However, the analytical application of this system was not described. Also, a fluorescent Eu(III) chelate incorporated into biotinylated liposomes (energy donor) and a fluorescent dye labeled streptavidin (energy acceptor) have been proposed in a competitive assay for biotin determination using luminescence resonance energy transfer as the detection system [14]. Although the detection limit of the method is very low, the preparation of both the donor and the acceptor reagents is laborious and, in addition, its application to the analysis of real samples was not demonstrated.

## 2. Experimental

### 2.1. Apparatus and instruments

An SLM Aminco (Urbana, IL, USA) AB2 luminescence spectrometer provided with a 150 W continuous xenon lamp and a 7 W pulsed xenon lamp, and furnished with a 176-052-QS Hellma (Hellma Hispania, Barcelona, Spain) flow cell, with an inner volume of 18 μL, was used to monitor the RLS signals. A Perkin-Elmer Lambda 35 UV/VIS spectrometer (Perkin Elmer, Beaconsfield, UK) was used to obtain absorbance spectra. A four-channel peristaltic pump with rate selector (Gilson Minipuls-3, Vilier le Bel, France), a low pressure injection valve (Reodyne, Tecnokroma, Barcelona Spain), and Omnifit (Cambridge,UK) teflon tubing of 0.5 mm I.D. were used for the construction of the hydrodynamic manifold. A desktop laboratory MPW-350R centrifuge (MPW Med. Instrument, Warsaw, Poland) with cooled chamber of rotating, and equipped with an angle rotor HSL-11199 (45°, 12 × 12 × 1.5 mL, max. speed = 18,000 rpm, 24088 RCF and $r_{min/max}$ = 3.5/6.25) was used. A VorTemp 56 LA-S2056 shaking incubator (Labnet Int. Woodbrige, USA) and an ultrasound bath were used. A Büchi rotavapor R-205 (Flawil, Switzerland)) was used to evaporate the organic solvent and form the liposomes.

The characterization of the synthesized nanomaterials was performed by conventional optical and transmission electron microscopy (TEM). Images were acquired in the first case using a bright-field UIS2 Optical System Microscope (Olympus, Hamburg, Germany, http://www.olympus.de/microscopy/, *BX51*, Model *U MDOB3*) equipped with a DP20 Digital Camera, which provides high precision images of 2 Mpixels in UXGA (1600 × 1200), operating at 15 frames per second. TEM images were obtained using a CM-10 Philips Microscope (Philips Research, Eindhoven, The Netherland, http://www.research.philips.com) with 0.5 × 0.34 nm resolution and equipped with a digital megaview III camera. Copper grids (200C-FC) coated with a Formvar® carbon film 200 mesh supplied by Aname (Madrid, Spain, http://www.aname.es) were used as support in TEM experiments.

### 2.2. Reagents

All chemicals used were of analytical grade. 1,2-Distearoyl-*sn*-glycerol-3-phosphatidylcholine sodium salt (18:0 PC, DSPC), 1,2-Dioleoyl-sn-glycerol-3-phosphoethanolamine-*N*-(biotinyl) sodium salt (18:1 biotinyl-DOPE), 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-*N*-(cap-biotinyl) sodium salt (18:1 biotinyl-cap-DOPE) were purchased from Avanti Polar Lipids (Alabama, USA). Cholesterol (CH), tetrachloroauric acid ($HAuCl_{4)}$ trihydrate, 1-dodecanethiol (DT), sodium tetrahydrideborate, avidin and biotin were purchased from Sigma. Trisodium citrate dihydrate, sucrose and other common reagents were also purchased from Merck. A solution containing 25 mM disodium hydrogen phosphate (Merck) and adjusted to a pH 7.4 was used as buffer solution. Methanol and chloroform were used for preparing the liposomes. Aqueous solutions were prepared using deionized water purified with a Milli-Q system (Millipore, Bedford, Ma, USA).

