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Liposome-based immunostrip for the rapid detection of *Salmonella*

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Abstract *Salmonellae* are ubiquitous human pathogens, which pose a danger to the elderly and children. Due to the increased number of outbreaks of human illness associated with the consumption of contaminated products in the USA and many other countries, there is an urgent need to develop rapid assays to detect common food-borne pathogens. This study demonstrates the feasibility of using a detectable label comprising methyl blue (MB), a visible dye, entrapped inside liposomes. Immunoliposomes tagged with anti-*Salmonella* common structural antigens (CSA) antibody encapsulating MB dye were prepared and used as the signal amplifier for the development of a field-portable colorimetric immunoassay to detect *Salmonellae*. Tapping mode atomic force microscopy (TMAFM), a scanning probe technique, was utilized to demonstrate the presence of anti-*Salmonella* antibody at the thus-prepared liposome. A plastic-backed nitrocellulose strip with two immobilized zones formed the basis of a sandwich assay. The first zone was the antigen capture zone (AC zone), used in a sandwich (noncompetitive) assay format; the other was the biotin capture zone (BC zone), used as a quality control index for the strip assay. During the capillary migration of the wicking reagent containing 80 μL of immunoliposomes and 40 μL of the test sample (heat-killed *S. typhimurium*), sample pathogens with surface-bound immunoliposomes were captured at the AC zone, while the unbound immunoliposomes continued to migrate and bind to the anti-biotin antibodies coated on the BC zone. The color density of the AC zone was directly proportional to the number of *Salmonella typhimurium* in the test sample. The detection limit of the current assay with heat-killed *Salmonella typhimurium* was 1,680 cells. The cross-reactivity of the proposed immunoassay was also investigated, and pathogens including *E. coli* O157:H7 and *Listeria* genus specific caused no interference with the detection of *Salmonella typhimurium*.

Keywords *Salmonella typhimurium* · Point-of-care
diagnostics · Lateral flow immunoassay · Pathogen detection

Introduction

Pathogens such as *Salmonellae*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Vibrio cholerae*, parasites, and viruses are likely to contaminate fresh produce through raw or improperly composted manure, irrigation water containing untreated sewage, or contaminated wash water. The Centers for Disease Control and Prevention (CDC) estimates that 76 million cases of food-borne illnesses or food poisoning occur every year in the USA, which can amount to $35 billion annually in medical costs and lost productivity [1].

Salmonellosis, a disease caused by *Salmonellae*, affects an estimated two million Americans each year and is common throughout the world. In 1994, an outbreak of salmonellosis that affected an estimated 224,000 persons in the USA was attributed to a large supply of contaminated

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ice cream [1–4]. *Salmonella* species are Gram-negative, flagellated, facultatively anaerobic bacilli possessing three major antigens: H, or flagellar antigen; O, or somatic antigen; and Vi antigen (possessed by only a few serotypes) [5, 6]. Pathogenic *Salmonellae* ingested in food survive passage through the gastric acid barrier and invade the mucosa of the small and large intestine to produce toxins.

Salmonellae consist of a range of very closely related bacteria, each with its own unique impact on the human body. The three main serotypes of *Salmonella enterica* are *Typhimurium*, *Enteritidis*, and *Typhi*. Of these, *S. typhimurium* is the most common cause of food poisoning by *Salmonella* species in recent years. In the clinical laboratory, *Salmonellae* are usually grown isolated on brilliant green sulfa agar, bismuth sulfite agar, XLT4 agar, double modified lysine iron agar, or XLD agar, or DCA [7, 8]. They form moist colonies about 2 to 3 mm in diameter and are incapable of fermenting lactose and producing H_2S.

Identification of the widespread outbreaks of food illness is a considerable challenge for public health officials. The most difficult outbreaks to detect are those that span a large geographical area. In an effort to make outbreaks easier to pinpoint, more-rapid assays of investigation are continuously being developed. New technologies such as PCR [9–16], nucleic acid-based assays [14, 15], or DNA fingerprinting [16, 17] make it easier for local officials to compare strains of pathogens found in cases across the country. Another type of method, antibody-based assays [18–33], has also been reported to apply in the detection of *Salmonella* to simplify the time-consuming, labor-intensive conventional culture methods (usually requiring more than 5 days).

