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Biosensor for selective detection of *E. coli* in spinach using the strong affinity of derivatized mannose with fimbrial lectin

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ABSTRACT

Escherichia coli (*E. coli*) contamination in foods and water resources represents a major threat for human health and the environment. This work exploits the strong affinity of mannose-containing oligosaccharides with the fimbrial lectin of *E. coli* to design novel biosensors. Modified carbohydrate ligands were synthesized by introducing phenyl residues and aliphatic chains to mannose via reductive amination in order to increase both the affinity and selectivity to *E. coli* compared to other pathogenic bacteria. The synthesized ligands include *p*-thiolphenyl aminomannose (PTAM), *p*-carboxyphenyl aminomannose (PCAM), 1-deoxy-1-aminomannopyranoside (DAMP), glucosamine and low molecular weight chitosan bonded to mercapto undecanoic acid. The structures of the ligands were confirmed using ^{1}H NMR and 1H, ^{13}C, COZY NMR, and ESI/MS. The ligands were immobilized onto gold electrodes and SPR surfaces using-mercaptoundecanoic acid with glycine as deactivating agent. Two detection mechanisms were tested: (i) metal-enhanced electrochemical detection (MED) and (ii) label-free surface plasmon resonance (SPR) detection. The introduction of phenyl residues and aliphatic side groups to the mannose-containing oligosaccharides produced extremely high affinity for *E. coli* with detection limit of 1 cfu/mL. The relative selectivity of these ligands for *E. coli*, *Citrobacter freundii*, *Staphylococcus epidermidis were* 100%, 2.6% and 8.6% respectively. The biosensors were validated using spinach leaves at 3.0 cfu/mL. The work provides a generic biosensor for other pathogenic bacteria by enabling multivalent binding, immediate recognition for pathogens as well as inhibition of bacterial growth.

1. Introduction

Bacterial pathogens pose a significant threat to humans, animals and agricultural health (Call et al., 2003). The CDC estimates that over 48 million people are infected annually with 128,000 hospitalization and 3000 deaths (www.cdc.gov). The major pathogens responsible for these illnesses include *E. coli*, Salmonella, Listeria, Staphylococcus and Norovirus, mostly from food, water and recreational facilities. Contamination has been reported in numerous food-based samples such as processed foods, raw agricultural food products, and even non-agricultural raw materials (Ackers et al., 1998; Bell et al., 1994; Michino et al., 1999). Recently, there was a cantaloupe outbreak in Colorado, USA, which claimed to be the deadliest food outbreaks in more than a decade (Washington, A.P. In Cantaloupe outbreak is deadliest in a decade, 2011).

Current methods used for the detection of *E. coli* are fluorescently-labeled antibodies (Yamaguchi et al., 2003), DNA probes and microarray (Call et al., 2003; Disney and Seeberger; 2004), carbohydrate functionalized nanodots (Tseng et al., 2011), fluorescently-labeled polymers (Disney et al., 2004), fluorescence microarrays (Yu et al., 2002), polymerase chain reaction (PCR) and SPR (Torun et al., 2012). The standard techniques to count different pathogenic species such as *E. coli* include selective plate counting, ELISA (Hu et al., 2009), and PCR (Green and Field, 2012). However, some of these methods rely on selective growth of bacteria, which can take several days to obtain results. Though some methods such as DNA microarrays and immunosensors are faster, they require high user expertise, and expensive antibodies and storage.

With the rising tide of food and raw material imports from countries with varying levels of safety controls, there is an urgent need to develop methods capable of detecting the presence of *E. coli* at infectious doses. The infectious doses of *E. coli* vary between 10 and 100 cfu in food samples (Teunis et al., 2004), and in some cases less than 10 cfu (Gomes et al., 2009) may be infectious. The desirable method should: (i) be rapid, selective and sensitive, (ii) require minimal sample preparation and (iii) promote strong affinity recognition to decrease the risk of false-positive results.

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A large number of bacterial toxins, viruses and bacteria target carbohydrate derivatives on the cell surface to attach and gain entry into the cell (Bouckaert et al., 2005; Disney and Seeberger, 2004). Bacteria also have specialized proteins on their surfaces that enable them to bind to simple sugars such as mannose as well as more complex carbohydrates. The virulence of different bacterial pathogens has been found to correlate with their binding affinity for the carbohydrates (Evanko, 2005; Disney and Seeberger, 2004). Literature has shown that the introduction of hydrophobic side groups such as aliphatic chain and phenyl residues to mannose increases the affinity of mannose to FimH lectin (Bouckaert et al., 2005; Schwardt et al., 2011). However, this idea has yet to be implemented for the development of novel biosensors.

Previously, we reported the development of dissolved oxygen (DOX-96) sensor system coupled with pattern recognition techniques for the identification and classification of 11 types of bacteria (Karasinski et al., 2007). DOX system was successfully applied for bacterial pathogens such as *E. coli, Bacillus subtilis, Bacillus cereus* and *Alcaligenes faecalis* (Karasinski et al., 2005). It is the objective of this work to demonstrate that modifying mannose with phenyl residues; aliphatic side chains and heterocyclic interspacer increases its affinity to fimbrial lectin on E-coli surfaces. We introduced hydrophobic groups to mannose via reductive amination which has increased both the affinity and selectivity of mannose to *E. coli* in comparison to *Citroobacter freundii* and *Staphylococcus epidermidis*. This is the first study in which mannose is directly used to detect *E. coli* at infectious doses with unique specificity towards *E. coli*. Validation of the developed method was performed using baby spinach samples with high reliability.

