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Signal-Enhancing Thermosensitive Liposomes for Highly Sensitive Immunosensor Development

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Liposomes are potential candidates as nanovesicles for the development of detection systems with improved sensitivity and detection limits, due to their capacity to encapsulate diverse types of signal enhancing molecules. An amperometric immunosensor exploiting enzyme encapsulating thermosensitive liposomes for the ultrasensitive detection of carcinoembryonic antigen (CEA) is reported. Five different bioconjugation methods to link an anti-CEA antibody to horseradish peroxidase (HRP) encapsulating liposomes were studied and compared to HRP−Ab conjugate. ζ-Potential measurements of liposomes before and after each modification method as well as following incubation with CEA were used as a tool to monitor the success of modification and probe the affinity of the liposome linked antibodies. The use of different lysing conditions (temperature vs detergent) was evaluated, with the application of temperature providing an extremely effective means of liposome lysis. Finally, thermosensitive liposomes modified using biotin−streptavidin and *N*-succinimidyl-*S*-acetylthioacetate (SATA)/sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-1-carboxylate (Sulfo-SMCC) chemistries were used to detect CEA and compared in terms of their stability, background signal, and limit of detection. Detection limits of 2 orders of magnitude lower than that obtained with the HRP−antibody reporter conjugate were obtained (0.080 ng CEA/mL and 0.0113 ng CEA/mL), with 11-fold and 9-fold amplification of signal, for the biotin−streptavidin and SATA/Sulfo-SMCC modified liposomes respectively, clearly demonstrating the powerful potential of enzyme encapsulating liposomes as signal enhancement tools.

Serum tumor markers are of increasing interest for the early diagnosis and/or follow up of cancer patients,[1,2] and the carcinoembryonic antigen (CEA) is one of the most studied biomarkers, being related to several cancer types including liver, colon, breast, and colorectal cancer.[3,4] CEA is also present in healthy tissue in very low concentrations, with the CEA serum concentration for healthy male and female nonsmokers being 3.4 and 2.5 ng·mL^{-1}, respectively, while for healthy smokers the serum concentration doubles and it is markedly higher in individuals with the specific cancer types listed previously.[4] In a recent review, CEA was shown to be highly sensitive for the monitoring of reappearance of the disease after therapy or surgery and considerably less expensive than radiology and endoscopy, which are typically used for patient follow-up.[4] A highly sensitive, cost-effective, rapid, and easy-to-use analytical tool for the monitoring of CEA levels would thus be very useful for the early diagnosis and follow-up monitoring. Immunosensors address these requirements and also facilitate analysis at the point of care, showing significant potential advantage over the laboratory based immunoassays currently used for analysis of CEA.[5,6] As depicted in Figure 1, electrochemical immunosensors exploiting enzyme labels for signal generation are analogous to their enzyme linked immunosorbent assay, with the exception that the capture antibody is immobilized directly on the surface of the transducer and signal generation is due to the oxidation/reduction of an electrochemical substrate/product of the enzyme label.[5] While there are many reports of the application of electrochemical immunosensors in clinical diagnostics, the sensitivity and achievable limit of detection is restricted by the number of enzyme labels present on the reporting antibody, which is normally one enzyme per antibody. There are an increasing number of reports demonstrating the possibility of signal amplification strategies using various approaches, including the use of inorganic nanoparticles,[7,8] liposomes,[9,10] and/or hybrid materials.[11]

Liposomes are promising candidates as molecular signal enhancement tools, coupling their capacity to encapsulate biomolecules in the aquatic core or within the lipid membrane, with

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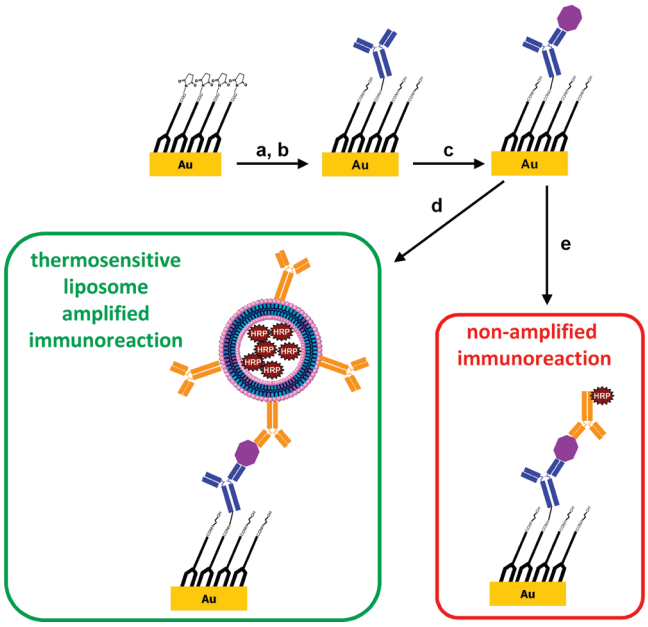

Figure 1. Liposome based sandwich immunosensor for CEA detection: (a, b) Modification of the bare gold electrode by 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide ester (DT2) and further activation of the carboxyl groups of the chemisorbed dithiol using EDC/NHS; (c) immobilization of primer antibody on electrode surface and addition of the target protein; (d) amplified immunosensor using enzyme encapsulating liposomes, (e) traditional nonamplified enzyme immunosensor.

