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Investigation of NeutrAvidin-tagged liposomal nanovesicles as universal detection reagents for bioanalytical assays

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Abstract

Ligand-tagged liposomes, obtained by covalent conjugation of ligands to the liposomal surface, have been widely used as detection reagents in bioanalytical assays. A non-covalent conjugation method where IgG was attached to protein G-tagged liposomes has been recently utilized. To enlarge the application of non-covalent methods to a greater variety of ligands, including peptides, proteins, and nucleic acids, we developed and optimized a new method for the preparation of NeutrAvidin-tagged liposomes with subsequent attachment of biotinylated ligands. Two assays were used to investigate the feasibility of NeutrAvidin-tagged liposomes. The first assay was a competitive immunoassay for detecting rabbit antibodies, while the second assay was a sandwich hybridization assay for detecting a synthetic target: a DNA fragment of *Erwinia amylovora*. To produce the immunoliposomes for the detection of rabbit IgG, NeutrAvidin was covalently tagged to the liposomal surface at four different starting molar percentages (0.1, 0.2, 0.4, and 0.8). The biotinylated goat anti-rabbit IgG at three different molar ratios of biotin to IgG (5, 10, and 20) were then attached to the NeutrAvidin-tagged liposomes by using two different molar ratios of goat anti-rabbit IgG to NeutrAvidin (1 and 5). After the comparison of all 24 combinations, the best result was obtained with the 0.1 starting molar percentage of NeutrAvidin, 20 as the molar ratio of biotin to goat IgG, and 1 as molar ratio of IgG to NeutrAvidin. Under these optimized conditions, the limit of detection (LOD) for rabbit IgG was 38 pmol/mL. Moreover, the best combination for the sandwich hybridization assay was with the 0.1 starting molar percentage of NeutrAvidin-tagged liposomes and when the molar ratio of biotinylated reporter probe to NeutrAvidin was equal to 1. The LOD for the synthetic target DNA fragment of *E. amylovora* was ca. 30 pmol/mL. Both assays could be completed in about 30 min without the requirement of sophisticated equipment or techniques. Therefore, these two assays have successfully demonstrated the feasibility of NeutrAvidin-tagged liposomal nanovesicles as a universal reagent for the attachment of different types of biotinylated ligands in a fast and easy coupling process. In addition, these ligand-tagged liposomes have the potential for wide use in different types of bioanalytical assays.

Keywords: Antibody; Biotin; NeutrAvidin; Nucleic acid; Liposomal nanovesicles; Liposomes

1. Introduction

A bioanalytical assay is a procedure to identify and quantify a biological substance based on a specific, functional, or biological response to a test. Therefore, bioanalytical assays can be applied in various fields for different purposes. For example, they have been utilized for investigating the presence of contaminating microorganisms [1], toxicants [2], or allergens [3–5] in food samples including drinking water. In ecotoxicology, bioanalytical assays are used for measuring the level of environmental pollutants, such as polychlorinated biphenyls (PCBs) in water [6], cadmium in soil [7], and benzene in air [8]. Additionally, in clinical diagnosis, the measurement of cytokines in plasma is one of the most sensitive and specific infection indicators in newborns with neonatal sepsis [9].

Bioanalytical assays can be classified into two major types: protein-based and nucleic acid-based. Examples of protein-based methods include: enzyme-linked immunosorbent assay (ELISA), radio immunosorbent assay (RIA), and immunoblotting. These protein-based methods usually entail

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the specific interaction between antibodies and antigens [10]. The second group of assays, nucleic acid-based methods, are applied to detect a specific sequence of DNA or RNA. First, the targeted sequence is amplified by polymerase chain reaction (PCR), reverse transcription (RT)-PCR, or nucleic acid sequence-based amplification (NASBA). After amplification, amplicons are identified by capillary sequencing analysis, agarose-gel electrophoresis with ethidium bromide staining, or hybridization with specific probes [11].

Detection reagents in bioanalytical assays are used for quantifying the assay results, and can be produced by conjugating markers to analytes in a competitive immunoassay, to antibodies in a sandwich immunoassay [12], or to a nucleic acid probe in a hybridization assay [13]. The first marker molecules applied in binding assays were radioisotopes, because of their inherent sensitivity. However, due to their short half-life (P^{32}, 14.3 days; I^{125}, 60 days), potential health hazards and waste disposal problems, radioisotope labels were gradually replaced by either enzymes or fluorescent labels [14]. Alkaline phosphatase (AP) [15] and horse-radish peroxidase (HRP) [16] are the most frequently used enzyme labels, while fluorescein [17] and rhodamine [18] are examples of common fluorescent probes. In conventional bioanalytical assays, a detection reagent is conjugated to a few molecules of enzyme or fluorescent probe. In this study, the liposome label replaces those traditional methods since liposomes can encapsulate hundreds of thousands of molecules of the red dye marker, sulforhodamine B (SRB) in its aqueous cavity, which allows the assay results to be visually and immediately detected without further processing [19,20]. In addition, liposomes are very stable [21] and a variety of ligands can be conjugated to the liposomal surface, making liposomes versatile detection reagents in bioanalytical assays [22].