Liposomes [34–36] have been used intensively in our laboratory for encapsulating various signal-generating molecules, such as fluorophores, DNA, and photoproteins, for the development of immunodetection systems [37–44]. Immunodetection techniques, which utilize immunological reactions to measure the presence of a target substance, offer sensitivity, speed, and simplicity of operation, providing potential solutions to the need for more-rapid identification of pathogens.

Despite notable advances in food-processing industries, food-borne illnesses continue to be a significant growing public health problem. In laboratory diagnosis, most microbiological assays rely on a single phenotype to selectively isolate a pathogen, which is a slow process. Therefore, as part of our project on the development of rapid assays for the detection of multi-pathogenic bacteria such as *Salmonellae* and *E. coli* O157:H7, we present here a simple and rapid immunostrip assay for *Salmonella* detection, which has potential as a rapid and inexpensive point-of-care diagnostic assay.

Materials and methods

Reagents and materials

All inorganic chemicals and organic solvents used were reagent grade or better. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), and dipalmitoylphosphoethanolamine (DPPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-(κ-Maleimidoundecanoyloxy)sulfosuccinimide ester (sulfo-KMUS), *N*-ethylmaleimide, hydroxylamine hydrochloride, and succinimidyl-*S*-acetylthioacetate (SATA) were purchased from Pierce Chemicals Co. (Rockford, IL, USA). Methyl blue ($C_{37}H_{27}N_3Na_2O_9S_3$) was purchased from Waldeck GmbH & Co. KG Division Chroma (Muenster, Germany). Biotin-X-DHPE (*N*-((6-(biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) was purchased from Molecular Probes (Eugene, OR, USA). Affinity-purified polyclonal antibody (goat anti-*Salmonella*, CSA-1), heat-killed *Salmonella typhimurium*, *E. coli* O157:H7, and *Listeria* genus specific were purchased from Kirkegaard-Perry Laboratories, Inc. (Gaithersburg, MO, USA). Nitrocellulose membranes were purchased from Millipore (Bedford, MA, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of dye-encapsulated liposomes

Liposomes were prepared by a reversed-phase evaporation [45–47] method described previously [40], involving a water-in-oil emulsion. Briefly, the lipid mixture consisted of DPPC, cholesterol, DPPG, DPPE, and Biotin-X-DHPE in a molar ratio of 5:5:0.5:0.25:0.005. This was first dissolved in a solvent mixture consisting of chloroform, isopropyl ether, and methanol (4:4:2), followed by 1-min sonication at 45 °C under nitrogen. One milliliter of 30 mM methyl blue was then added to the lipid mixture. After sonication of the solution for a further 5 min with occasional swirling, the organic solvent was removed by evaporating at 45 °C, leaving a dark blue, gel-like suspension of liposomes. An additional 1 mL of methyl blue was added, followed by another 5 min of sonication at 45 °C. The liposomes preparation was incubated in a 45 °C water bath for 30 min before passing through 2- and 0.4-μm polycarbonate filters to produce a homogenous suspension of uniform size. Any unencapsulated dye or trace of organic solvent was removed from the liposomes preparation by gel filtering on a 1.5×25-cm Sephadex G-50-150 column at room temperature, followed by dialysis (MWCO, 12–14 kDa) at 4 °C in the dark.

Derivatization of liposomes with sulfo-KMUS

One milligram of sulfo-KMUS was first dissolved in 10 μL of dimethyl sulfoxide (DMSO) solution and added to the dialyzed liposome preparation. The mixture was allowed to react at room temperature for 2.5 h. The reaction was stopped by reacting with Tris-HCl (pH 7.8) for 15 min at room temperature on the shaker, followed by dialysis (MWCO, 12–14 kDa) against 10 mM HEPES buffer, pH 7.5, containing NaCl and sucrose at 4 °C in the dark.