### 2.3. Procedures

#### 2.3.1. Liposome hybrids preparation

The first step for the preparation of the liposome hybrids was the synthesis of negatively charged AuNPs, which were obtained by the conventional Turkevich method [15]. Briefly, 5 mg of $HAuCl_4$ trihydrate were dissolved in 47.5 mL of deionized water in a round-bottom flask. A volume of 2.5 mL of $3.4 \times 10^{-2}$ M trisodium citrate dihydrate was added drop wise in two minutes and then was vigorous stirred under reflux at 100 °C for 30 min. The final aqueous solution, containing approximately 0.25 mM AuNPs, was kept stable in refrigerator at 4 °C until use.

AuNP-liposome hybrids were prepared by using a modification of the conventional rapid solvent evaporation (RSE) method [16]. A volume (100 μL) of a lipid mixture composed by DSPC, biotinyl-DOPE or biotinyl-cap-DOPE, and CH (75:12.5:12.5) was diluted to 1.0 mL with a chloroform/methanol (880:120) mixture and was put into a 50 mL round-bottom flask. A volume (3 mL) of about 0.25 mM negatively charged AuNPs was mixed with 150 μL of DT (98% w/v) to obtain hydrophobic NPs, and 5 mL of 25 mM disodium hydrogen phosphate buffer solution (pH 7.4). This solution was carefully added along the flask wall and the liposomes were formed in the aqueous phase. The organic solvent was removed in the rotary evaporator at 40 °C and 300 rpm under vacuum pressure. After 30 min, a pale-violet opalescent fluid was obtained which contained a wide variety of liposomes.

The liposome population was homogenized by using two physical treatments, mechanical shaking and sonication. The liposome suspension (2 mL) was shaken in a shaking incubator at 40 °C, which is a temperature close to the transition temperature, and at 250 rpm for 30 min, stopping each 10 min for 2 min. This liposome

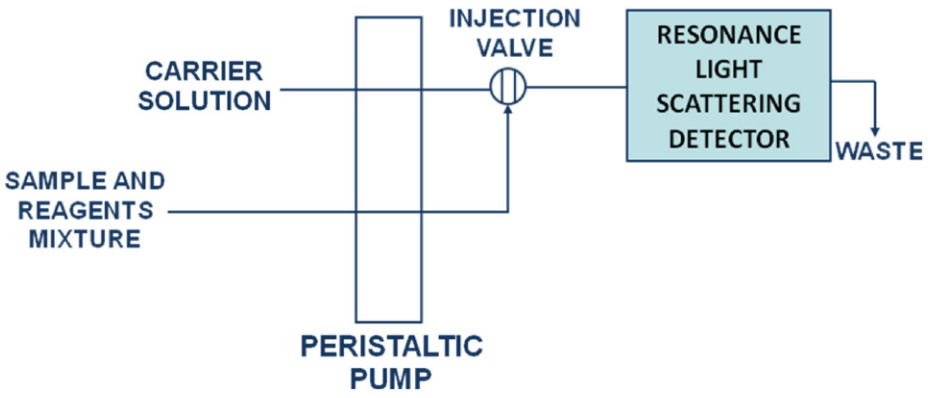


**Fig. 1.** Scheme of the flow system.

suspension was partially submerged in a bath sonicator and treated for a period of 10 min, stopping each 2 min for 10 s. The suspension changed in its appearance from milky to a clear suspension.

A sucrose density gradient centrifugation (SDGC) system was used to separate un-trapped DT-AuNPs from filled and empty liposomes. A volume of 0.6 mL of the liposome suspension was added to a non-linear density gradient performed in 5-layers (10%, 20%, 25%, 30% and 50% sucrose) of 0.15 mL each one, using a 100% sucrose solution. The system was centrifuged at 5352.8*g* for 15 min obtaining an upper zone, which contained the un-trapped AuNPs, an intermediate zone with the liposome hybrids, which showed a reddish appearance, and a lower zone with the empty liposomes.