Methods for producing ligand-tagged liposomes can be classified into three groups: direct insertion, covalent conjugation, and non-covalent conjugation. The first group incorporates amphiphilic ligands, such as gangliosides into the bilayer membrane during the preparation of liposomes [2,23] or by post-insertion with the preformed liposomes [24,25]. In the second group, ligands are covalently conjugated to the liposome surface. For example, the maleimide derivatives on the liposomal surface react with thiol groups of ligands to produce a thioether bond [26], and the *N*-hydroxysuccinimide (NHS) ester derivatives of phospholipids are coupled with ligands through an amide bond [27]. Another example is periodate-oxidized liposomes that contain glycolipid moieties and are used to conjugate proteins or amine-containing ligands through reductive amination [28]. The third group of methods uses non-covalent conjugation, in which the ligand attachment is by specific recognition, such as nucleic acid hybridization [29], protein A or G binding to the Fc fragment of antibodies [30], or avidin to biotinylated molecules [31,32]. The capture molecules (nucleic acids, protein A/G, or avidin) are pre-attached to the liposomal surface and therefore can actively capture target ligands (nucleic acids, antibodies, or biotinylated molecules, respectively) on the liposomal surface.

Biotin is a naturally occurring vitamin with a molecular weight of 244 Da. Due to its relatively small size, biotin can be conjugated to a variety of ligands including carbohydrates, peptides, proteins, antibodies, or DNA/RNA without significantly altering their structure or biological function [33–35]. There are three commonly used biotin-binding proteins: avidin, streptavidin, and NeutrAvidin. Avidin (~67 kDa) is a glycoprotein found in egg white with a basic isoelectric point (p*I*) of 10–10.5 [36]. However, avidin has an issue of non-specific binding due to its content of carbohydrate groups and basic p*I* [37]. Streptavidin (60 kDa) is isolated from *Streptomyces avidini* with no carbohydrate content and has a mildly acid p*I* of 5 [38]. NeutrAvidin (~60 kDa) is produced by deglycosylation of avidin without losing biotin-binding affinity [39]. Due to the absence of carbohydrate content and its near neutral p*I* (6.3), NeutrAvidin has reduced non-specific binding, resulting in an improvement in assay sensitivity [40]. Therefore, in this study NeutrAvidin was bound to the liposomal surface for the production of ligand-tagged liposomes.

Two different bioanalytical assays were used to investigate the feasibility and performance of NeutrAvidin-tagged liposomes. One is a competitive lateral flow immunoassay for the detection of rabbit IgG performed by attaching biotinylated goat anti-rabbit IgG to NeutrAvidin-tagged liposomes and by immobilizing rabbit IgG on the test line of the nitrocellulose (NC) membrane test strips. Rabbit IgG in the sample competed with rabbit IgG coated on the test line. Therefore, the signal intensity of test lines is inversely proportional to the amount of rabbit IgG in the sample. The other example is a sandwich hybridization lateral flow assay for the detection of synthetic targets of a DNA fragment of *Erwinia amylovora*, a fire blight pathogen causing a destructive bacterial disease of apples and pears by killing blossoms, shoots, limbs, and sometimes, entire trees [41]. In this assay, the reporter probe biotinylated at the 5′ end was conjugated to NeutrAvidin-tagged liposomes, while the biotinylated capture probe was immobilized with streptavidin on the test line of previously prepared test strips. In this sandwich hybridization assay, the signal intensity of test line is directly proportional to the concentration of the target DNA in the sample.

In this work, we optimized the preparation of immunoliposomes with four different starting molar percentages (0.1, 0.2, 0.4, and 0.8) of NeutrAvidin on the liposomal surface, three different molar ratios of biotin to IgG (5, 10, and 20), and two different molar ratios of IgG to NeutrAvidin (1 and 5). After optimization, we successfully demonstrated the feasibility of using NeutrAvidin-tagged liposomes as universal reagents for the facile preparation of detection reagents in bioanalytical assays, as exemplified by the anti-rabbit IgG–NeutrAvidin-liposomal nanovesicles in a competitive immunoassay and DNA probe-NeutrAvidin-liposomal nanovesicles in a sandwich hybridization assay.

2. Experimental

2.1. Materials

EZ-Link Sulfo-NHS-LC-LC-Biotin, EZ-Link maleimide activated NeutrAvidin, *N*-succinimidyl-S-acetylthiopropionate (SATA), hydroxylamine hydrochloride, *N*-ethylmaleimide (NEM), and Blocker Casein were purchased from Pierce (Rockford, IL). Dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylglycerol (DPPG), and the Mini Extruder were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). HiFlow Plus 120 NC membranes were obtained from Millipore (Bedford, MA). Polycarbonate (PC) membranes of 0.2 and 0.4 μm pore-size were from Whatman International Ltd. (Maidstone, UK). Rabbit IgG and goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Predator (polyethersulfone) membranes were obtained from Pall/Gelman Company (Port Washington, NY). All general chemicals and buffer reagents were purchased from Sigma (St. Louis, MO). Organic solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). The synthetic target and biotinylated probes were synthesized by Operon Biotechnologies Inc. (Huntsville, AL).