2. Materials and methods

2.1. Materials

Aminophenyl disulfide, *p*-amino benzoic acid, D-(+)mannose, D-(+)-glucosamine hydrochloride, low molecular weight chitosan, dimethylamine borane, silver nitrate ($AgNO_3$), *potassium ferrocyanide* ($K_3Fe(CN)_6$, agar plates, corning centrifuge tubes, Mueller Hinton broth, Mueller Hinton agar, Tryptic soy broth and Tryptic soy agar were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), acetic acid, hydrogen peroxide (H_2O_2) and potassium chloride (KCl) were purchased from J.T Baker Chemicals (Philipsburg, N.J). Acetic acid-d4 and deuterium oxide (D_2O) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 1-deoxy-1-aminomannopyranoside was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). *E. coli* ATCC® 25922™, *C. freundii* ATCC® 8090™ and *S. epidermidis* ATCC® 12228™ were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Silver and platinum wires were purchased from BASi analytical instruments (West Lafayette, IN, USA). A T-cut gold-quartz crystals (9 MHz, area = 0.2 cm^2) were purchased from International Crystal Manufacturers (OK, USA).

2.2. Methods

2.2.1. Modification of mannose

Hydrophobic side groups were introduced to mannose in order to enhance its affinity to *E. coli* (**Scheme 1**) by following the procedure previously applied to different carbohydrates other than mannose (Seo et al., 2007).

Briefly, D-(+) mannose was dissolved in water at 100 mM while aminophenyl disulfide and *p*-aminobenzoic acid were dissolved in acetic acid at 50 mM and 100 mM, respectively. The mannose with aminophenyl disulfide or *p*-amino benzoic acid solutions were mixed and incubated in sealed tubes for 1 h at 20 °C for the amination reaction step. Then, freshly prepared, 100 mM dimethylamine borane reducing agent was added to the reaction and the tube incubated unsealed for 1 h at 20 °C for the reduction step in the case of aminophenyl disulfide. The tube was then sealed and heated at 50 °C under streaming nitrogen gas for the condensation step. The mixture was finally cooled in ice-bath and filtered to obtain the modified mannose. In the case of *p*-aminobenzoic acid, the reduction step was carried out overnight in fume hood in unsealed tube. As acetic-acid evaporates, *p*-carboxyphenylamino mannose single crystals were formed. All modified mannoses were preserved at −20 °C under light and moisture blocking conditions. NMR characterization revealed the obtained product is stable for 14 months.

2.2.2. Characterization of the modified mannose derivatives

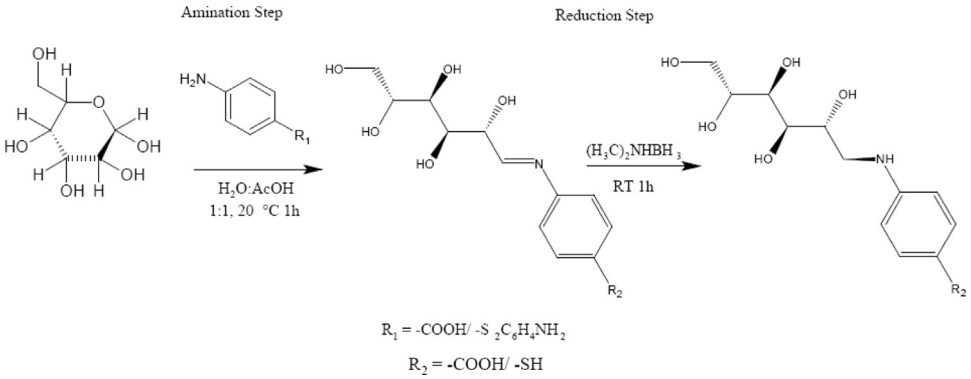
The structures of the modified mannose were confirmed using ^1H NMR are results shown in Supplementary section 1. The conditions of ESI/MS were explained in Supplementary data while ESI/MS spectra are given in Fig. 3.

2.2.2.1. SPR characterization. Synthesized modified mannose ligands were used to target FimH lectin; the ligands include *p*-thiolphenyl aminomannose (PTAM), *p*-carboxyphenyl aminomannose (PCAM) and 1-deoxy-1-aminomannopyranoside (DAMP). SPR characterization of the ligands was initially performed and was followed by *E. coli* detection:

2.2.2.1.1. p-Thiolphenyl aminomannose (PTAM). Thiolated mannose was immobilized on the SPR sensor gold disk by utilizing the thiol group from the aminophenyl disulfide. 1 mM of *p*-thiolphenyl aminomannose prepared in the 1:1 acetic acid:water was applied to gold surface with ligand-immobilization sequence. In this respect, Channel 1 was used for the modified mannose while Channel 2 was used for the unmodified mannose.

2.2.2.1.2. p-Carboxyphenyl aminomannose (PCAM). In the case of *p*-carboxyphenyl aminomannose, the 11-mercaptoundecanoic acid (MUA) gold surface was modified with L-lysine to introduce primary amino which was activated by EDC/NHS. 1 mM *p*-carboxyphenyl aminomannose, dissolved in 1:1 acetic acid:water, was applied to activated surface; glycine was used as deactivating agent.

2.2.2.1.3. 1-Deoxy-1-aminomannopyranoside (DAMP). For 1-deoxy-1-aminomannopyranoside, the gold surface was activated with MUA, followed by modified with *p*-amino carboxybenzoic acid. 1-deoxy-1-aminomannopyranoside was used as deactivating agent of uncoupled EDC/NHS-activated carboxyl groups of MUA. Then, 1-deoxy-1-aminomannopyranoside was introduced to the system via EDC/NHS mediated amide bond formation. However, D-glucosamine and chitosan were directly introduced to MUA modified gold surface via EDC/NHS chemistry. In all cases, the sensor surface was stabilized using 0.1 M NaOH as dissociation buffer and 50 mM pH 4.5 acetate buffers as coupling buffer. The auto sampler was prepared and the solutions put in the microtiter plate as dictated by the sequence. The analysis was programmed as follows: baseline (120 s), ligand coupling (900 s); deactivation (600 s); and regeneration (120 s). The running buffer (PBS pH 7.2) was used for the baseline when a new measurement cycle was started. Deactivation time was 0 s for case 1 and 600 s for cases 2 and 3. SPR characterization of the modified sugars is shown in S2.