#### 2.3.2. Competitive avidin–biotin assay

The assay was based on the competition between the analyte (biotin) and AuNPs-biotinyl-cap-liposome hybrids for binding the avidin sites and the measurement of the RLS signal at 525 nm ($\Delta\lambda$=0, 4/4 slit ratio and a detector gain of 10 V). The analytical parameter used was the difference in the RLS signal obtained in the presence and absence of biotin. Closed vials were prepared by mixing 100 μL of 25 mM phosphate buffer solution (pH 7.4), 300 μL of the AuNP-biotinyl-cap-liposome hybrids suspension, 100 μL of standard biotin or sample solutions, in a biotin concentration range of 0.001–20 μg $mL^{-1}$, and 100 μL of 5 μM avidin solution. These solutions were stirred and sequentially transported to the detector using an on-line flow system and the buffer phosphate as carrier (Fig. 1). The length of the coil reactor between the injection valve and the detector was 25 cm. Each standard or sample solution was assayed by carrying out six successive injections of 50 μL each one. Each aliquot required 30 s for loading, 30 s for transporting to the detector and 1 min for acquiring the RLS measurement.

#### 2.3.3. Analysis of food samples

The determination of biotin in food samples requires the use of an acid hydrolysis step to release biotin, as it is mainly bound to proteins. Four food samples (chicken liver, yeast, roasted peanuts and toast bread), purchased from a local market, were analyzed. Each sample was weighed (1 g) and homogenized in water, maintaining a weight ratio of one part of food to four parts of water. The acid hydrolysis consisted on the addition of 1.5 mL of 3 M hydrochloric acid to 0.5 mL of the aqueous homogenate. The mixture was vortexed and incubated at 100 °C for 120 min. After incubation, samples were cooled to room temperature and centrifuged at 550*g* for 10 min. An aliquot (1 mL) of the supernatant was adjusted to pH 7.4 with NaOH, filtered through a 0.45 μm filter, and 100 μL of this solution were treated as described above.

## 3. Results and discussion

### 3.1. Study of the aggregation process

The aggregation of AuNPs-biotinylated liposome hybrids with avidin was studied by obtaining the synchronous spectra ($\Delta\lambda$=0 nm). Two liposome hybrids obtained using biotinyl-DOPE and biotinyl-cap-DOPE, in which "cap" is a 6-carbon spacer that avoids or minimizes potential steric hindrance, were assayed. Fig. 2 shows the synchronous spectra obtained for both AuNPs-liposome hybrids in the absence and presence of avidin. As can be seen, the best RLS signals are obtained for the biotinylated derivative that includes the spacer, which shows that it facilitates the avidin–biotin interaction. The synchronous spectra show a wavelength range of about 400–490 nm in which high RLS signals are obtained, but this spectral zone suffers of non-specific RLS signals that can be ascribed to uncontrolled phenomena associated with the emission of the light source in the spectrofluorimeter and with the potential formation of non-specific aggregates. However, these spectra also show a shoulder in the range 520–560 nm, which is ascribed to the dispersion of the AuNPs and the aggregation process. Thus, a wavelength of 525 nm was chosen to obtain the RLS measurements for the development of the affinity method.

### 3.2. Optimization of variables

#### 3.2.1. Variables affecting the synthesis and purification of AuNP-liposome hybrids

As indicated above, negatively charged AuNPs were synthesized by the Turkevich method [15] using $HAuCl_4$ and sodium citrate. These NPs were directly assayed to obtain liposome hybrids but the efficiency of the encapsulation was very low. In order to improve this efficiency, the surface of the negatively charged AuNPs was modified using DT to obtain hydrophobic AuNPs soluble in aqueous medium. The study of the optimum DT volume, in the range of 50 and 200 μL, was carried out by monitoring the corresponding absorption spectra, obtaining a maximum absorbance value at 520 nm when the DT volume was 150 μL. The mean size diameter of these NPs was 30 ± 10 nm, which was measured by obtaining TEM images.

Biotinylated liposome hybrids containing AuNPs were prepared as described above, using the RSE method in conjunction with both shake and ultrasound treatments. A previously described method [16], which was modified respecting to the type of phospholipids

and the internalized material, was used to obtain these liposomes. The study of the variables affecting the method and the values chosen are depicted in Table 1. The composition of the phospholipid mixture and the chloroform and methanol volume ratio are critical variables to obtain the liposome hybrids. The best results were obtained using a phospholipid mixture (100 μL) compose by DSPC, biotinyl-DOPE or biotinyl-cap-DOPE, and CH (75:12.5:12.5), which was diluted to 1.0 mL with a chloroform/methanol (880:120) mixture. This solution was mixed as indicated above with a solution containing 3 mL of about 0.25 mM negatively charged AuNPs, 150 μL of DT (98% w/v) and 5 mL of 25 mM phosphate buffer solution (pH 7.4).