the straightforward biofunctionalization of the phospholipid polar head groups with, for example, antibodies, facilitating the realization of multifunctional vesicles.[12] Although liposomes were first reported as signal amplifiers in immunosensors in 1980, there have been a limited number of reports detailing their exploitation for increased sensitivity and decreased detection limit.[13,14] Viswanathan et al. used potassium ferrocyanide-encapsulated and ganglioside (GM1)-functionalized liposomes for the detection of cholera toxin (CT), where the CT is detected by a "sandwich-type" assay on the electronic transducers, where the toxin is first bound to the anti-CT antibody and then to the GM1-functionalized liposome. The potassium ferrocyanide molecules are released from the bound liposomes on the electrode by lyses with methanolic solution of Triton X-100 and were able to detect as low as 10^{-16} g of cholera toxin (equivalent to 100 μL of 10^{-15} g mL^{-1}). The authors used the same concept for the detection of CEA, achieving a detection limit of 1×10^{-12} g mL^{-1}.[15,16] Ou et al. described a novel rolling circle amplification (RCA) immunoassay based on DNA-encapsulating liposomes, liposome-RCA immunoassay, exploiting antibody-modified liposomes with DNA primer probes encapsulated, which following lysis were used to initiate a linear RCA reaction, generating a long tandem repeated sequence that was detected using fluorescence. This combination of amplification via the use of liposomes followed by a secondary RCA amplification, facilitated the detection of prostate specific antigen (PSA) over a 6-decade concentration range from 0.1 fg mL^{-1} to 0.1 ng mL^{-1} with a limit of detection as low as 0.08 fg mL^{-1}.[17] In another strategy reported by the same authors, alkaline phosphatase (ALP)-encapsulated and detection antibody-functionalized liposomes were used as the reporter antibody. The model analyte PSA was first captured by anti-PSA capture antibody immobilized on the electrode and then sandwiched with the functionalized liposomes. The bound liposomes were then lysed with surfactant to release the encapsulated ALP, which served as secondary signal amplification means, and a detection limit as low as 0.007 ng mL^{-1} was obtained.[9]

In all reports, the encapsulating liposomes are lysed using lysing agents such as Triton-X; however, these surfactants can have a detrimental effect on encapsulated biological material, such as enzymes,[18,19] and potential alternatives are the use of liposomes with leakage triggered by environmental factors such as temperature or pH.[20] Thermosensitive liposomes have been widely reported for drug delivery;[21] there are, however, no reports to date of their use as tools for signal amplification, either for immunoassays or for immunosensors.

The objective of the work reported here is the development of an amperometric immunosensor exploiting enzyme encapsulating thermosensitive liposomes for the ultrasensitive detection of carcinoembryonic antigen (CEA). A method developed by our group, based on pH jump, was used to prepare the liposomes, and different bioconjugation methods including covalent and bioaffinity based interactions for the binding of these horse radish peroxidase (HRP)-encapsulating liposomes with an anti-CEA monoclonal antibody were evaluated. The different methods were applied to the detection of low levels of CEA and compared in terms of stability, background signal, and limit of detection, and the approach was demonstrated to be very effective in facilitating an enhancement in signal, with a concomitant decrease in detection limit.

EXPERIMENTAL SECTION

Reagents and Materials. Sodium dihydrogen phosphate (NaH_2PO_4; anhydrous, reagent grade, ACS), disodium hydrogen phosphate (Na_2HPO_4; anhydrous, reagent grade, ACS), sodium hydroxide, hydrochloric acid, glycerol (99.5%, reagent grade, ACS), and hydroxylamine hydrochloride were purchased from Scharlau Chemie SA. PBS-Tween (10 mM) preprepared lyophilized buffer (NaCl 0.138 M, KCl 0.0027 M) Tween 20 0.05%, L-cysteine, (97%), sodium acetate, Triton X-100, horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), glutaraldehyde, chloroform, ferric chloride ($FeCl_3 \cdot 6H_2O$), ammonium thiocyanate (NH_4SCN), sulfuric acid, hydroquinone (98%), hydrogen peroxide, dimethylsulfoxide (DMSO), maleimide activated streptavidin, maleimide activated HRP, and Sephadex G-100 were purchased from Sigma-Aldrich.

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Phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoylphosphoethanolamine (DPPE), biotin-capped-1,2-dipalmitoylphosphoethanolamine (Biot-DPPE), and lyso-palmitoylphosphatidylcholine (lyso-PPC) were supplied by Avanti Polar Lipids Inc. as powders and were used without further purification. *N*-Hydroxysuccinimide (NHS) (98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC), and 2-mercaptoethanol were purchased from Acros Organics. Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-1carboxylate (Sulfo-SMCC), *N*-succinimidyl-*S*-acetylthioacetate (SATA), Microcon nominal weight cut off centrifugal filters (YM-10, YM-30, YM-50, YM-200), and affinity column Freezyme Conjugation purification kit were supplied by Pierce Chemicals. Monoclonal antibodies (anti-CEA) and carcinoembriyonic antigen (CEA) used were provided by Fujirebio Diagnostics AB. One millimolar stock solutions of 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide (DT2) (SensoPath Technologies (Bozeman, MT)) were prepared in ethanol and stored at −20 °C.

Liposome Preparation and Purification. *Curvature-Tuned Preparation of HRP-Encapsulated Liposomes.* The liposomes were prepared using a method recently developed in our laboratories.[22] Briefly, 10 mL of 0.1 M sodium phosphate buffer solution (PBS, pH 7.4) was heated in a 15 mL glass round bottomed flask to the predetermined temperature of 45 °C. This flask was placed in a water jacket, which was connected to an UltraTerm 200 Model (P-Selecta) Thermocycler to maintain a constant temperature. From this buffer, 4 mL was taken and used to directly hydrate a 50 mg lipid mixture composed of DPPC, DPPE, and lyso-PPC with a constant molar ratio (86.1:1.9:12.0). The mixture was vortexed for ∼2 min with glass beads and then placed in a water bath to maintain the temperature. To the PBS remaining in the glass flask, 300 μL of glycerol and 20 mg of the horseradish peroxidase (HRP) enzyme were added, followed by the hydrated lipids, and the mixture was left to stir for 15 min at a constant temperature under an argon environment. The pH of the buffer was then increased to pH 11 using NaOH and immediately returned to pH 7.4 using HCl. All of the above steps were carried out under argon. The mixture was then left in the same conditions for a fixed period of time (∼25 min) before being allowed to cool to room temperature and finally was stored in the fridge at 4 °C until used.