2.2. Preparation and characterization of NeutrAvidin-tagged liposomes

Liposomes were prepared by a hydration/(freezing and thawing)/extrusion method [1]. First, DPPE–ATA was prepared by conjugating DPPE to SATA as described previously [20]. The mixture of DPPC, DPPG, cholesterol, and DPPE–ATA in a molar ratio of 45.4:4.5:46:4 was dissolved in a solution of 6 mL chloroform, 1 mL methanol, and 0.5 mL DPPE–ATA, and dried in a rotary evaporator. The dried lipid film was hydrated by the addition of 3 mL of 0.15 M SRB solution (in 0.02 M HEPES, pH 7.5, osmolality 535 mmol/kg). The lipid solution was processed with 5 freeze/thaw cycles, and then extruded through 0.4 and then 0.2 μm pore-size PC membranes. Unencapsulated SRB was removed by gel filtration using a Sephadex G-50 column with Tris-buffered saline (TBS: 0.02 M Tris with 0.15 M NaCl, 0.01% NaN_3, pH 7.5) containing sucrose (osmolality 535 mmol/kg).

NeutrAvidin-tagged liposomes were made by conjugating EZ-Link maleimide activated NeutrAvidin to sulfhydryl liposomes, produced from ATA liposomes by deprotection with hydroxylamine, with the starting mol% of NeutrAvidin to surface lipids of 0.1, 0.2, 0.4, and 0.8. Each reaction was incubated overnight at 4 °C. After quenching the reaction with 1 M NEM for 4 h at room temperature, NeutrAvidin-tagged liposomes were separated from unbound NeutrAvidin on a Sepharose CL-4B column with Tris-buffered saline (0.02 M Tris with 0.15 M NaCl, 0.01% NaN_3, pH 7.5, osmolality 535 mmol/kg).

The phospholipid concentration of the liposomes was determined by Bartlett's phosphorus assay [42], and the size of the liposomes was measured by laser diffraction particle size analysis in an LS particle size analyzer (Coulter Scientific Instruments, Hialeah, FL). The Bio-Rad protein assay was run to determine the final protein concentration on the liposomes by using EZ-Link maleimide activated NeutrAvidin as the standard. Due to the background signal from liposomes in the Bio-Rad protein assay, untagged liposomes were also analyzed at the same lipid concentration as the tagged liposomes. The signal of untagged liposomes was subtracted from that of NeutrAvidin-tagged liposomes to give a net signal for the attached NeutrAvidin ($[\text{NeutrAvidin}]_a$). The conjugation efficiency of NeutrAvidin to the liposomal surface was calculated from the following equation:

$$\frac{[\text{NeutrAvidin}]_a \times 100}{[\text{NeutrAvidin}]_0} = \text{conjugation efficiency (\%)}$$

where $[\text{NeutrAvidin}]_0$ is the starting amount of NeutrAvidin and $[\text{NeutrAvidin}]_a$ is the amount of attached NeutrAvidin.

2.3. Biotinylation of IgG and characterization of biotinylated IgG

The 10 mM EZ-Link Sulfo-NHS-LC-LC-Biotin solution was prepared immediately before use since the NHS ester is easily hydrolyzed to become non-reactive. The IgG sample was diluted to 2 mg/mL in phosphate buffered saline (PBS: 20 mM 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4 with 150 mM NaCl, pH 7.4) and then mixed with the appropriate volume of 10 mM biotin reagent in order to provide 5, 10, and 20 molar ratios of biotin to IgG. This biotinylation reaction was performed for 30 min at room temperature. To determine the number of biotin molecules per IgG, the modified IgG sample was run through a Sephadex G-25 column (0.75 cm × 10 cm) to remove the unbound biotin reagents. After desalting, the modified IgG sample was analyzed by the EZ Biotin Quantitation Kit (Pierce) to determine the number of biotin molecules per IgG as described by the supplier.

2.4. Conjugation of biotinylated IgG to NeutrAvidin-tagged liposomes

Three different batches of biotinylated IgG, with the starting molar ratios of biotin to IgG of 5, 10, and 20, were added into the NeutrAvidin-tagged liposome solution with either 5:1 or 1:1 molar ratio of IgG to NeutrAvidin. The reaction mixture was incubated for 20 min at room temperature. The size of the IgG–NeutrAvidin-tagged liposomal nanovesicles was then determined in the particle size analyzer.