The influence of the temperature in the RSE method was studied in the range of 30–60 °C, which covered the transition temperature of the phospholipids used. The efficiency on liposome production was evaluated using optical microscopy by recounting 100 of the liposomes and distributing them as percentage between small, large and giant unilamellar liposomes and multi-vesicular liposomes. The percentage of first type of liposomes, which are the most stable liposomes, increased up to a temperature of 40 °C, remaining constant at higher temperature values. Thus, this temperature was selected as optimal. The study of the stability of the liposome suspension showed that it increased when the suspension is refrigerated at 4 °C. Table 1 shows also the optimization of the variables involved on the mechanical shaking and sonication treatments applied to homogenize the size of the liposomes. The final result after these treatments was a liposome suspension consisting in a mixture of filled (*F*) and empty (*E*) liposomes with a significant presence of phospholipid residues.

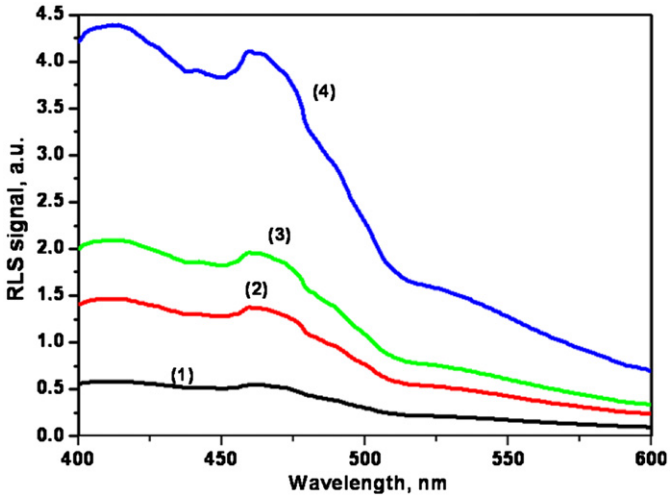


**Fig. 2.** Synchronous spectra obtained for solutions containing AuNP-biotinylated-liposome hybrids (1,3) and AuNP-biotinylated-cap-liposome hybrids (2,4) in the absence (1,2) and the presence (3,4) of avidin (80 μg $mL^{-1}$).

The purification of the liposome suspension, to separate filled liposomes from empty liposomes, phospholipid residues and un-trapped AuNPs, was initially assayed using conventional centrifugation, in which 1.5 mL of the suspension was treated at different centrifugation speeds, between 2007.3 and 20,073*g* at 4 °C, and assaying variable centrifugation times, between 15 and 30 min. A centrifugation of 20,073*g* for 30 min provided the separation between filled and empty liposomes, but the efficiency of the process was very low. This efficiency could be increased using higher centrifugation speed and time, but these experimental conditions also increased the liposome destruction. This limitation was overcome using a SDGC method in which 0.6 mL of the liposome suspension was added to a non-linear density gradient performed in 5-layers of 0.15 mL. Different density gradient profiles were performed in triplicate varying the sucrose percentage from 10% to 95% by using a 100% sucrose solution. Also, the centrifugation speed, in the range 2676.4–20,073*g*, and the centrifugation time, in the range 5–30 min were optimized. The best results were obtained for a density gradient formed by layers of 10%, 20%, 25%, 30% and 50% sucrose, with centrifugation speed at 5352.8*g* for 15 min. Liposome hybrids, which showed a reddish appearance, were located in the intermediate zone corresponding to 25% sucrose, while un-trapped AuNPs and empty liposomes appeared in the top zone and in the lowest zone, respectively.