Preparation of SATA-modified antibodies

Two milligrams of goat anti-*Salmonella* antibody (Ab) were dissolved in 0.1 mL of 10 mM phosphate-buffered saline (PBS) at pH 7.2. A 1.0-mg sample of SATA in DMSO (10 μL) was added to the Ab solution. The mixture was then allowed to react on a shaker for 30 min at room temperature, followed by dialysis (MWCO, 6–8 kDa) against 10 mM PBS (pH 7.2) containing ethylenediamine-tetraacetic acid (EDTA) at 4 °C for 3.5 h. Concurrently, the acetylthioacetyl-antibody was deprotected by adding hydroxylamine hydrochloride (100 μmol) to obtain free sulfhydryl groups. Subsequently, the thiolated Ab was purified by gel filtration on Sephadex G-25. Fractions containing sulfhydryl (SH)-derivatized protein were collected by measurement of the absorbance at 280 nm.

Conjugation of maleimide-derivatized liposomes with SH-containing antibody

The coupling of thiolated Ab to maleimide-derivated liposomes was achieved by incubating overnight at 4 °C. Unreacted sulfhydryl groups on the antibody and the unreacted sulfo-succinimidyl groups on the sulfo-KMUS were subsequently capped with *N*-ethylmaleimide and Tris base. The antibody-tagged liposomes were separated from unreacted SH-derivatized antibody on a Sepharose CL-4B column equilibrated with Tris-buffered saline (TBS) (pH 7.0) containing sucrose. The desired fraction of liposomes was collected, followed by dialysis at 4 °C in the dark for improved stability.

Stability study and characterization of liposomes

The intactness of liposomes means the maintenance of their integrity, which can be determined by measuring absorbance from MB before and after lysis. According to our previous studies, almost instantaneous and total lysis of liposomes was observed at room temperature when a solution of 50 mM *n*-octyl β-D-glucopyranoside (OG) was

added [39]. The diameter of the liposomes was measured with a Malvern nano-S90 particle size analyzer (Malvern, Worcestershire, UK) using the manufacturer's method. In addition, tapping mode atomic force microscopy (TMAFM), a scanning probe technique that exerts only minimal force on soft materials, was utilized to demonstrate the presence of anti-*Salmonella* antibody at the thus-prepared liposome. TMAFM measurements were carried out with a NanoScope IIIa controller (Veeco Metrology Group, Santa Barbara, CA). Images were acquired using a 10-μm scanner and monolithic silicon cantilevers (NCHR, NanoWorld, Switzerland) whose force constant and the tip curvature were nominally 42 nN m^{-1} and <10 nm, respectively. Samples for TMAFM were prepared by placing a drop of liposome solution on freshly cleaved mica. To remove the excess solution and physisorbed lipid, the films on mica were rinsed with water and then dried by a stream of $N_2(g)$. The microscope was housed in a Plexiglas chamber where dry N_2 was purged throughout the experiments and the humidity was kept lower than 2%.

Preparation of test strips and assay procedures

Immobilization of anti-*Salmonella* antibodies and anti-biotin antibodies on the plastic-backed nitrocellulose membrane was done by manual pipetting. Affinity-purified polyclonal antibody to the *Salmonella* CSA was isolated from a serum pool of goats immunized with different serotypes of *Salmonella*. This antibody is broadly reactive to *Salmonella* and recognizes most *Salmonella* serotypes. The membrane was first cut into 10×8.8-cm sheets, followed by dot-blotting 2 μL of anti-*Salmonella* antibody solution at a concentration of 2 mg mL^{-1} or 4 mg mL^{-1} and 1 μL of anti-biotin antibody (1 mg mL^{-1}), leading to a final amount of 4 μg or 8 μg of anti-*Salmonella* antibody and 1 μg of anti-biotin antibody per strip. The antibody-coated dots were allowed to air-dry in the hood for 5 min and further dried under vacuum (10 psi) at room temperature for 1 h. The coated nitrocellulose sheet was immersed in a blocking solution consisting of 0.5% poly (vinylpyrrolidone) and 0.03% casein in TBS at pH 7.5 for 1 h on a rotating shaker, followed by drying under vacuum (10 psi) at room temperature for at least 6 h. The prepared sheet was then cut into 0.5×8.8-cm strips using a paper cutter, producing strips with the antigen capture (AC) zone 1.5 cm and biotin capture (BC) zone 3.5 cm above the bottom of the strip. The prepared strips were stored at 4 °C until use.