Liposome Purification. Size exclusion chromatography (SEC) was used to physically separate the liposomes from unencapsulated HRP. A size exclusion chromatography column was prepared using Sephadex G-100. The column was left to equilibrate overnight before being washed with 100 mL of 0.1 M PBS buffer solution. The liposome solution was then added to the column, and eluate aliquots were collected for further analysis.

Liposome Characterization. *Size Measurements by Photon Correlation Spectroscopy.* The mean diameter of the liposome emulsions was measured using Zeta Sizer 3000H (laser He−Ne (633 nm), detector angle 90°) equipment from Malvern Instruments, Inc. The standard deviations were calculated from the mean of the data of a series of experiments ($n \geq 3$).

Visualization of Liposome Size and Shape Using Transmission Electron Microscopy (TEM). A drop of sample was added to a 200-mesh copper grid with a thin film of Formvar polymer and left at room temperature until a dried film was obtained. TEM analyses were performed using a Transmission Electron Microscope JEOL 1011 operated at 80 keV with an ultrahigh-resolution pole piece providing a point resolution of 2 Å. Micrographs 1024 pixels × 1024 pixels in size were acquired using a Megaview III multiscan-CCD camera. Images were analyzed by iTEM image analysis platform.

ζ-Potential Measurements. ζ-Potential measurements were carried out to characterize the change in the surface charge of the liposomes before and after modification, as well as following addition of CEA as a means of monitoring the affinity of the anti-CEA−liposome linked antibody for CEA after modification. Liposome samples were diluted in 2 mL of Milli-Q water, subsequently incubated for 30 min at room temperature, and finally analyzed after purification from nonattached proteins.

Evaluation of Lipid Content Using Stewart's Method.[23] In order to measure the lipid content, a small amount of each sample (50 μL) was taken and mixed with 500 μL of chloroform (to break up the liposomes into individual lipids) and 1 mL of ammonium ferrothiocyanate, which binds to the phosphoryl group of the lipids, leading to the formation of a pink colored complex. The samples were then vortexed and centrifuged at 1000 rpm for 10 min, and the absorbance of each aliquot was measured at 496 nm using a Cary UV−vis spectrophotometer, and using a previously prepared calibration curve, the lipid contents were estimated.

Liposome Modification. *Conjugation of Biotin-Capped-DPPE Liposomes with Streptavidin Coupled Anti-CEA (Method I).* First, liposomes were prepared using biotin-capped-DPPE in the place of DPPE. Anti-CEA monoclonal antibody (1 mg) was dissolved in 0.1 M PBS (pH 7.4) to a final volume of 1500 μL. A 10 μL aliquot of a solution containing 1 mg of SATA was then dissolved in 100 μL of dried DMSO and added to the antibody solution, and the mixture was gently stirred for 30 min at room temperature, protected from light. A deacetylation solution (100 μL) consisting of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl (pH 7.2) was added to the SATA−antibody mixture and allowed to react for 2 h at room temperature, again protected from light. Subsequently, 1 mg of maleimide activated streptavidin was added to the SATA modified antibody in a 1 to 5 molar ratio of Ab to lipid, and the solution was incubated for 2 h at 37 °C. To deactivate unconjugated maleimide groups, an aliquot of 2-mercaptoethanol was added to the solution to a final concentration of 0.15 mM and stirring continued for 15 min. Finally, the conjugate was purified and concentrated by passing through a YM-50 nominal weight cutoff filter, and the conjugate washed three times with 0.1 M PBS (pH 7.4). The protein concentration was calculated by measuring the absorbance at 280 nm and using the relationship of [Protein] mg/mL = $A_{280} \times 1.38$. The mixture was left to incubate for 2 h at 4 °C under continuous stirring, and the resulting conjugate was passed through a YM-100 nominal weight cutoff filter and stored at 4 °C.

SATA/Sulfo-SMCC (Method II). First, maleimide activated liposomes were prepared by adding 40 μL of Sulfo-SMCC (4.8 mg/mL in PBS pH 7.4) to a 1 mg/mL liposome solution and incubating for 2 h at 4 °C. Meanwhile, anti-CEA monoclonal antibody (1 mg) was dissolved in 0.1 M PBS (pH 7.4) to obtain a final volume of 1500 μL. A 10 μL aliquot of a solution containing

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1 mg of SATA was then dissolved in 100 μL of dried DMSO and added to the antibody solution, and the mixture was gently stirred for 30 min at room temperature, protected from light. A deacetylation solution (100 μL) consisting of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl (pH 7.2) was added to the SATA−antibody mixture and allowed to react for 2 h at room temperature, again protected from light. The resulting solution was purified using YM-50 nominal weight cutoff filter, and the protein concentration was again measured by absorbance. Subsequently, thiol-activated anti-CEA monoclonal antibody and maleimide activated liposomes were again mixed with a molar ratio of 1:5, and the solution incubated for 2 h at 4 °C. To deactivate unconjugated maleimide groups, an aliquot of 2-mercaptoethanol was added to the solution to a final concentration of 0.15 mM and gentle stirring continued for 15 min. Finally, the conjugate was passed through a YM-100 nominal weight cutoff filter and was washed three times with 0.1 M PBS (pH 7.4).