### 3.2.2. *Variables affecting the avidin–biotin system*

As Fig. 1 shows, a flow injection system was used to automatize the measurement step of the competitive assay. Avidin, biotin and biotinylated hybrid liposomes solutions, in a final volume of 600 μL, were placed in closed vials and transported to the detector, using a phosphate buffer solution as carrier, to obtain the analytical response. Both, hydrodynamic and chemical

**Table 1**
Optimization of variables affecting the liposome formation.

| Synthesis step | Variable | Range studied | Optimal value |
|---|---|---|---|
| **Liposome formation** | AuNPs (0.25 mM), mL | 0.5–5 | 3 |
| | Phospholipids volume, μL | – | – |
| | DSPC (0.1 M) | 50–80 | 75 |
| | Biotinyl-DOPE or biotinyl-cap-DOPE (0.1 M) | 5–15 | 12.5 |
| | CH (0.1 M) | 5–15 | 12.5 |
| | DT, μL | 50–200 | 150 |
| | Buffer volume, mL | 1–20 | 5 |
| | Chloroform, μL | 600–900 | 880 |
| | Methanol, μL | 100–200 | 120 |
| | Temperature, °C | 30–60 | 40 |
| | Evaporation time, min | 5–30 | 20 |
| | Rotatory speed, rpm | 50–500 | 300 |
| **Shaking treatment** | Temperature, °C | 20–100 | 40 |
| | Time, min | 5–40 | 30 |
| | Stop cycle, min | 5–15 | 10 |
| | Stop time, min | 1–5 | 2 |
| | Shaking speed, rpm | 100–300 | 250 |
| **Ultrasound treatment** | Sonication cycle, min | 5–30 | 10 |
| | Stop cycle, min | 1–5 | 2 |
| | Stop time, s | 1–30 | 10 |

variables were optimized, as shown in Table 2, which includes the variables studied, the ranges assayed and the values chosen. All the assays were carried out at 25 °C to avoid the degradation of the liposomes at higher values and the difference in the RLS intensity obtained in the presence and absence of biotin was used as the analytical parameter.

A flow-rate of 1 mL $min^{-1}$ and an injection volume of 50 μL were chosen, which allow the transport of enough reaction plug to the detector in 30 s, obtaining a suitable RLS signal. A 25 cm length was selected as the distance between the injection valve and the detector with the minimal dispersion of the pick-up suspension. Each solution was injected 6-times and each measurement was obtained in 1 min. The optimum pH for the development of the competitive system, studied in the range of 5–10, was 7.4. A 25 mM phosphate buffer solution was used to adjust this pH value and, also, as carrier solution to transport the reactant mixture from the injection valve to the detector. The study of the optimization of the avidin concentration, in the range of 0.25–20 μM, showed that the RLS signal increased until a 5 μM concentration, remaining practically constant at higher concentration values.

**Table 2**
Optimization of variables affecting the avidin–biotin system.

| Type | Variable | Range studied | Optimal value |
|---|---|---|---|
| Hydrodynamic | Flow rate, ml $min^{-1}$ | 0.2–2 | 1 |
| | Sample injection, μL | 20–100 | 50 |
| | Length of $L_1$ reactor, cm | 20–100 | 25 |
| | Measurement time, min | 0.5–5 | 1 |
| Chemical | pH | 5–10 | 7.4 |
| | [$Na_2HPO_4$] buffer, mM | 5–50 | 25 |
| | [Avidin], μM | 0.25–20 | 5 |
| | Liposome volume, μL | 50–400 | 300 |

### 3.3. Features of the method

The calibration graph was constructed using the optimum values of the variables established in the previous section and carrying out the RLS measurements at 525 nm. Each sample was assayed using six successive injections of 50 μL each one. Table 3 summarizes the figures of merit, which include the equation parameters for the Boltzman logistic relationship [17], the dynamic range of the calibration graph, the limits of detection (LOD) and quantification (LOQ) [18], and the precision of the method, expressed as RSD% values.

The regression analysis was performed following the residual analysis method, which revealed a significant sigmoid correlation between the RLS measurement and the analyte concentration. The dynamic range of the calibration graph is 0.001–20 μg $mL^{-1}$ ($n=14$) and the $IC_{50}$ is 1.1 μg $mL^{-1}$. Calibration data were also adjusted to a linear relationship in a concentration range between 0.01 and 2.0 μg $mL^{-1}$ with a regression coefficient of 0.9899 ($n=7$).