The format for the proposed immunostrip assay consists of immunoliposomes solution, test sample, and nitrocellulose test strips with immobilized anti-*Salmonella* and anti-

biotin antibodies. The assay was performed by dispensing 40 μL of the sample pathogenic organism in PBS and 80 μL of an immunoliposomes solution into a 1×7.5-cm glass test tube with gentle mixing for 30 min. A test strip was then inserted into the test tube, and the chromatographic process was begun. After the solution front reached the upper end of the test strip, it was removed and air-dried. The color intensity of each zone on the test strip was estimated visually or quantified by using a scanning reflectance photometer (KGW Enterprises, Inc., Elkart, IN, USA)

Selectivity of immunoliposomes

The evaluation of the cross-reactivity of immunoliposomes with the target *Salmonella typhimurium* and negative controls such as *E. coli*. O157:H7 and *Listeria* genus specific was then conducted. The anti-*Salmonella* antibody-coated test strips were inserted into the test tubes that contained immunoliposomes and the target antigen or negative control pathogenic organisms. The color intensity of each zone on the test strips was estimated visually or quantified by scanning densitometry.

Safety consideration

Salmonella typhimurium is a harmful bacterium that should be handled with care. Heat-killed *S. typhimurium*, other pathogenic organisms, and organic solvents used in the modification and production of conjugated liposomes and in the performance of the assay were handled in either a laminar flow hood or chemical hood with surgical gloves. All *S. typhimurium* or other harmful pathogen-contaminated labwares were autoclaved before being discarded.

Results and discussion

Characteristics and stability of MB-encapsulated multivalent immunoliposomes

A multistep conjugation procedure for the preparation of MB-encapsulated, anti-*Salmonella* antibody-tagged liposomes (immunoliposomes) was demonstrated. Methyl blue, also known as cotton blue or helvetia blue, is a chemical compound commonly used as a stain in histology. In the current study, MB was chosen as the liposomal encapsulant due to its high water solubility. Sulfo-KMUS is a sulfhydryl-reactive and amine-reactive heterobifunctional cross-linker that conjugates thiolated immunoglobin G (IgG) and the primary amine group of DPPE on the liposome bilayer. The maleimide group of sulfo-KMUS is highly specific for coupling to sulfhydryl-containing molecules, thus directing the conjugation to the second molecules. The resulting biofunctionalized liposomes were proven to be more-stable complexes (>15 weeks) using TMAFM.

Extrusion of the liposome preparations through polycarbonate filters was helpful for obtaining a homogeneous size of the liposomes, resulting in an even capillary migration on the test strips. Liposomes passed 20 times through a 0.4-μm polycarbonate filter had a mean diameter of 236 nm. The 236-nm liposomes were used in all subsequent experiments. The characteristics of liposomes are described as followed. With liposomes of 236-nm diameter it is possible to calculate that the average volume of a single liposome is 6.88×10^{-12} μL and the volume entrapped (assuming a bilayer thickness of 4 nm) is 6.21×10^{-12} μL. Assuming the MB dye concentration inside the liposomes was equal to the original MB dye solution used, and comparing the absorbance of lysed liposomes to that of standard MB solutions, it is possible to calculate that there were about 3.56×10^{12} liposomes per mL and that each liposome contained ca. 1.33×10^5 molecules of dye. If the average surface area of the DPPC molecules is 71 Å^2, and that of cholesterol is 19 Å^2 [48], it is estimated that ca. 4,800 molecules of anti-*Salmonella* CSA antibody were on the outer surface of each liposome, given that 2.5 mole% of sulfo-KMUS-derivatized liposomal DPPE successfully reacted with SH-derivatized IgG. Although it is likely that the high-protein-loaded particles would cause aggregation of antigens with changes of their flow properties, this was not observed in the current study.