Modification Using NHS and EDC (Method III). In this method, 1 mg of anti-CEA monoclonal antibody was diluted in 1 mL of acetate buffer (pH 5), and an excess of NHS and EDC dissolved in PBS was added. The mixture was kept at 4 °C, under stirring conditions for 30 min, and excess unbound NHS/EDC was then removed using a YM-50 nominal weight cutoff filter; the antibody concentration was measured using absorbance. The liposome buffer was exchanged from PBS (pH 7.4) by centrifuging for 10 min at 10 000 rpm and resuspending the pellet (liposomes) in 1 mL of acetate buffer (pH 5). The lipid content was measured as previously described, and the liposomes were suspended in acetate buffer, mixed with activated antibody again in a 1:5 mol/mol ratio, and left to incubate overnight at 4 °C. Modified liposomes were then purified using a YM-100 nominal weight cutoff filter, and the lipid content was again estimated.

Sodium Periodate Method (Method IV). In this method, in the first step, monosaccharide residues in the antibody are oxidized with periodate to produce aldehyde groups, and in the second step, the aldehyde groups are allowed to react with amino groups in the liposomes; finally, the Schiff bases formed are reduced. In more detail, the anti-CEA monoclonal antibody (1 mg) was dissolved in 250 μL of water, and 50 μL of freshly prepared sodium periodate (100 mM) was added and the solution stirred for 20 min at room temperature, protected from light. The activated antibody was then dialyzed overnight against 1 mM acetate buffer, pH 4.4. Meanwhile, the liposome buffer solution was exchanged by centrifugation as previously described, and the liposome pellet was reconstituted in 10 mM sodium carbonate buffer. The pH of the dialyzed antibody solution was adjusted to pH 9.0 by addition of 0.2 M sodium carbonate buffer (pH 9.5), and the liposome solution was immediately added, again at a 1:5 (mol/mol) lipid to antibody ratio, and the mixture stirred for 2 h at room temperature. Freshly prepared sodium borohydride solution (25 μL of a 4 mg/mL solution) was then added, and the mixture again stirred for 2 h, this time at 4 °C. The modified liposomes were then purified using a YM-100 nominal weight cutoff filter.

Glutaraldehyde Method (Method V). In this method, liposomes are first activated using excess glutaraldehyde. The liposome−glutaraldehyde mixture was then left at room temperature (25 °C) for 10 min before being passed through a YM-100 nominal weight cutoff filter to remove excess glutaraldehyde. The liposomes activated with glutaraldehyde were then mixed with the anti-CEA monoclonal antibody again at a 1:5 (mol/mol) lipid to antibody ratio. Antibody stock (2.5 mg/mL) was diluted in 0.1 M PBS to give a final concentration, which was dependent on liposome lipid content. Liposome−antibody mixtures were incubated overnight at 4 °C.

Preparation of Horseradish Peroxidase (HRP)−Anti-CEA Conjugate.[24] Horse radish peroxidase (HRP) was conjugated to the reporter anti-CEA monoclonal antibody using the maleimide activation method described in Method I above, replacing the streptavidin with the HRP. Purification of the resulting conjugate was carried out with the metal chelate affinity chromatography Freezyme Conjugation purification kit. Finally, the pure conjugate was concentrated by passing through a YM-50 nominal weight cutoff, and the conjugate was washed three times with 0.1 M PBS (pH 7.4).

Electrochemical Instrumentation. All the electrochemical measurements were performed using a PGSTAT12 potentiostat (Autolab) controlled with the General Purpose Electrochemical System software, with built-in frequency response analyzer FRA2 module. A three-electrode configuration of Ag/AgCl-3 M NaCl as a reference (CH Instruments., model CHI111), Pt wire as a counter (BAS model MW-1032), and bare or modified Au (BAS model MF-2014, 1.6 mm diameter) as working electrode was used.

Electrode Preparation. Bare gold electrodes were thoroughly cleaned prior to surface modification. The gold surface was polished with alumina powder of 0.3 μm and sonicated in ethanol and Milli-Q water twice for 5 min each. The electrodes were then placed in cold Piranha's solution (1:3 v/v H_2O_2 to H_2SO_4) for 5 min and once again sonicated twice in ethanol and Milli-Q water for 5 min each. (*Caution: Piranha's solution is highly corrosive and violently reactive with organic materials; this solution is potentially explosive and must be used with extreme caution).* The electrodes were dried using nitrogen and electrochemically cleaned in 0.5 M H_2SO_4 solution via cyclic voltametry for 40 cycles at 10 mV/s and characterized in 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl (pH 7.2). Clean bare electrodes were incubated with 1 mM of 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid *N*-hydroxysuccinimide ester for 3 h at room temperature. The carboxyl groups of the chemisorbed dithiol were activated by with a mixture of 200 mM EDC and 50 mM NHS solution for 40 min. Three micrograms per milliliter of the anti-CEA capture antibody solution in 10 mM acetate buffer (pH 5.0) was then added to the washed electrode and left for 2 h at room temperature. Active ester sites were blocked by incubating the modified electrodes in 10 mM ethanolamine solution in PBS (pH 8.0) at room temperature for 30 min. CEA analyte solutions were prepared in 0.1 M PBS (pH 7.4), and electrodes were incubated in these solutions for 30 min before their incubation with secondary anti-CEA-liposome or anti-CEA-HRP solutions for 40 min at room temperature. Electrode regeneration was carried out treating the electrodes in 10 mM glycine solution (pH 2.5) for 5 min.