Scheme 1. Two steps modification of mannose via reductive amination in order to introduce *p*-thiol aniline and *p*-amino benzoic acid.

2.2.3. Metal enhanced detection (MED) biosensor

MED based *E. coli* detection was performed for *p*-thiolphenyl aminomannose. MED is a label-free electrochemical detection method (Aluoch et al., 2005; Jennings and Laibinis, 1996; Yan and Sadik, 2001; K'Owino et al., 2003, 2007; Mwilu et al., 2009; Noah et al., 2011a, 2011b). MED measurements were performed in a conventional three electrode cell using Princeton Applied Research potentiaostat equipped with WinEchem data acquisition software. A T-cut gold-quartz crystals (9 MHz, area = 0.2 cm^2) purchased from International Crystal Manufacturers (OK, USA) were used as the working electrode, silver wire as the reference electrode while platinum wire was used as the counter electrode. The gold quartz crystals were cleaned in freshly prepared piranha solution (30% H_2O_2:H_2SO_4, 1:3V/V), rinsed with water and ethanol and finally blow dried with nitrogen gas. A well-type electrochemical cell, made from Teflon with a capacity of 300 μL was used for the electrochemical measurements. All QCM experiments were performed using a QCA 917- quartz crystal analyzer.

2.2.4. SPR-based biosensor for the detection of E. coli

PCAM, PTAM and DAMP were used as ligands for SPR experiments. Different concentrations (1.0×10^{11} cfu/mL) of *E. coli* were prepared in sterile PBS (pH 7.4) buffer from the stock solution and used for the interaction with the immobilized modified mannose on the surface of the SPR disk. PBS buffer was used as the baseline and wash buffers, while 0.1 M NaOH was used as the dissociation and regeneration solution. The auto-sampler and the microtiter plate were then prepared for the interaction using the following protocol: baseline (120 s); association (900 s); dissociation (600 s); and regeneration (120 s), if otherwise specified.

2.2.5. Selectivity

Selectivity was evaluated by using *C. frenduii* and *S. epidermidis*, which have been reported to exhibit mannose binding capability (Jones et al., 1995; Kristian et al., 2008). In this case, PTAM and DAMP were used because they provide distinct structures of importance in carbohydrate–lectin interaction. SPR response for similar concentrations of these three bacteria and cross-reactivity studies were evaluated at different concentrations for both ligands.

2.2.6. Real sample application

Inoculation of *E. coli* to spinach leaves was performed using prior literature with some modification (Linman et al., 2010). Fresh baby spinach leaves were purchased from a local grocery store (Weiss, Vestal, NY) and stored at 4 °C until usage. Fig. 1 shows the procedures employed for the spinach testing. Fresh baby spinach leaves containing no visible cracks or scratches were soaked into 70% ethanol for 30 s, and then rinsed with excess pure water. The spinach leaves were dried using nitrogen gas flushing at mild flow rate. *E. coli* at 10^5–10^9 cfu/mL from nutrient broth medium was inoculated onto the leaves, followed by a 3-h incubation at 37 °C in incubator. The number of inoculated *E. coli* was determined by optical density method. After incubation, unbound or weakly bound *E. coli* were removed by PBS rinsing, while the attached *E. coli* were recovered using buffered peptone rinsing. 1 mL of the recovered *E. coli* was centrifuged at 5000 rpm for 5 min, and the resulting samples were utilized for SPR detection.

3. Results and discussion

3.1. Sensor concept and design of modified mannose ligands

The sensor concept is based on the hypothesis that the introduction of phenyl residues and aliphatic chains to mannose via reductive amination increases both the affinity and selectivity to *E. coli* compared to other pathogenic bacteria (Fig. 2a). The resulting modified carbohydrate-based sensors could accommodate multivalent binding. These modified surfaces should be applicable to any transducer surface including optical, electrochemical or QCM surfaces.

The structures of modified ligands and surface immobilization onto SPR surface and gold electrodes are shown in Fig. 2b. In (a), PTAM was synthesized from *p*-thiolaniline and mannose to enable it to bind directly to gold surface. PTAM contains no mercapto undecanoic acid. In (b), glucosamine was immobilized onto gold electrodes and SPR surfaces via mercapto undecanoic acid modified gold surface using glycine as deactivating agent. In (c), DAMP directly binds to mercaptoundecanoic acid modified gold surface using glycine as deactivating agent. In (d), PCAM was synthesized from *p*-aminobenzoic acid and mannose. This enabled it to bind to mercapto undecanoic acid modified gold surface via L-lysine linkage. Glycine was used as deactivating agent. In (e), *p*-aminobenzoic acid was first linked to mercapto undecanoic acid modified gold disk. This was followed by introduction of 1-deoxy-1-aminomannopyranoside using glycine as deactivating agent. Finally, in (f), chitosan was introduced to mercapto undecanoic acid modified gold disk using glycine as deactivating agent.