2.5. Competitive lateral flow immunoassay for detecting rabbit IgG

The design of the competitive lateral flow immunoassay for detecting rabbit IgG, using biotinylated goat

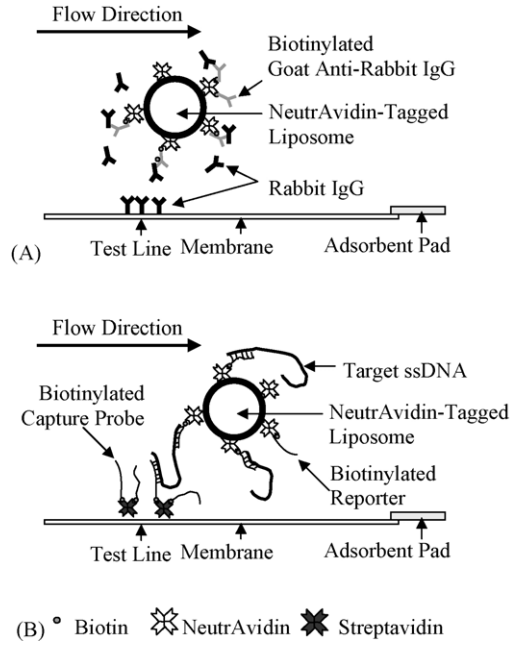

Fig. 1. The simplified scheme for competitive lateral flow immunoassay for the detection of rabbit IgG (A) and sandwich hybridization assay for the detection of ssDNA fragment (B).

anti-rabbit IgG–NeutrAvidin-tagged liposomal nanovesicles as the detection reagent, is shown in Fig. 1A.

2.5.1. NC membrane test strip preparation

The NC membrane test strips for the lateral flow assay were prepared by coating 40 μg/cm^2 of rabbit antibody in a test line (2.0 cm from the proximal end) on the HiFlow Plus 120 NC membrane, using a Linomat IV TLC Applicator (Camag Scientific, Wrightsville Beach, NC). Rabbit IgG was dissolved in PBS with 5% methanol to have a final concentration of 4 mg/mL. After coating, the membrane was dried at 37 °C for 30 min in a ventilated convection oven, and then immersed in a blocking solution containing 1% polyvinylpyrrolidone (PVP), 0.01% gelatin, 0.002% Tween 20 in PBS, for 10 min with shaking. This blocked membrane was dried by absorption using a paper towel and then in a ventilated convection oven at 37 °C for 60 min. After drying, the membrane was stored at 4 °C with desiccants. Before running the lateral flow assay, the blocked membranes were cut into test strips (5 mm × 50 mm) and a filter paper pad was attached to the top of the test strip, as shown in Fig. 1A.

2.5.2. Assay format

In the assay, 0.1 mol% NeutrAvidin-tagged liposome solution was mixed with biotinylated goat anti-rabbit IgG (NeutrAvidin:biotinylated IgG = 1:1, mole) and 10 μL of rabbit IgG sample, and then added to a glass tube (12 mm × 75 mm) containing 10 μL of Blocker Casein (1% casein in TBS). This reaction mixture was incubated for 20 min at room temperature with gentle shaking. A test strip was then inserted into the glass tube. After the sample solution was totally absorbed into the test strip, an additional 30 μL Blocker Casein was added to eliminate the background signal on the NC membrane by washing away the non-specifically bound liposomes and membrane. Once the Blocker Casein solution was completely absorbed into the strip (about 10 min), the test strip was taken out. The signal intensity of the test line was qualitatively estimated visually, or quantitatively measured by scanning the test strips using an Epson Expression 636 color image scanner (Torrance, CA), and the scanned images were then converted into gray scale readings by Scan Analysis densitometry software (Biosoft, Ferguson, MO).

2.6. Sandwich hybridization lateral flow assay for detecting ssDNA fragment

The design for the detection of ssDNA fragment by the sandwich hybridization lateral flow assay using the biotinylated reporter probe-NeutrAvidin-tagged liposomal nanovesicles as the detection reagent is shown in Fig. 1B.

2.6.1. Synthetic target and probes

The single-stranded, 60-mer synthetic target is a short fragment (531–590 nt) of the 699 bp internal transcribed spacer region (ITS) of the 16S–23S rRNA (rDNA) used for field identification of strains of *E. amylovora* [43]. Complementary to this target were designed the 23-mer capture probe (539–561 nt) and 20-mer reporter probe (565–584 nt). These oligos were synthesized, biotinylated at the 5′ end, and purified as salt-free probes by Operon Biotechnologies Inc. Probes were dissolved to a final concentration 300 nmol in 0.1 M phosphate buffer, pH 7.5 supplemented with 1 mM EDTA and 0.1% sodium azide, and stored at −20 °C.

2.6.2. Test strip preparation

A mixture of streptavidin and biotinylated oligonucleotides-capture probe (molar ratio 3:1) was incubated for 15 min at room temperature. The mixture was applied 20 mm above the bottom edge of the Predator membrane (20 cm × 6 cm) by using Linomat TLC Applicator. Coating was performed at 40–70% relative humidity and room temperature. The membrane was dried for 5 min at room temperature and the oligonucleotide was immobilized by drying at 55 °C for 2 h under reduced pressure (15 psi). The coated membrane was blocked in the blocking solution (0.002 M Tris, 0.15 M NaCl, 0.5%, w/v, PVP MW 10,000 and 0.015%, w/v, casein—Hammarsten quality MW 75,000–100,000; pH 7.0) by gently shaking for 30 min at room temperature. The blocked membrane was first dried using a paper towel and then dried for 3 h at room temperature under reduced pressure (15 psi), and stored in vacuum-sealed plastic bag at 4 °C. The membrane was cut into 5 mm strips immediately prior to use. Each strip contained 60 pmol of biotinylated oligonucleotide bound by 20 pmol of streptavidin.