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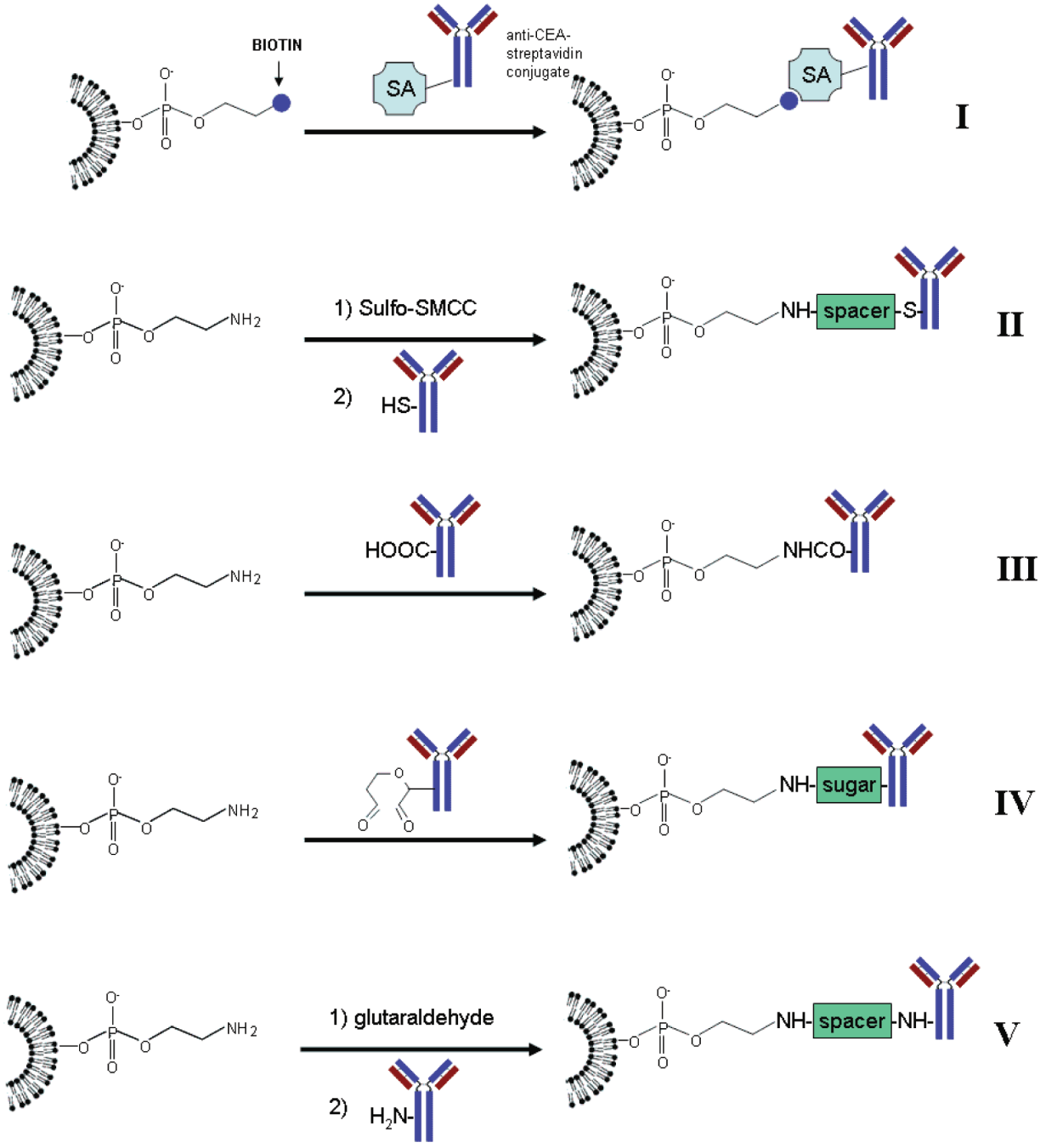
Scheme 1. Flow Scheme of Modification Methods Studied



RESULTS AND DISCUSSION

Characterization of Antibody Modified Liposomes. A known lipid formulation was used to prepare thermosensitive liposomes. Depending on the concentration of DPPE/biotin-capped DPPE used in the formulation, the liposome size was found to be tunable (results not shown). In this study, an optimized formulation resulting in a monodisperse population of liposomes with diameter of 200 ± 13 nm was used (Figure 1). The prepared liposomes were modified with anti-CEA using five different modification methods: the first method exploiting the use of biotin-capped DPPE in the formulation and subsequent attachment to the streptavidin linked anti-CEA antibody and the other four being based on the covalent binding of the anti-CEA antibody to the amine group of the DPPE lipid using different functionalities and activation methods (Scheme 1). The modification methods were compared in terms of the size, shape, and stability of the resulting liposome−anti-CEA complexes, as well as for their affinity for the CEA antigen.

As a means of characterization, transmission electron microscopy (TEM) was carried out on the liposomes before and after modification to probe the shapes formed (e.g., aggregates of liposomes). The liposomes modified with glutaraldehyde were observed to be shapeless and bulky with a significantly increased size of the vesicles (see Figure 2e) while all other methods resulted in evenly shaped liposomes with an increased bilayer thickness from 4 to 12 nm and an increase in the diameter in the order of 16−20 nm, as exemplified in Figure 2 where the modified liposomes resulting from Methods I−V are shown. Glutaraldehyde links amine groups, and it is highly probable that a polymer of