The precision of the method, expressed as percent of the relative standard deviation (%RSD), was established using a mathematical model in which all of the regression and residual parameters were included [19]. Two sets of standard solutions ($n=6$), prepared at the minimal and maximal error zones of the calibration graph, corresponding to the $IC_{50}$ value and the quantification limit (1.1 and 0.001 μg $mL^{-1}$, respectively) were assayed, obtaining RSD% values of 2.1% and 4.9%, respectively. The estimated sampling frequency under the working conditions was 9 $h^{-1}$.

**Table 3**
Features of the method.

| Calibration graphs | Linear ($y=A+Bx$)[a] | Boltzman (logistic)[a,b] |
|---|---|---|
| Equation parameters | $A=22.9$ ($\pm 0.3$) | $A_1=23.8$ ($\pm 0.1$) |
| | $B=-8.7$ ($\pm 0.9$) | $A_2=5.77$ ($\pm 0.05$) |
| | $r^2=0.9899$ ($n=7$) | $x_0=1.1$ ($\pm 0.1$) |
| | – | $p=0.8$ ($\pm 0.2$) |
| | – | $X^2=0.0045$ |
| | – | $r^2=0.9998$ ($n=14$) |
| LOD, μg $mL^{-1}$ | 0.001 | 0.0003 |
| LOQ, μg $mL^{-1}$ | 0.01 | 0.001 |
| Linear range, μg $mL^{-1}$ | 0.01–2.0 | 0.001–20[c] |
| RSD, % | | |
| 0.00 1μg $mL^{-1}$ | –[d] | 4.9 |
| 1.1 μg $mL^{-1}$ | 3.6 | 2.1 |

[a] $y$=normalised signal; $x$=concentration (μg $mL^{-1}$.
[b] Logistic Boltzman expression: $y=A_2+[(A_1-A_2)/(1+(x/x_0)^p]$.
[c] Dynamic range for the Boltzman relationship.
[d] Uncalculated value.

### 3.4. Application of the method

The usefulness of biotinylated liposome hybrids containing AuNPs as analytical reagents has been checked by determining biotin in several food samples. Table 4 summarizes the results obtained, which are compared with the concentration range usually found for biotin in the food samples analysed. A recovery study was also carried out by adding two amounts of biotin to the samples to obtain final concentrations of 0.01 and 0.5 μg/ml. The results obtained are also shown in Table 4, in which can be seen that the recovery values range between 88.2% and 105.2%.

## 4. Final remarks

The proposed method shows for the first time the usefulness of AuNP-biotinylated liposome hybrids as enhancement reagents in a competitive binding assay for biotin determination in food samples. Although other competitive assays based on the use of biotinylated liposomes have been described [14], their practical

**Table 4**
Application of the method.

| Sample | Type | Dilution factor | Usual range values (ng $g^{-1}$)[a] | Concentration found (ng $g^{-1}$) | Recovery (%)[b] | |
|---|---|---|---|---|---|---|
| | | | | | 1st add | 2nd add |
| 1 | Yeast | 100 | 150–200 | 153.6 | 88.2 | 99.5 |
| 2 | Chicken liver | 50 | 1600–1800 | 1632 | 93.2 | 105.2 |
| 3 | Roasted peanut | 70 | 175–200 | 190.8 | 101.9 | 97.4 |
| 4 | Toast bread | 100 | 12–18 | n.q.[c] | 94.6 | 100.5 |

[a] Biotin content in food samples (http:/lpi.orst.edu/infocenter/ vitamins/biotin/biotin.html).
[b] 0.01 and 0.5 μg $mL^{-1}$ for the first and second addition, respectively.
[c] Analyte concentration lower than LOQ.

usefulness by their application to the analysis of real samples has not been shown. The use of AuNPs entrapped into biotinylated liposomes allows the measurement of RLS intensity as analytical parameter for biotin quantification, reaching a detection limit similar or better to those described using liquid chromatography or other competitive assays [9–11]. Although a batch format can be used for the method development, a flow format was chosen to automate and facilitate the repetitive measurement of a relatively high number of aliquots of a sample, using very low sample volumes, which improves the precision of the method and increases the sample throughput.

## Acknowledgements

We gratefully acknowledge financial support from the Spanish MICINN (Ministerio de Ciencia e Innovación) (Grant No. CTQ2009-08621/BQU), the Junta de Andalucía (Grant No. PO9-FQM-493) and the FEDER-FSE program.

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