3.2. Characterization of modified mannose ligands

Mass spectrometry with ESI ion source was used to investigate the molecular weight of the modified mannoses. The molecular weights of modified ligands were predicted from ChemDraw (CambridgeSoft, MA). In addition, MS/MS peaks revealed possible fragmentation which overlaps with ChemDraw prediction. These two molecules differ in their *p*-groups; –COOH group displaces –SH group. The molecular weight differences between these two

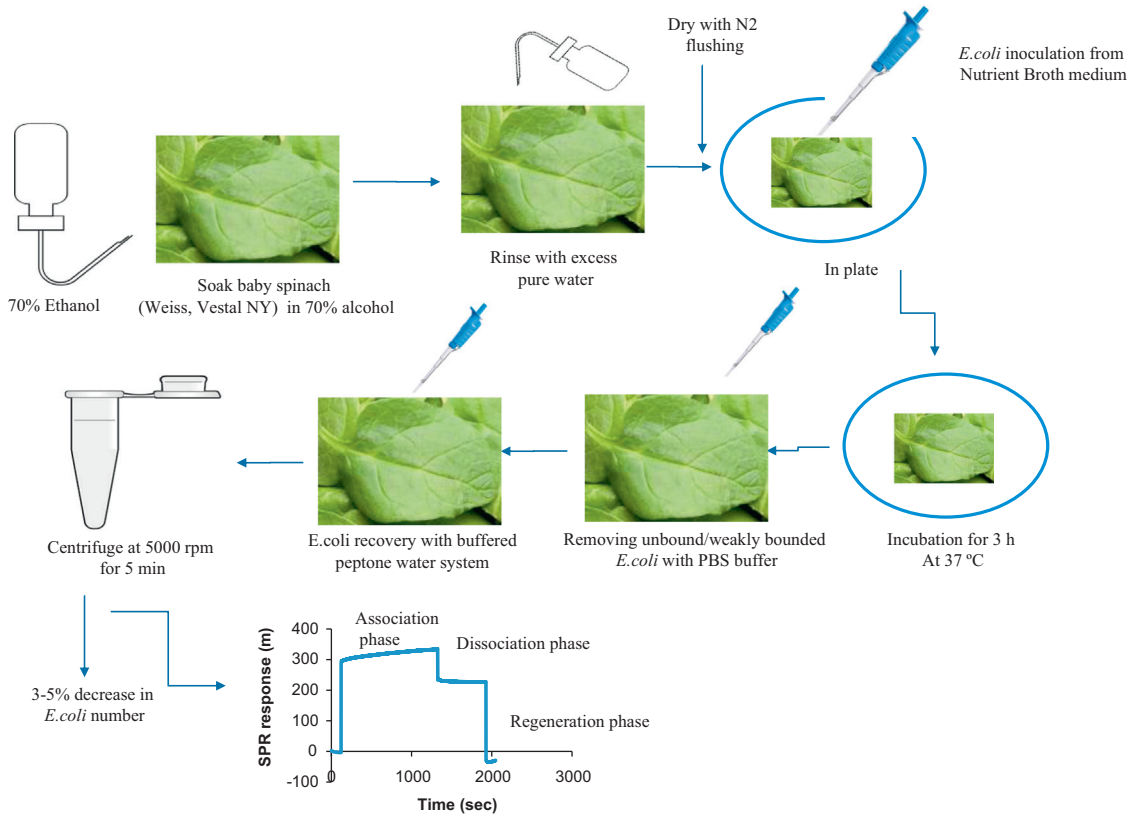


Fig. 1. Biosensor validation for *E. coli* detection using baby spinach samples.

molecules were around 12, and the MS data clearly showed the peaks as around 290 m/z and 302 m/z for former and latter molecules, respectively. MS data for both molecules gave MS/MS peaks around 180 which belonged to mannose. Fig. 3 shows the MS spectra obtained for (a) PCAM and (b) PTAM: MS/MS data gave peaks around 258 m/z and 278 m/z indicating partial or total cleavage of carboxyl group for *p*-carboxyphenyl aminomannose.

NMR characterization of *p*-carboxyphenyl aminomannose and *p*-thiolphenyl aminomannose revealed that D-mannose lost its anomeric shifts at 4.93 and 5.21 and the products gave shifts between 6.7 and 7.9 belonging to phenyl rings. Additional details of NMR characterization are discussed in Supplementary section. NMR data confirmed that mannose had been successfully modified with the selected linkers.

3.3. Electrochemical detection of E. coli

We tested the MED concept in the development of a label-free MED biosensor for detection of *E. coli* which carries type 1 Fimbriae specific to mannose binding (Schwardt et al., 2011). We modified mannose by introducing thiol groups for immobilization on the QCM crystal used in the experiments. We then utilized this modified mannose as a receptor to directly detect *E. coli*. The signal was based on the redox peaks from silver underpotentially deposited on a QCM crystal. The thiolated mannose was immobilized on the silver modified gold surface. When different concentrations of *E. coli* (6.25×10^2–6.25×10^7 cfu/ml) were introduced into the medium, the molecular recognition occurring was evidenced by a corresponding change in the redox properties of the silver monolayer. Changes in the redox currents were dependent on the concentrations of the analyte. The electrode was then characterized using QCM and CV. The redox activity of the silver deposited on the gold surface was monitored by CV. Both cathodic and anodic peak currents indicated a decrease in the intensity as the concentration of *E. coli* was increased. This further confirmed that *E. coli* bound to the modified mannose immobilized on the gold sensor surface leading to insulation of the electroactive part of the electrode and hence the decrease in the current. The extent of insulation was found to be proportional to the concentration of *E. coli* (Fig. 4a).

A plot of the change in the anodic peak current versus the *E. coli* concentration was linear between 6.25×10^2 and 6.25×10^6 cfu/ml. The lowest concentration detected was 6.25×10^2 cfu/ml but from the data it is clear that much lower concentrations can be detected (Fig. 4*a*). The detection time was 10 min for *E. coli*-ligand saturation. These results indicated that MED provides fast, reliable and sensitive method in detection of bacterial pathogens.