The presence of antibody on the outer surface of liposome was confirmed by TMAFM. A literature report [49] showed that the adhesion of liposome on mica led to lipid bilayers where the phosphatidyl group adsorbed on the hydrophilic mica surface and the hydrophobic aliphatic chain drove the formation of the second layer in the hydrophilic aqueous environment. The left-hand image of Fig. 1 shows that, without incorporating *Salmonella* antibody, the morphology of the lipid film was smooth. The difference in height between terraces was ca. 5.5 nm, thinner than two times the fully extended length of DPPC (ca. 3.4 nm) [50] and in a good agreement with literature reports [51–53]. The right-hand of Fig. 1 is typical for films prepared from liposome with *Salmonella* antibody. The film exhibited protrusions of 1.9±0.5 nm in height, similar to the 3.0±1.0 nm found in a recent AFM study [54]. The diameters of the protrusions are 44±9 nm. It is quite common in AFM images for the lateral dimensions of isolated objects to appear a lot larger than expected because of the finite size of the tip curvature. The protrusions were associated with the antibody because such protrusions did
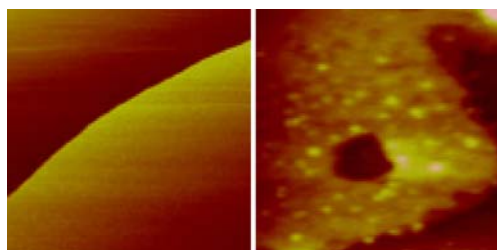
Fig. 1 TMAFM images revealing morphological features of lipid films adhered on mica. The *left-hand* and *right-hand images* were obtained, respectively, from liposomes without and tagged with *Salmonella* antibody. The *contrast* in the *left-hand image* indicates a height difference of ca. 5.5 nm between lipid bilayers (*lower right*) and mica substrate (*upper left*). The *right-hand image* exhibits protrusions of an apparent height of 1.9 nm above the bilayers, suggesting the intact nature of the antibody at the liposome. Image size 650×650 nm

Fig. 3 Scanned image of the assay performed with different concentrations of heat-killed *S. typhimurium*. **a** contains no *S. typhimurium*, **b** 2×10^4 cells, **c** 2×10^5 cells, **d** 1×10^6 cells, **e** 2×10^6 cells, **f** 1×10^7 cells, **g** 2×10^7 cells

not present in plain liposome. The TMAFM results confirm that the proposed multistep conjugation strategy can be used as the platform conjugation procedure for the preparation of antibody-tagged liposomes (immunoliposomes).

Assay performance

The assay involves an immobilized-antibody zone (antigen capture zone, AC zone) in the membrane strip that is exposed to the target antigen, *Salmonella typhimurium*, in a sample solution (Fig. 2). The multivalent immunoliposomes subsequently bind to the bound antigen in the AC zone, while free, unbound immunolipsomes continue to migrate to the biotin capture zone (BC zone). Thus the intensity of
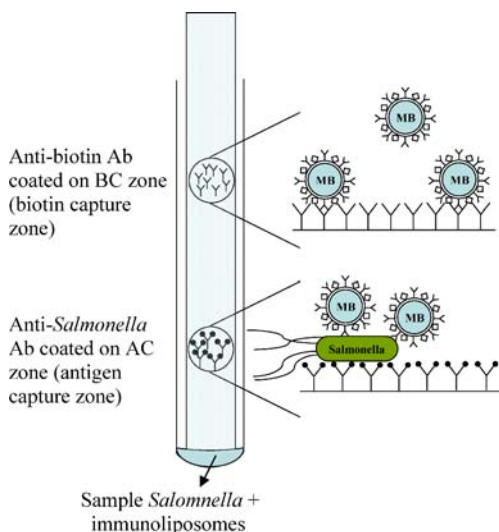
the blue color exhibited by the bound immunoliposomes on the AC zone is directly proportional to *S. typhimurium* present in the sample, and the color exhibited on the BC zone serves as a QC index for the test trips, as shown in Fig. 3. Since MB is a visible dye, the color intensity may be measured semiquantitatively by visual examination. However the use of a QuadScan reflectance photometer (KGW Enterprises, Inc., Elkhart, IN), which is equipped with a high-intensity incandescent lamp to illuminate specific zones to give a reflectance value, can provide more-