2.6.3. Biosensor assay

The mixture of 5 μL of master mix (50% formamide, 4 × SSC, 0.2% Ficoll, 0.2% sucrose), 1 μL (0.5 μmol) of synthetic target or water (as negative control), and 1 μL (2.0 μmol) of biotinylated reporter probe were mixed in a microcentrifuge tube by pipeting. The hybridization mixture was preincubated first at 95 °C for 5 min and after that at 37 °C for 15 min. After incubation, 3 μL of NeutrAvidin-tagged liposomes were added, mixed and the solution transferred into borosilicate glass tubes (12 mm × 75 mm). The membrane strip (with 20 pmol of streptavidin) was inserted into tube, and after the entire hybridization mixture was absorbed by the strip, 35 μL of running buffer (10% formamide, 10 × SSC, 0.2% Ficoll, 0.2% sucrose) were added to the tube and allowed to traverse the entire length of the strip. The 20 × SSC solution was prepared from 3 M NaCl, 0.3 M sodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$), pH 7.0.

3. Results and discussion

3.1. Effect of NeutrAvidin mol% on its conjugation efficiency and liposome size

Four different starting mol% (0.1, 0.2, 0.4, and 0.8) of NeutrAvidin to the surface lipids of liposomes were used to study the effect of NeutrAvidin mol% on its conjugation efficiency and liposome size after conjugation. In Table 1, liposomes with the starting mol% of NeutrAvidin as 0.1, 0.2, 0.4, and 0.8 had their final mol% of NeutrAvidin determined to be 0.076, 0.166, 0.174, and 0.365, respectively. The conjugation efficiency of NeutrAvidin to the liposomal surface increased from 76% to 83% as their starting mol% of NeutrAvidin increased from 0.1 to 0.2. However, the additional increases of the starting mol% of NeutrAvidin (0.4 and 0.8) showed considerably reduced conjugation efficiencies to 43% and 46%, respectively. However, at 0.4 mol% start, the available surface space on liposomes for binding NeutrAvidin begins to decrease. This reduction causes difficulty in attaching maleimide groups on NeutrAvidin to the small sulfhydryl (–SH) groups on liposomal surface, resulting in a decrease in the conjugation efficiency. Our data also indicated that liposome size correlated with the starting mol% of NeutrAvidin. The size of NeutrAvidin-tagged liposomal nanovesicles increased from 241 ± 19.1 to 741 ± 66.8 nm as the starting mol% of NeutrAvidin increased from 0.1 to 0.8. This expansion of liposome size may be due to two reasons. One is the increasing number of NeutrAvidin molecules on the liposomal surface since the values of tagged mol% of NeutrAvidin increased from 0.076 to 0.365. The more likely reason is that a higher number of NeutrAvidin can increase the possibility of forming NeutrAvidin bridges between liposomes, i.e., aggregation, due to the fact that the number of maleimide groups per NeutrAvidin is greater than 1.

Table 1
Effect of NeutrAvidin molar percentage on its conjugation efficiency to the liposomal surface and liposome size

Starting NeutrAvidin mol%	Final NeutrAvidin mol%	Conjugation efficiency (%)	Liposome diameter (nm)
0	0	NA	241 ± 19.1
0.1	0.076	76	392 ± 39.9
0.2	0.166	83	500 ± 45.0
0.4	0.174	43	547 ± 31.7
0.8	0.365	46	741 ± 66.8

3.2. Effect of molar ratio of biotin/IgG and IgG/NeutrAvidin on liposome size

Before attaching ligands to NeutrAvidin-tagged liposomal nanovesicles, biotinylation on these ligands is required. In this study, the detection of rabbit antibody was used as a model, in which goat anti-rabbit IgG was biotinylated and then attached to NeutrAvidin on the liposomal surface to produce IgG–NeutrAvidin-tagged liposomes (immunoliposomes). During the production of immunoliposomes, multi-biotinylated IgG may become protein bridges between NeutrAvidin-tagged liposomes, resulting in liposomal aggregation. If the size of the liposomal aggregate is larger than the pore-size of the NC membrane, this aggregate would be immobile on the NC membrane test strip. This condition can cause a high background signal on the NC membrane and produce a significant reduction of assay sensitivity. In the extreme situation, liposomal aggregation would occur as soon as the biotinylated IgG was added. To investigate the effect of biotin/IgG molar ratio on liposomal aggregation, three different biotin/IgG starting molar ratios (5, 10, and 20) were used for biotinylation. After biotinylation, each batch of biotinylated IgG was mixed with 0.1, 0.2, 0.4, and 0.8 starting mol% of NeutrAvidin-tagged liposomal nanovesicles with two different molar ratios (1 and 5) of IgG/NeutrAvidin. A particle size analyzer was used for measuring the size of the "IgG–NeutrAvidin-tagged liposomes" in order to estimate the degree of liposomal aggregation. The best conditions for the production of IgG–NeutrAvidin liposomes (immunoliposomes) were selected from these 24 combinations, as shown in Table 2.