3.4. SPR-based biosensor for the detection of E. coli

SPR is a surface-sensitive analytical technique based on the ability to detect small changes in refractive index induced by molecular adsorption at a noble metal film (Lin et al., 2006; Torun et al., 2012). Biospecific interactions were monitored via angular spectral or phase characteristics of the reflected light (Liang-Ping et al., 2005; Lin et al., 2006). Fast, selective and sensitive detection of pathogens are crucial in terms of prevention pathological-microorganism mediated food outbreaks. Lectin–carbohydrate interactions have been widely studied in biosensor applications in order to develop biosensors (Disney et al., 2004; Sadik and Yan, 2007). Particularly for *E. coli*, mannose–FimH interaction has been preferred to meet these requirements.

Two different detection ranges were chosen for SPR; *PTAM* was evaluated in the range of 1.25×10^2–1.25×10^7 cfu/mL with detection limit of 1.25×10^3 cfu/mL. In addition, increasing the range up to 10^{11} cfu/mL showed that SPR has a wider detection range and can go up to 10^{11} cfu/mL. In contrast to this, MED-based detection showed narrower linear range. Even though the LOD of MED-based

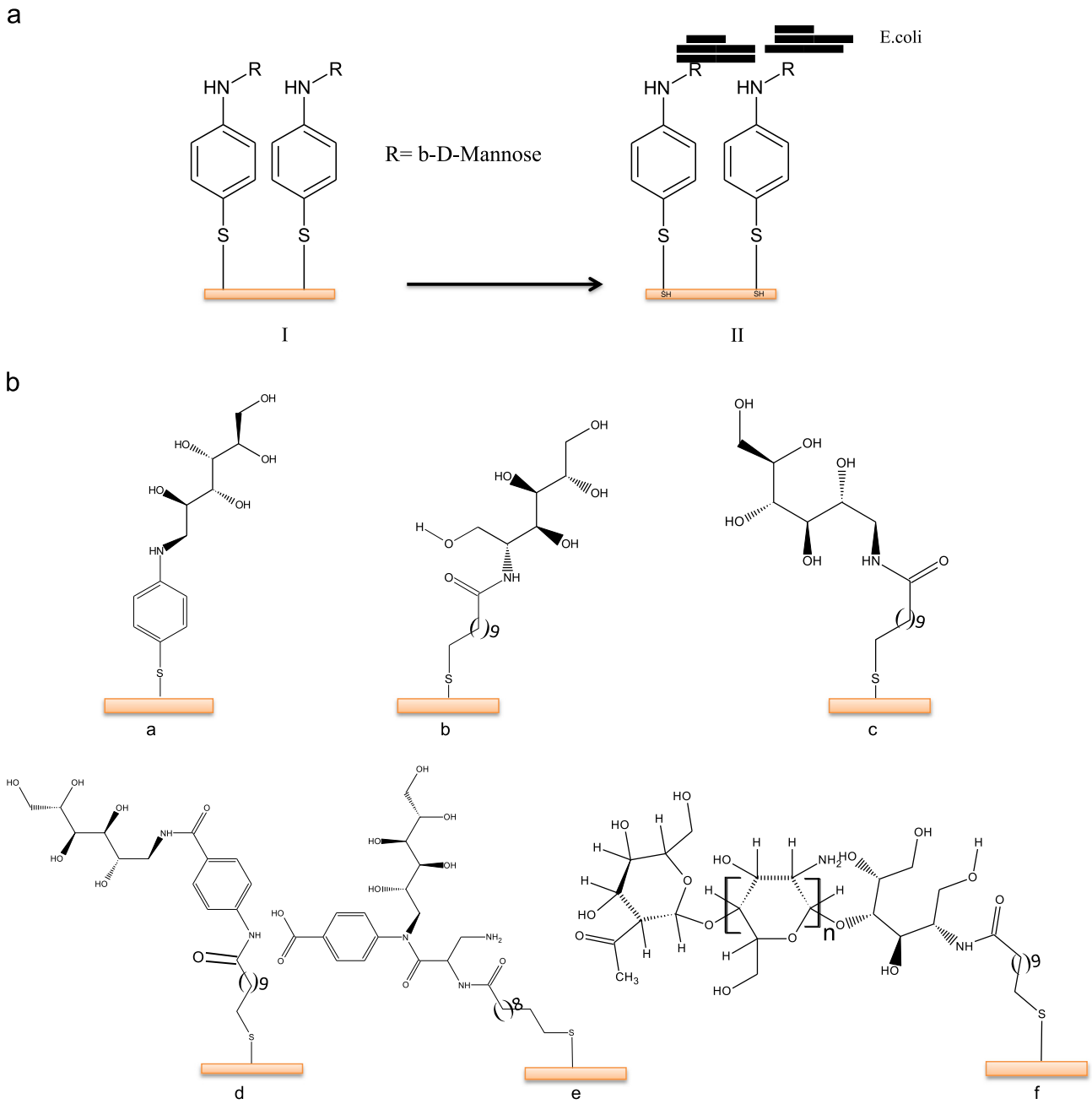


Fig. 2. (a): Proposed concept for *E. coli* using modified mannose via reductive amination to increase both the affinity and selectivity, (b) structures and linkage procedures employed for modified mannose ligands for *E. coli* (a) PTAM; (b) modified glucosamine, (c) DAMP bonded to mercaptoundecanoic acid modified gold surface; (d) *p*-aminobenzoic acid bonded to mercapto undecanoic acid modified gold disk using DAMP (e) PCAM bonded to mercapto undecanoic acid modified gold surface via L-lysine linkage, and (f) chitosan bonded to mercapto undecanoic acid modified gold disk.