Fig. 2 Sandwich binding of sample *Salmonella* between the immunoliposomes and anti-*Salmonella* antibodies immobilized in the AC zone (drawing is not to scale)
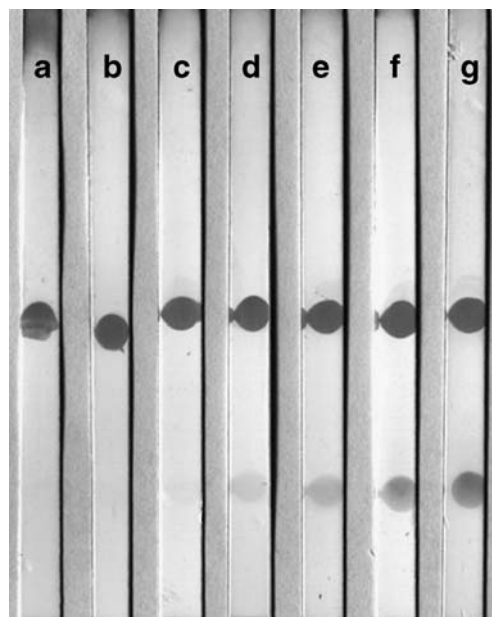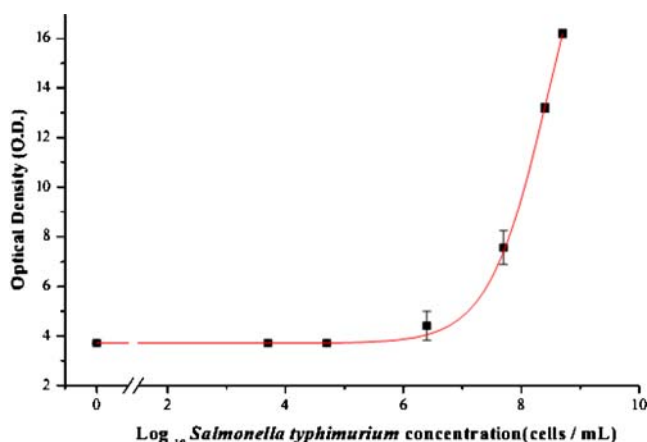

Fig. 4 Dose–response curve for the detection of heat-killed *S. typhimurium*. Each *point* represents the mean of three measurements; *error bars* represent ± 1SD (the *datum point at zero* represents signals from blank)
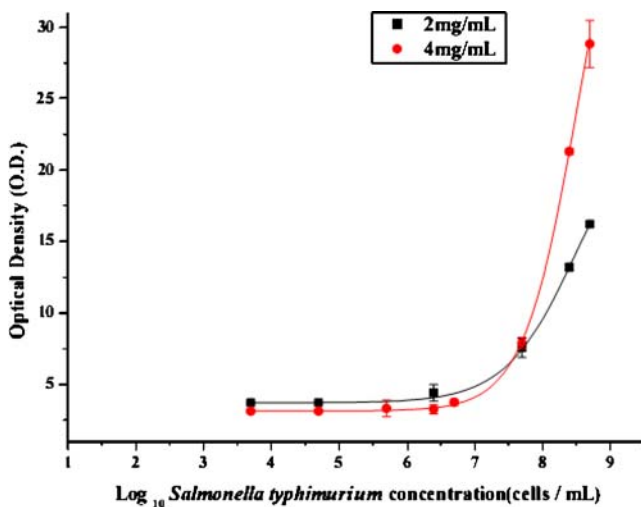
Fig. 5 Effects on the assay performance of antibody concentration immobilized on the nitrocellulose membrane (■ 2 mg mL^{-1}, ● 4 mg mL^{-1})