All immunoliposomes with 0.4 and 0.8 starting mol% of NeutrAvidin precipitated as soon as the addition of biotinylated anti-rabbit IgG was added, no matter the molar ratio of IgG/NeutrAvidin or biotin/IgG. This precipitation could be the result of forming antibody bridges between NeutrAvidin-tagged liposomes, resulting in an immediate liposomal aggregation. After biotinylation, the estimated biotin number per anti-rabbit IgG was 2.6, 6.8, and 8.1 for the starting molar ratio of biotin/antibody as 5, 10, and 20, respectively. Therefore, all batches of biotinylated antibodies had the potential to be a protein bridge between NeutrAvidin-tagged liposomes. Liposomes tagged with higher starting NeutrAvidin mol%, such as 0.4 and 0.8, have greater chance of

Table 2
Effect of NeutrAvidin molar percentage and molar ratio of IgG/NeutrAvidin and biotin/IgG on liposome size

Starting NeutrAvidin mol%	Final NeutrAvidin mol%	IgG/NeutrAvidin molar ratio	Biotin/IgG molar ratio	Liposome diameter (nm)
0.1	0.076	1/1	5	402±38.7
0.1	0.076	1/1	10	397±39.6
0.1	0.076	1/1	20	380±39.1
0.1	0.076	5/1	5	367±37.3
0.1	0.076	5/1	10	351±27.0
0.1	0.076	5/1	20	505±41.6
0.2	0.166	1/1	5	PPT*
0.2	0.166	1/1	10	PPT
0.2	0.166	1/1	20	PPT
0.2	0.166	5/1	5	433 ± 24.7
0.2	0.166	5/1	10	423 ± 26.6
0.2	0.166	5/1	20	364 ± 36.7
0.4	0.174	1/1	5	PPT
0.4	0.174	1/1	10	PPT
0.4	0.174	1/1	20	PPT
0.4	0.174	5/1	5	PPT
0.4	0.174	5/1	10	PPT
0.4	0.174	5/1	20	PPT
0.8	0.365	1/1	5	PPT
0.8	0.365	1/1	10	PPT
0.8	0.365	1/1	20	PPT
0.8	0.365	5/1	5	PPT
0.8	0.365	5/1	10	PPT
0.8	0.365	5/1	20	PPT

PPT*: precipitation.

capturing biotinylated antibodies. Therefore, they form liposomal aggregates more easily and precipitate.

Liposomes tagged with 0.2 mol% of NeutrAvidin precipitated only at the molar ratio of IgG/NeutrAvidin of unity with all three (5, 10, and 20) different biotin/IgG molar ratios. However, precipitation was avoided when a five-fold excess of biotinylated IgG was added to NeutrAvidin. Therefore, by increasing the amount of biotinylated IgG, liposomal precipitation could be prevented. This absence of precipitation can be explained as the result of the absence of antibody bridges, since the majority of biotin-binding sites on NeutrAvidin was saturated by individual biotinylated antibodies. On the other hand, the molar ratio of biotin/IgG had no significant impact on preventing liposomal aggregation. Additionally, 0.1 mol% of NeutrAvidin-tagged liposomes showed no precipitation under every combination of the molar ratios of biotin/IgG and IgG/NeutrAvidin, possibly due to the very low NeutrAvidin mol% on the liposomal surface. For the development of immunoliposomes, both 0.1 and 0.2 starting mol% of NeutrAvidin-tagged liposomes could be used. However, starting with 0.2 mol% of NeutrAvidin-tagged liposomes required a higher molar ratio of IgG/NeutrAvidin to prevent the liposomal precipitation. The higher amount of antibodies can significantly increase the cost of the preparation of the IgG–NeutrAvidin-tagged liposomal nanovesicles (immunoliposomes). Because of that consideration, we decided to make immunoliposomes with starting with 0.1 mol% of NeutrAvidin and with the IgG/NeutrAvidin molar ratio of 1. Thus, the liposomal aggregation by NeutrAvidin bridges is more dependent on the mol% of NeutrAvidin on the liposomal surface than on the molar ratio of IgG/NeutrAvidin. However, the molar ratio of biotin/IgG has no significant effect on the liposomal aggregation.

3.3. Optimizing the biotin/IgG ratio for preventing liposomal aggregation

Adding extra biotin molecules into IgG–NeutrAvidin-tagged liposomes is another way to prevent liposomal aggregation. In this study, the starting molar ratios of biotin/goat IgG were 5, 10, and 20 and, after biotinylation, the attached biotin numbers per IgG were estimated 2.6, 6.8, and 8.1, respectively. Therefore, unbound biotin molecules can be used to occupy the rest of available biotin-binding sites on NeutrAvidin. In this way, there is no requirement for adding extra biotin molecules, and IgG samples did not need to be run through gel filtration chromatography or dialyzed to remove unbound biotin molecules, resulting in a simpler and faster process for producing immunoliposomes.