E. coli detection was better, SPR was chosen for further work due to its automated-nature and ease of regeneration. Actually, 1.25×10^3 cfu/mL is such a high LOD value, and even glucosamine and chitosan gave similar LOD values as 8×10^3 and 1.1×10^4 cfu/mL. Glucosamine and chitosan have similar chemical structures to N-acetyl glucosamine, which has affinity to FimG (Westerlund-Wikstrom and Korhonen, 2005), thus explaining the high affinity.

We are not aware of any prior work in which, glucosamine and chitosan were reported for this purpose. It is possible that by utilizing 11-mercaptoundecanoic acid as linker significantly increased the SPR resolution so these molecules provided unexpectedly low LOD values (Table 1). This observation motivated us to introduce a long linker to the phenylated mannose in order to increase resolution without affecting the selectivity. It has been reported that introducing linkers between ligands and the gold surface improves resolution (Torun et al., 2012). In this respect, *PCAM* was evaluated in the range of $17–17 \times 10^5$ cfu/mL with 17 cfu/mL obtained as LOD. As detailed in literature, the presence of oxo-group enhances mannose–FimH binding potency (Bouckaert et al., 2005; Rabbani et al., 2010; Schwardt et al., 2011). Despite the presence of oxo-group in between L-lysine and *PCAM*, there was no oxo-group between D-mannose and phenyl ring; reductive amination did not allow this introduction. To overcome this problem, *p*-aminobenzoic acid and 1-deoxy-1-amino mannopyranoside were sequentially introduced to MUA modified gold surface (Fig. 2b). As expected, the introduction of oxo-group enhanced the affinity through possible hydrophobic interactions with side groups around the tyrosine gate.

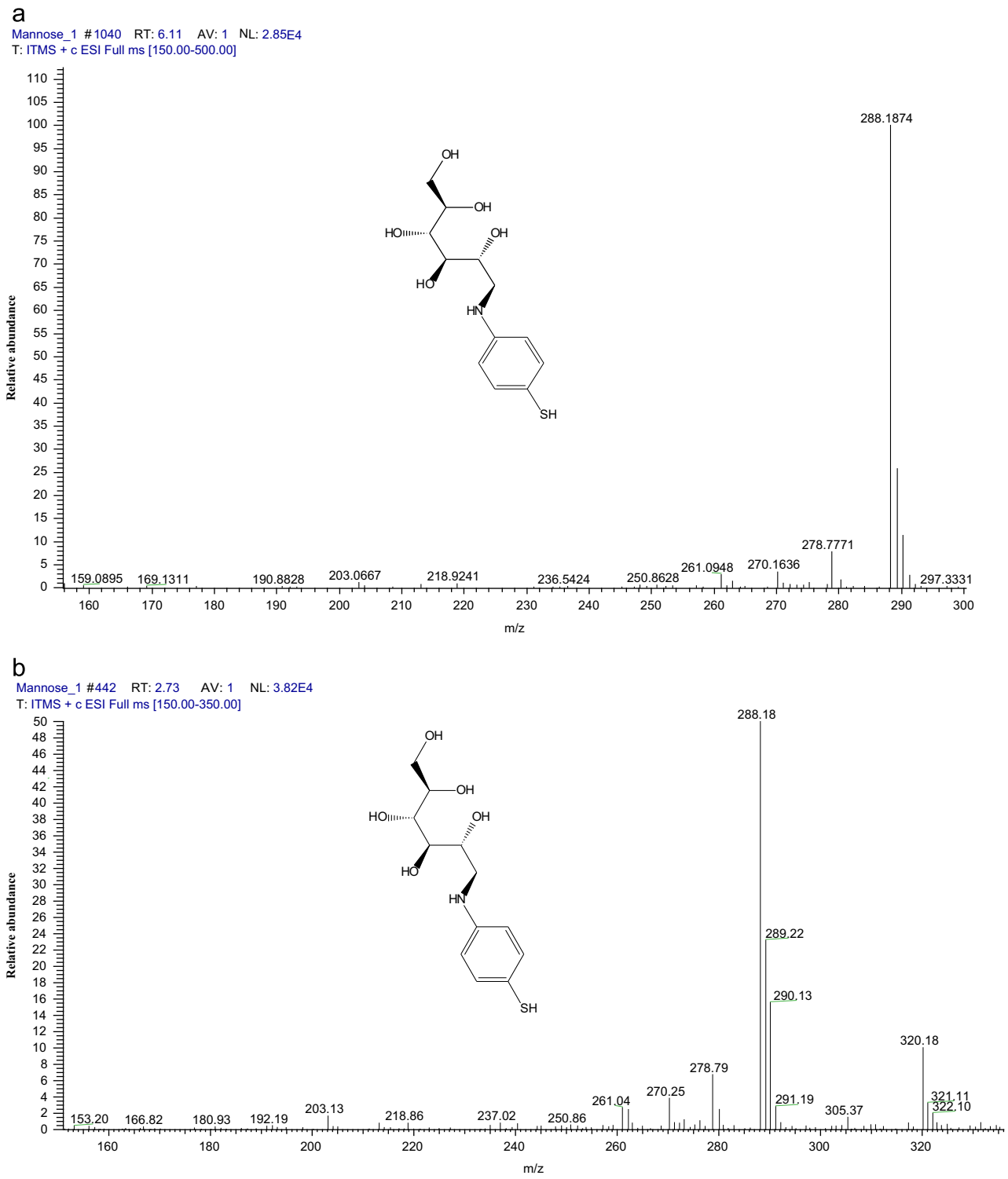


Fig. 3. (a) Typical MS spectra of *p*-carboxyphenyl aminomannose and (b) *p*-thiophenyl aminomannose.