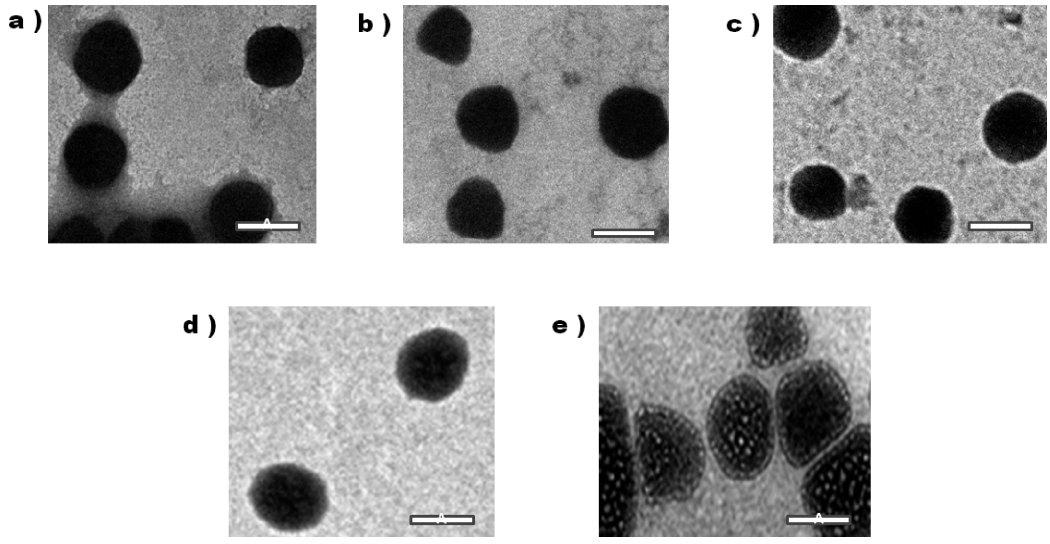


Figure 2. TEM images of liposomes modified using Methods (a) I, (b) II, (c) III, (d) IV, and (e) V. Scale bar 200 nm.

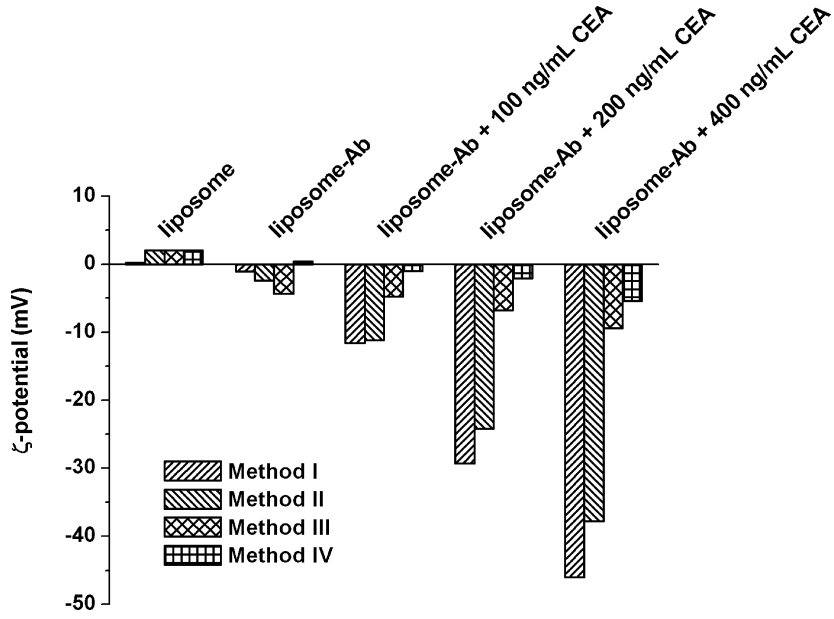


Figure 3. ζ-Potential values of liposomes biofunctionalized using Methods I, II, III, and IV. Measurements were obtained before and after modification with anti-CEA and following incubation with increasing concentrations of CEA.

liposomes were formed with liposomes being conjugated to each other and not to the antibody. In the other methods, differential functional groups are linked, e.g., maleimide with thiol and amine with carboxyl, avoiding this problem.

For each method, the modification was monitored with ζ-potential measurements in order to see the effect of each layer on the total charge of the particle surface. In Figure 3, results obtained with the antibody functionalized liposomes prepared according to Methods I, II, III, and IV are shown, and as can be seen, following functionalization with the anti-CEA monoclonal antibody, a decrease in the ζ-potential was obtained. Following incubation with the target antigen CEA, with increasing concentrations of the CEA, there was a shift in ζ-potential to more negative values. This is an expected result since proteins are negatively charged at a pH higher than their p*I* value. In this case, the highly acidic nature of CEA leads to a negative charge at pH 7.4, resulting in a change on the surface of the liposomes which increases depending on the concentration of CEA localized on the surface. These results demonstrated the great potential of ζ-potential measurements to be used as a versatile technique, not only confirming the functionalization of the liposomes with antibody and subsequent binding of the target antigen but also providing comparative data for the evaluation of the different methods. From the ζ-potential results, Methods I and II are the preferred methods for biofunctionalization of the liposomes, despite the fact that lower levels of antibodies appear to be linked to the surface, presumably due to a preferred orientation of the antibody for binding to the target antigen.

Temperature vs Detergent Triggered Enzyme Release. Triton-X100 is one of the most commonly used detergents for breaking the vesicle membrane and promoting the leakage of the encapsulated material from the liposomes. However, Triton X-100 is known to have a denaturing effect on enzymes, having a pronounced effect on their activity.[18]

As an alternative to the use of detergents to lyse liposomes, the use of thermosensitive liposomes (DPPC/DPPE/lyso-PPC =

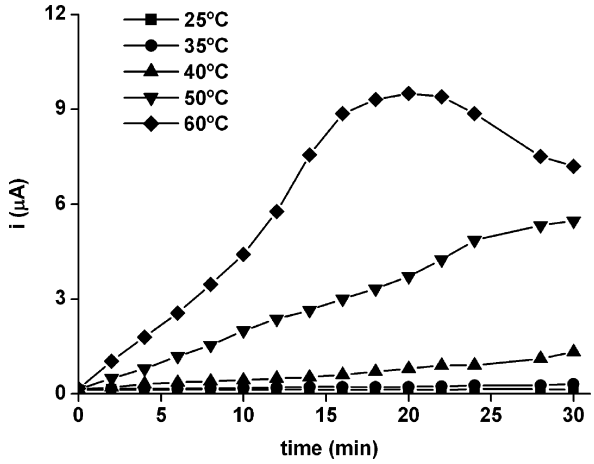


Figure 4. Analysis of thermo-sensitivity and release kinetics of biofunctionalized liposomes using amperometry prior to their modification.