To determine the optimal molar ratio of biotin/IgG for preventing liposomal aggregation, a capture lateral flow immunoassay was applied, in which rabbit IgG was coated on the test line on the NC membrane strip and biotinylated anti-rabbit IgG was conjugated to the starting 0.1 mol% of NeutrAvidin-tagged liposomes. The performance of lateral flow assay was determined by the value of the signal/noise ratio, in which the signal is the color intensity on the test line and the noise is the background on the NC membrane test

strips. The higher value of signal/noise gives higher assay sensitivity. The value of signal/noise increased in proportional to the molar ratio of biotin/IgG. The biotin/IgG molar ratios of 5 and 10 had higher background caused by liposomal aggregation. If the number of free biotin molecules is not enough to fill up biotin-binding sites on NeutrAvidin, biotin molecules on the anti-rabbit IgG would be used to fill up the remaining biotin-binding sites on NeutrAvidin. This biotin–NeutrAvidin interaction results in the biotinylated IgG becoming a protein bridge between liposomes. The resulting liposomal aggregation can reduce the flow of samples on the NC membrane as soon as the size of liposomal aggregation becomes larger than the pore-size of the NC membrane, and a pink background is produced on the NC membrane. The highest value of signal/noise occurred at molar ratio of biotin/IgG 20, and this was optimal for producing antibody-tagged liposomes for a lateral flow assay, utilizing the NC membrane with pore-size of around 9–12 μm.

3.4. Detection of rabbit IgG

To demonstrate the feasibility of immunoliposomes made by IgG–NeutrAvidin liposomes, a competitive immunoassay for the detection of rabbit IgG was applied in a lateral flow assay (LFA). In this assay, rabbit IgG was immobilized on the test line of the NC membrane test strip and biotinylated anti-rabbit IgG was conjugated to NeutrAvidin-tagged liposomes using the previously optimized conditions (Fig. 1). The rabbit IgG in the sample competed with the immobilized rabbit IgG for the limited antigen binding sites on the anti-rabbit IgG molecules attached to NeutrAvidin-tagged liposomes. For this assay, the signal intensity on the test line is inversely proportional to the concentration of rabbit IgG in the sample.

To determine the limit of detection (LOD), increasing concentrations of rabbit IgG were spiked in PBS and subsequently detected by the LFA to generate a dose–response curve, shown in Fig. 2. A three-parameter sigmoidal function was calculated from this curve with a high R^2 value of 0.997. The LOD, defined as the concentration equivalent to the mean of the blank rabbit IgG samples minus 3 standard deviations (S.D.), was calculated to be 38 pmol/mL of rabbit IgG. This work demonstrated the feasibility of using NeutrAvidin-tagged liposomes to conjugate biotinylated antibodies for the production of immunoliposomes as the detection reagent in immunoassays.

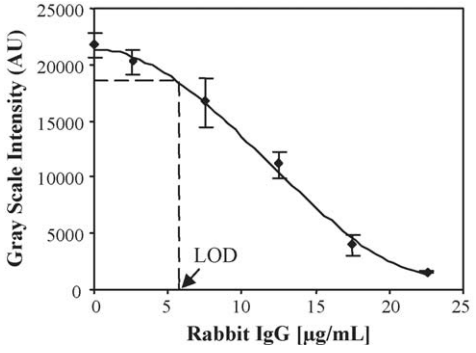

Fig. 2. Dose–response curve for the detection of rabbit IgG by a competitive lateral flow immunoassay. The curve was generated by a serial dilution of rabbit IgG samples (0, 2.5, 7.5, 12.5, 17.5, and 22.5 μg/mL) by measuring the gray scale intensity (GSI) of the test line on the LFA strips. A three-parameter sigmoidal function ($R^2 = 0.997$) was calculated from this dose–response curve with a limit of detection of 5.7 μg/mL (=38 pmol/mL).

3.5. Detection of ssDNA fragment

In recent years, the detection of nucleic acids, including DNA and RNA, has been increasing in importance. Because of the uniqueness of nucleic acid sequences, one organism can be discriminated from others [44]. Three types of markers have been used in nucleic acid assays: DNA, messenger RNA (mRNA), and ribosomal RNA (rRNA). For maximizing analytical sensitivity, the specific sequence of the targeted nucleic acid would be amplified [45]. Most DNA amplification is performed using PCR [46], while mRNA and rRNA are amplified using either reverse transcriptase PCR (RT-PCR) or nucleic acid sequence-based amplification (NASBA) [47,48].