According to Rabbani et al. (2010), the IC_{50} of mannose to FimH decreased from 2.4 μM to 0.102 μM and 0.065 μM after the introduction of phenyl ring and n-heptyl group, respectively. Moreover, it is possible that the carbohydrate recognition domain of FimH of *E. coli* is large and deep enough to capture the ligands used in this study (Schwardt et al., 2011). Therefore, it was predicted that high affinity will be obtained via the introduction of phenyl and aliphatic linker in between the gold surface and the mannose moiety. As predicted, we found that SPR gave linear response between 2.5 and 2.5×10^5 cfu/mL with 2.5 cfu/mL recorded as the LOD with average correlation coefficient of 0.9784 R^2 range for $N=5$ experiments. The current approach shows the lowest LOD value with perfect reusability and regeneration.

3.5. Selectivity

In addition to sensitivity, high selectivity of the method is expected based on carbohydrate-lectin interaction. In this study *C. frenduii* and *S. epidermidis* were used to evaluate the specificity

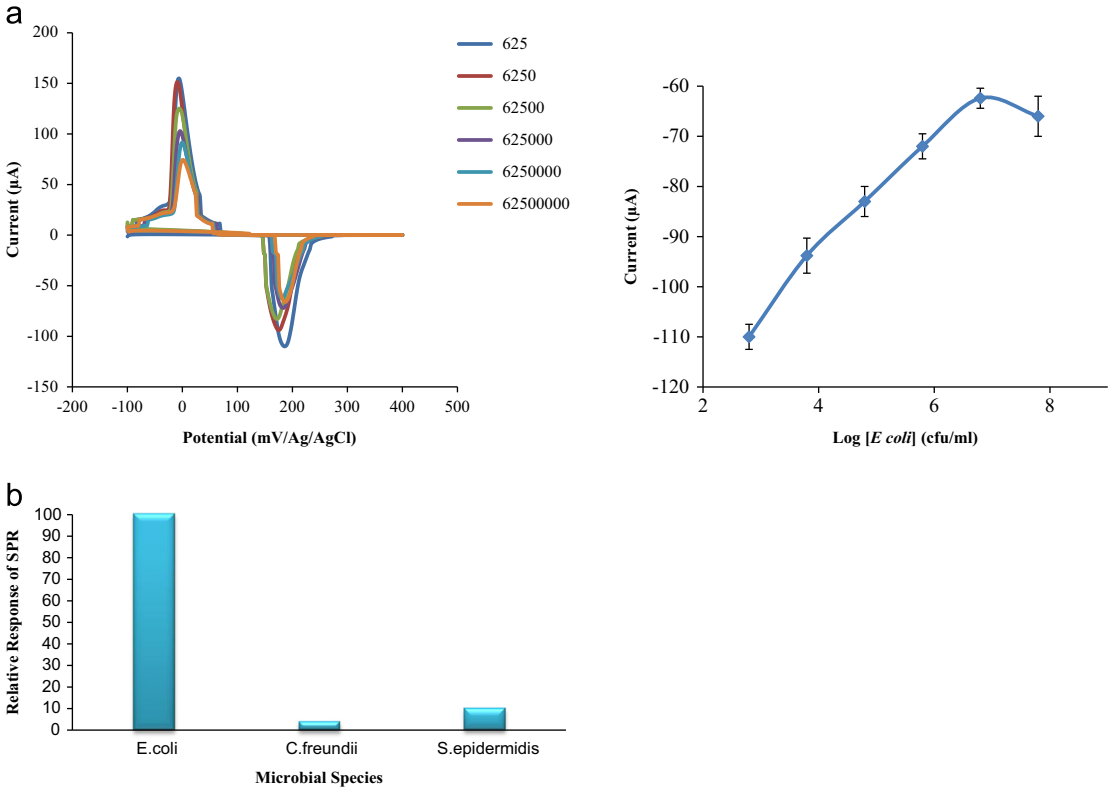


Fig. 4. (a) Voltammograms obtained after incubating the mannose modified electrodes with different concentrations of *E. coli*. (b) Calibration curve using a plot of the anodic peak current versus *E. coli* concentration. Linear detection range was obtained as 6.25×10^2–6.25×10^6, (b) Selectivity of thiolated modified mannose for *E. coli* compared to *C. freundui* and *S. epidermidis* at PTAM modified mannose using SPR detection.

Table 1
Effect of ligands on biosensor sensitivity.

Ligand	LOD [cfu/mL]
PCAM	17.0
PTAM	1250
DAMP plus benzoic acid	2.5
DAMP only	1.0
Chitosan	1.1×10^4
D-Glucosamine	8×10^3

of the ligands binding to *E. coli* and *C. freundii* belong to the Enterobacteriaaceae family in which all members are capable of producing type 1 pili, and hence should exhibit mannose binding ability (Jones et al., 1995). *S. epidermidis*, a common skin related pathogen is a gram positive bacterium which has mannose binding outer-surface protein (Kristian et al., 2008). As seen in Fig. 4b, SPR gave strong selectivity for *E. coli* over *C. freundii* and *S. epidermidis* as the relative responses were 100, 4.0 and 10.3, respectively.