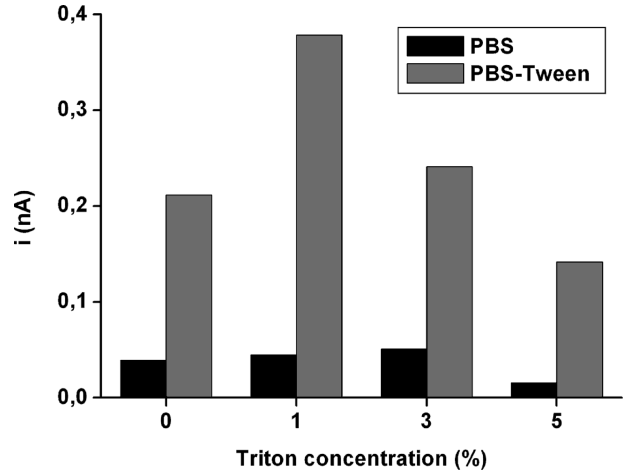


Figure 5. Time dependence of the amperometric signal for the HRP release from the liposomes prepared by Method I at increasing concentrations of Triton X-100 in PBS (10 mM) and PBS-Tween (10 mM).

86.1:1.9:12.0 mol/mol) was evaluated, and the release capacities of the prepared liposomes after biofunctionalization with Method I were analyzed at different temperatures. Amperometric measurements were carried out with modified electrodes after their incubation with liposomes at room temperature before and after the addition of a mixture of H_2O_2 and hydroquinone (HQ), followed by incubation at different temperatures, measuring the changes in current over 30 min. As can be seen in Figure 4, the highest signal was achieved at about 20 min at 60 °C, and a decrease in signal was obtained at longer time periods due to decreased activity of enzyme. These results are as expected as the T_M of the thermosensitive liposomes is 41 °C, and they are expected to be stable below this temperature, which is clearly reflected in Figure 4 where no or negligible release of HRP is observed until the temperature is elevated to 50 °C.

Control studies monitoring HRP activity colorimetrically were carried out with free HRP to see if the HRP is still active at 60 °C over time, and the results showed that the HRP had conserved 98% of its activity compared to that at 25 °C for 20 min while a decreasing activity was measured at increasing incubation times, in agreement with amperometric studies.

In order to compare to lysis using Triton X-100, amperometric measurements were carried out with increasing concentrations of Triton X-100 (0, 1, 3, and 5% v/v) in PBS (10 mM, pH 7.4) or PBS-Tween (10 mM, pH 7.4) and the signal was recorded over time following addition of TMB and hydrogen peroxide. It is expected that the presence of Tween should assist the lysis of the liposomes and as expected can be seen in Figure 5; the signal obtained is consistently higher in PBS-Tween. However, the signal obtained in all cases is markedly lower than that obtained using elevated temperature, and furthermore, increasing the concentration of the Triton X-100 results in decreasing signals. Control experiments monitoring HRP activity colorimetrically with increasing concentrations of Triton X-100 were in agreement with the electrochemical studies, demonstrating a possible denaturation of the released HRP, further highlighting the usefulness of thermosensitive liposomes for the encapsulation of enzymes for signal enhancement.

Evaluation of Signal Enhancement Using Biofuntionalized Thermosensitive Liposomes. HRP encapsulating liposomes modified with anti-CEA monoclonal antibody were analyzed in terms of their capacity to recognize the CEA target and amplify the signal. The various modification methods studied were compared to each other, as well as to a covalent HRP−anti-CEA conjugate. Recently, we have reported on the preparation of highly stable self-assembled monolayers of dithiols enabling rapid chemisorption of proteins on gold surfaces.[25,26] In this work, the same methodology was used to prepare monolayers of the anti-CEA capture antibody on dithiol-modified electrodes (see Figure 1). Gold electrodes modified with DT2 covalently linked to a catching anti-CEA primary antibody were incubated with 18.75 ng/mL CEA for 30 min and subsequently incubated with 20 μg/mL of the biofunctionalized liposomes, followed by elevating the temperature to 60 °C for 20 min. The electrochemical signal was measured before and after heat treatment. Following heat treatment, the solution was allowed to cool down to room temperature before the electrochemical measurement was carried out. A control experiment was also carried out to test the effect of the temperature on CEA, where a range of concentrations of CEA alone was heated to 60 °C for 20 min and was then measured using sandwich enzyme linked immunosorbent assay (ELISA), and the response was obtained compared with that of CEA that had not being heated; no difference in signal was observed.