accurate quantitation results obtained with various concentrations of *S. typhimurium*, as shown in Fig. 4. According to the International Union of Pure and Applied Chemistry (IUPAC), the operational definition of detection limit in this study is calculated at the concentration corresponding to a signal three times the standard deviation (SD) below the mean for a calibrator that is free of *Salmonella*. Therefore the very sensitive limit of detection (LOD) estimated at 3 SD was determined, namely 1,680 cells for heat-killed *S. typhimurium* with 99.7% confidence. The effect of immobilized antibody concentration on the assay performance was also investigated. As seen in Fig. 5, strips immobilized with 2 mg mL^{-1} of antibody showed a wider dynamic detection range for *S. typhimurium*. To evaluate the specificity of the assay, *S. typhimurium* and negative controls at 10^7 to 10^8 cells mL^{-1} were tested. As shown in Fig. 6, the assays did not show any significant cross-reactivity to the nonspecific pathogenic organisms (negative controls).

The detection limits of other immunobiosensing devices reported previously for *Salmonella spp.* are around 10^5 cells mL^{-1} using renewable amperometric immunoassay (assay time 90 min) [27] and 10^2 cells mL^{-1} using quartz crystal microbalance (QCM) [28] with simultaneous measurements of resonant frequency and motional resistance. It was also reported that 28 h was required to perform a dot blot immunoassay [32]. The immunostrip assay proposed here, however, has the advantages of lower cost, simplicity, and speed over other existing assay systems. The proposed assay does not need sophisticated instruments and is a potential alternative method for field screening of contaminated food samples.

Conclusions

Conventional methods for detection of *Salmonella typhimurium* is often labor-intensive, time-consuming, and costly. Unlike conventional ELISAs, our proposed immunostrip assay requires no enrichment, repetitive washing, or incubation steps and can be completed in 30 min. Results obtained by TMAFM confirmed that the proposed multistep conjugation strategy can be successfully used as the platform conjugation procedure for the preparation of antibody-tagged liposomes. The methyl blue dye-entrapped, anti-*Salmonella* antibody-tagged immunoliposomes were successfully demonstrated to be useful in a sandwich immunoassay, which holds promise as a simple, rapid, and inexpensive quantitative assay for screening food samples for *S. typhimurium* with densitometry. Future

Fig. 6 Specificity of immunoliposomes to *Salmonella*: scanned image of the assay performed with **a** *E. coli* O157:H7 (6× 10^7 cells), **b** *Listeria* genus specific (4×10^7 cells), **c** the heat-killed *S. typhimurium* (4× 10^7 cells)
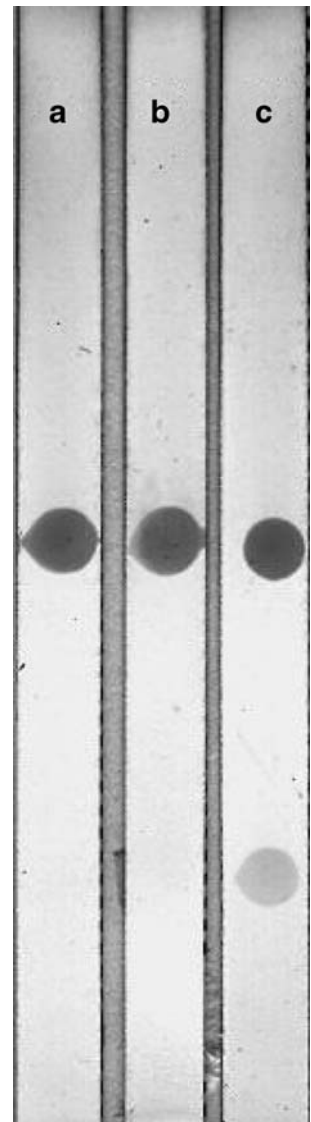
studies will focus on the development of the multianalyte immunodetecting system for *E. coli* O157:H7 and different *Salmonella* serotypes.

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