The introduction of these linkers to mannose can alter its binding to FimH lection (Schwardt et al., 2011), which could should result in high specificity of both ligands for *E. coli* recognition. When the microbes were exposed individually to PTAM, the selectivity for *E. coli* detection was 100% at 10^4 cfu/ml whereas the SPR response for *C. freundii* and *S. epidermidis* at 5×10^4 cfu/ml were 3.0 and 10.3 cfu/ml respectively. No responses were recorded for both *C. freundii* and *S. epidermidis* at10^3 cfu/ml. When mixtures of microbes were tested, all ligands still showed greater than 99% selectivity for *E. coli*, suggesting that the modification has produced highly selective recognition for *E. coli*. However, when DAMP was used directly, higher cross reactivity was noticed for *C. Freundii* (26.5%) and *S. epidermidis* (35%). Selectivity of the technique was so high despite the fact that *C. freundii* and *S. epidermidis* have mannose binding abilities. However, introducing different linkers to mannose can alter its binding abilities to FimH lectin. It is likely that our modified ligand does not fit into the mannose binding lections of the two comparable bacteriae. *S. epidermidis* showed higher affinity towards the ligand, and this is not surprising because *S. epidermidis* has mannose binding lectin which plays major role in its pathogenicity.

3.6. Real sample application

The detection of *E. coli* in food samples has become important with spinach-related *E. coli* outbreak in California (Linman et al., 2010). *E. coli* was spiked onto disinfected fresh baby spinach and was subsequently recovered by peptone (Fig. 1). The idea was to demonstrate the power of the detection system by using *E. coli* at high peptone concentration without peptone-related interference. We tested the disinfection quality of the leaves following peptone treatment by putting two leaves into agar for 48 h at 37 °C in incubator. Results show that no colony formation was observed. Standard curve of *E. coli* was generated by preparing a gradual increase in *E. coli* concentration using PBS buffer. PBS buffer was observed to consistently give negative response, which is expected for well-cleaned surfaces. Two different concentrations (3.4×10^8 and 1.13×10^7 cfu/mL) of recovered *E. coli* from spinach surface were evaluated with SPR for PTAM ligand. Dilution was performed using PBS and *E. coli* free peptone as blank. We compared the signal obtained from plate counting and SPR biosensor based on repetitive plate counting from the same standards, slightly different cfu/mL values was obtained. It should be noted that dilution was made with PBS buffer and to obtain low cfu/mL values higher amount of dilutions (e.g., 10^4–10^6) needs to be prepared in order to eliminate the peptone effect and make the medium more like PBS medium. *E. coli* was used at 200 cfu/mL and 3 cfu/mL

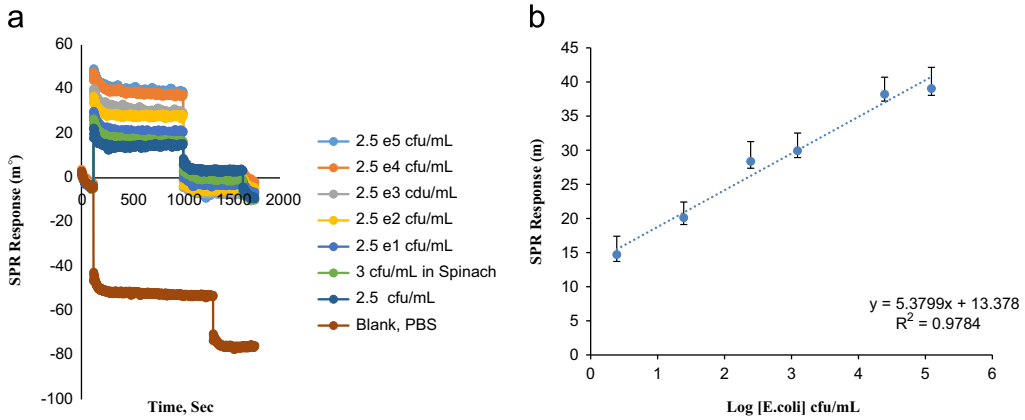


Fig. 5. (a) Calibration curve for SPR biosensor for *E. coli* detection in spinach sample via utilizing 1-deoxy-1-aminomannopyranoside (the ligand c shown in Fig. 2b). A linear detection range of 2.5–1.25 × 10^5 cfu/mL was obtained, and *E. coli* was spiked at 3.0 cfu/mL concentration onto spinach leaves. (b) Calibration curve obtained for *E. coli* within the range of 2.5–1.25 × 10^5 cfu/mL utilizing the ligand c.

concentrations for PCAM and DAMP respectively. Fig. 5 shows real-time SPR detection of *E. coli* recovered from *E. coli* spiked baby spinach leaves. A linear detection range was obtained between 2.5 and 1.25 × 10^5 cfu/mL, and *E. coli* was spiked at 3.0 cfu/mL concentration onto spinach leaves. (Fig. 5b) Calibration curve obtained for *E. coli* at the range of 2.5–1.25 × 10^5 cfu/mL via utilizing the ligand d. SPR provided excellent reusability even when real spinach samples were tested. The sensor allows multiple analyses with one calibration and 97% overlap was recorded at varying cfu/ml of *E. coli*.

4. Conclusion

We have synthesized modified mannose ligands in order to enhance the selectivity of carbohydrates as recognition platforms for *E. coli*. The ligands were subsequently employed for the development of SPR and electrochemical biosensors. Results showed that by introducing key functional groups to the phenyl-ring attached to mannose, a significantly decrease was observed to the limit of detection from 17 to 2.5 cfu/mL without affecting the selectivity. Results also showed that the different amino acids can influence its affinity for *E. coli*; which ultimately reduced the detection limit down to pathogenic levels of *E. coli*. It is possible that the strong interaction observed was derived from π–π interactions of phenyl substitution of the ligand and aromatic side rings of the amino acid located around entrance of the carbohydrate recognition domain and/or tyrosine gate (Bouckaert et al., 2005; Schwardt et al., 2011). To the best of our knowledge, this is the first study using mannose as a direct ligand to detect *E. coli* having one of the lowest detection limits at pathogenic levels. This approach could offer cheap, fast and sensitive alternatives to antibody-based and tedious plating counting technology for the detection of bacterial pathogens.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.05.008.

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