As can be seen in Figure 6, Methods I and II yielded the highest amplification factors with liposomes modified according to Method I demonstrating an 11-fold signal enhancement as compared to the HRP−anti-CEA conjugate and those modified according to Method II providing a 9-fold signal enhancement. This is in excellent agreement with the ζ-potential measurements shown in Figure 3. The reason for the superior performance of the biofunctionalized liposomes obtained using Methods I and II can be attributed to the conjugation methods used. As can be seen from Scheme 1, EDC/NHS (Method III) and periodate (Method IV) based methods are two-step methods, with the first step involving the activation of antibody and the second step involving the mixing of the activated antibody with liposomes. For the

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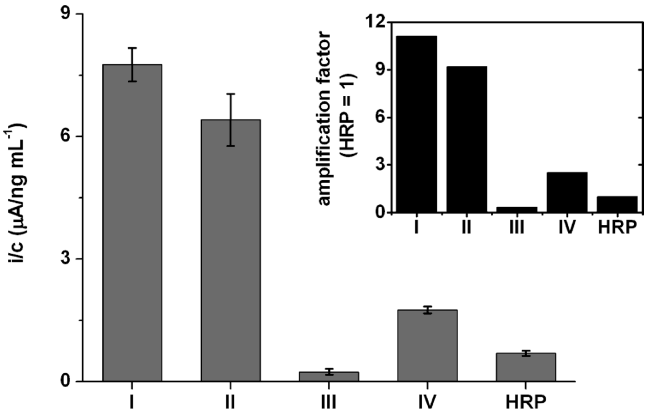

Figure 6. Effect of modification method: Signal enhancement capacities of anti-CEA modified liposomes prepared by Methods I–IV in the amperometric detection of 18.75 ng/mL CEA. The amplification factors are shown in the inset.

Table 1. Analytical Parameters Obtained for the Detection of CEA Using Anti-CEA Functionalized Liposomes and HRP–Anti-CEA Conjugate

	sensitivity (μA·mL/ng·cm^2)	EC_{50} (ng/mL)	LOD (ng/mL)
method I	7.765	17.96	0.080
method II	6.412	18.25	0.113
HRP–anti-CEA	0.697	4.416	2.159

antibody–liposome conjugates based on the bioaffinity of biotin–streptavidin and the covalent interaction via SATA/sulfo-SMCC, the bioconjugation process was carried out in three steps, where the liposomes were activated with one reagent while the antibody was separately activated with another reagent, followed by mixing of the activated moieties to form the conjugating bridge between liposomes and antibodies. The advantage of this approach is that the linkage between either SATA/sulfo-SMCC or biotin–streptavidin cannot be formed if one component is not present, and this protects against undesired cross-linking reactions, such as liposome–liposome and antibody–antibody self-conjugation.

Table 1 reports the analytical parameters, and Figure 7 shows the calibration curves obtained for the detection of CEA using liposomes prepared by Methods I and II and HRP–anti-CEA conjugate. As can be seen in Figure 7, linear dynamic ranges of CEA concentrations in the ranges of 0.59–37.5 and 1.17–37.5 ng·mL^{-1} were achieved using Methods I and II with R^2 values of 0.989 and 0.992, respectively. The limit of detection (LOD) values calculated from a linear regression equation using three times the standard deviation of blanks were 0.080 and 0.113 ng·mL^{-1}, for Methods I and II, respectively, which are significantly lower than that obtained with the HRP–anti-CEA conjugate. In addition, the electrochemical assay sensitivities obtained with the liposomes are improved in more than 1 order of magnitude with respect to the covalent HRP conjugate (Table 1).

Stability of Biofunctionalized Liposomes. The stability of the anti-CEA–liposome conjugates modified by Methods I and II were monitored over 30 days of storage in PBS (0.1 M, pH 7.4) at 4 °C in terms of change in electrochemical signal at CEA concentration of 18.75 ng·ml^{-1} and their size. Although there was slight increase in the liposome diameter (~4–5 nm), there was no significant change in the electrochemical signal (1.35% for Method I and 1.65% for Method II).

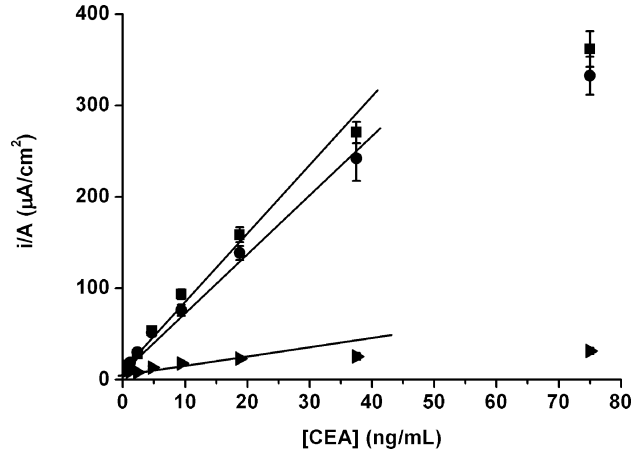

Figure 7. Amperometric calibration curves for the detection of CEA using liposomes modified via SATA/sulfo-SMCC (●) and biotin–streptavidin based methods (■). The calibration curve obtained with HRP–anti-CEA conjugate (arrowhead) is also shown for comparison.

CONCLUSIONS

An amperometric immunosensor using enzyme encapsulating thermosensitive liposomes for signal enhancement for the ultrasensitive detection of carcinoembryonic antigen (CEA) is reported. The biotin–streptavidin (Method I) and SATA-Sulfo-SMCC (Method II) approaches were demonstrated to be the most suitable biofunctionalization methods of the five methods studied for the modification of liposomes with anti-CEA Ab in terms of their stability, shape, and signal enhancement capacity. The ζ-potential of the prepared liposomes was measured before and after modification, and the change in the total surface potential was demonstrated to be useful for monitoring of the modification success or antigen capturing capacity of the liposome-linked antibodies. Heating thermosensitive liposomes to an optimized temperature rather than lysing with Triton X-100 facilitated a higher level of enhancement, maintaining the activity of the encapsulated enzymes. The use of thermosensitive liposomes modified using Methods I and II for amperometric detection of carcinoembryonic antigen provided 11- and 9-fold signal amplification, with significantly decreased detection limits as compared to HRP–anti-CEA-Ab, clearly demonstrating the potential of these nanotools for signal amplification.

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