The viability of pathogens is a crucial factor in determining their ability to pose an actual threat to public health. Since there is little correlation between cell viability and the presence of DNA [49], attention has turned to the use of mRNA or rRNA as markers of viability, as they have very short half-lives (seconds-to-minutes) and provide a better indication of cellular viability than DNA-based methods [45]. NASBA offers several advantages over RT-PCR. First, NASBA is performed at 41 °C without the need of a thermal cycler [50]. Moreover, the product of NASBA is a single-stranded antisense RNA, which can be directly hybridized with a labeled probe to reduce the assay time and simplify the entire process. To mimic the product of NASBA, a synthetic ssDNA fragment was used as the target in this study.

A sandwich hybridization format for the lateral flow assay was applied to detect the synthetic ssDNA fragment of the sequence of 16S–23S rRNA of *E. amylovora* [43]. In this assay (Fig. 1B), the biotinylated reporter probe was conjugated to NeutrAvidin-tagged liposomes and the biotinylated capture probe was immobilized through streptavidin on test strips. The sequence of the reporter probe was complementary to a portion of the synthetic target sequence. Because of the presence of a biotin molecule at the 5′ end, the reporter probe was attached to NeutrAvidin-tagged liposomes thereby forming the detection reagent. In the assay, the sample was first incubated with the reporter probe at 95 °C. This high temperature eliminated any secondary structure of the synthetic target and reduced non-specific interactions. During the incubation at 37 °C, the reporter probe hybridized with the complementary sequence of the synthetic target. Finally, NeutrAvidin-tagged liposomes were added to this mixture of synthetic target-reporter probe complex to perform a lateral flow assay through a test strip. If target is present in the sample, it first attaches to the reporter probe on

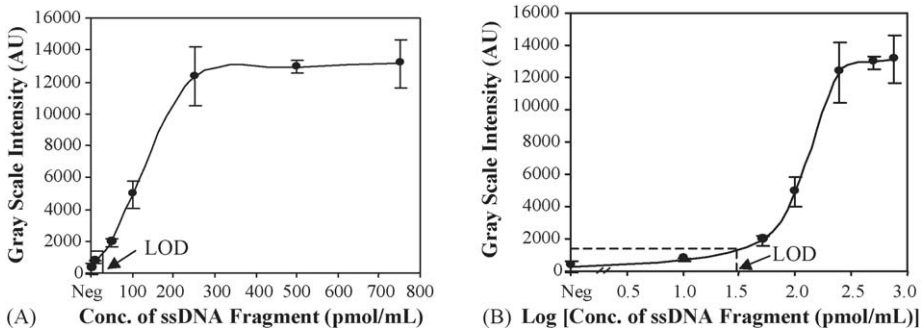

Fig. 3. Dose–response curves for the detection of the synthetic target of *Erwinia amylovora* DNA by a sandwich hybridization lateral flow assay. (A) The curve was generated from a serial dilution of synthetic target (0, 10, 50, 100, 250, 500, and 750 pmol/mL) by measuring the gray scale intensity (GSI) of the test line on the LFA strips. (B) A log scale dose–response curve of (A) showing the location of the LOD as approximately 30 pmol/mL (Neg: 0 pmol/mL of the synthetic target).

NeutrAvidin-tagged liposomes and is then captured by capture probe on the test line to form a visible red line on the test strip. Therefore, the signal intensity is directly proportional to the concentration of the synthetic target. Fig. 3 shows a dose–response curve for serially diluted synthetic target (0, 10, 50, 100, 250, 500, and 750 pmol/mL) samples obtained by the lateral flow assay using gray scale intensity (GSI) quantitation. The limit of detection was calculated as the concentration equivalent of the mean of the blank synthetic target samples plus 3 standard deviations. From this curve, the LOD was estimated to be approximately 30 pmol/mL of DNA fragment.

4. Conclusion

Data presented in this study demonstrated that NeutrAvidin-tagged liposomal nanovesicles could be universal reagents for a fast and easy method to prepare detection reagents for bioanalytical assays of different targets. By this method, a variety of biotinylated ligands, including antibodies and nucleic acids, can be quickly attached to the liposomal surface by the specific biotin–NeutrAvidin interaction. The optimal starting molar percentage of NeutrAvidin on liposomal surface was 0.1, since this small amount of NeutrAvidin prevented liposomal aggregation. To exemplify the feasibility of using NeutrAvidin-tagged liposomal nanovesicles, two assays were investigated in this study. The first assay was a competitive lateral flow immunoassay for the detection of rabbit IgG, and the second assay was a lateral flow sandwich hybridization assay for the detection of a synthetic target of *E. amylovora* DNA. Both assays could be performed in 30 min and gave very similar values for the LOD, which demonstrated the consistency of the assay sensitivity when using NeutrAvidin-tagged liposomal nanovesicles in a lateral flow assay.

In summary, these newly developed NeutrAvidin-tagged liposomal nanovesicles can be applied for many kinds of analytes in a lateral flow assay format with an analysis time of about 30 min. For example, any nucleic acid sequence can be identified, as long as the sequences for capture and reporter probe are known. Additionally, analytes whose antibodies have been developed can be detected in lateral flow immunoassays. Therefore, this universal reagent is ideal for quickly screening samples without requiring specific training or sophisticated equipment.

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