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Dendrimeric biosensor for detection of *E. coli O157:H7* in diet

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ABSTRACT
The focus of present research work was to ensure the safety of variety of products like milk, bread, ice-cream, curd, etc. by detecting harmful pathogen i.e. *E. coli O157:H7.* This study evaluated the presence of *E. coli O157:H7* in fresh food and water. Food samples and water were artificially contaminated with the known concentration of *E. coli O157:H7* and the concentration was estimated after 8 and 12h. Estimation of *E. coli O157:H7* was determined by adding prepared dendrimeric biosensors to detect *E. coil O157* using ELISA 96 Wells and the end point was estimated by measuring the pH change. The prepared biosensors showed the limit of detection in range of 10^{1}-10^{-8} CFU/mL. Estimated detection time for detecting *E. coli O157:H7* from diet was found to be 2h. It is concluded that modern biosensor-based method for estimating *E. coli O157:H7* in diet is sensitive, specific and precise.
Keywords: *Pathogen; biosensor; E. coli O157:H7; dendrimers.*

1. INTRODUCTION
Dendrimers are novel nanoparticulate systems that resemble the structure of tree [1]. These are gaining attention because they have large surface area than nanoparticles and monodispersed macromolecule made up of EDA and polymer like MM. They are also known as cascade molecules containing many functional group and concise molecular structure [2]. They are highly branched symmetric molecule with core at the center and prepared by two methods i.e. divergent and convergent. Dendrimers show several applications in different fields like biomedical field [3] as blood substitute, [4] drug delivery system, [5] imaging contrast agent, [6] solubility enhancer, photodynamic therapy etc. The stability of proteins and enzymes can be improved by immobilizing it onto the surface of dendrimers.
E.coli O157:H7 [7] is harmful pathogenic strain of *Escherichia coli* that mainly causes contamination in food. Consumption of food contaminated with this pathogen causes various food-borne acute and chronic problems like nausea, vomiting, watery diarrhoea, haemolytic anaemia, renal failure, gastroenteritis, haemorrhagic colitis, etc. There are several [8] conventional methods like plating, biochemical test, culturing, etc for detecting *E. coli O157:H7* but they are less sensitive and not reliable. Hence sensitive, precise, specific and reliable method is the need of today to detect this pathogen.

Biosensors are modern miniature, pocket size devices that can be used for the estimation of various biological as well as chemical substances [9]. Biosensors have many applications such as screening of drug, [10] diagnosis, monitoring safety of food, etc. Different nanoparticles [11] such as nanorods, nanoflowers, carbon nanotubes, dendrimers, nanodots, etc can be used for fabrication of biosensors for detecting food pathogens. In this research, we have synthesised dendrimeric polyvalent biosensors and provide large surface area for immobilization of concanavalin A and glucose oxidase on its surface. Dendrimers improves stability and durability of glucose oxidase and concanavalin A. So, the aim of the present work was to develop and characterize the dendrimeric biosensors for *E.coli O157:H7* detection in diet.

2. MATERIALS AND METHODS
2.1. Materials.
Calcium dihydrogen phosphate was obtained from Central Drug House, New Delhi, India. Glucose oxidase was purchased from SRL, Mumbai, India. Concanavalin A was purchased from HiMedia lab, Mumbai, India. Ethylene diamine (EDA) was purchased from LOBA Chemie, Mumbai, India. Methyl methacrylate (MM) was obtained from Otto Chemie, Mumbai, India. *E.coli O157:H7* was purchased from NCIM, Pune, India. Sorbitol MacConkey agar media was purchased from HiMedia lab, Mumbai, India. Enterohaeorrhagic E.coil, EHEC 0157 (E.coil O157) ELISA 96 Wells to *E.coli O157:H7* was purchased from Krishgen biosystems, Mumbai, India.
2.2. Methods.
2.2.1. Preparation of PAMAM dendrimers of generation 4 (G4).
Michael addition and amidation method were used to synthesize PAMAM dendrimers (G4). Ethylenediamine (EDA) and methylmethacrylate (MM) were taken in 1:1 ratio and allowed to react at 37º C, 180 rpm for 21 h. Further, it was again treated with 3ml of MM to form G0.5 dendrimers. G0.5 dendrimers were treated with 3ml EDA to form G1 dendrimers. G1 dendrimers were treated with 4ml of MM to form G1.5 dendrimers. G1.5 dendrimers were treated with 4ml of EDA to form G2 dendrimers. G2 dendrimers were treated with 5ml of MM to form G2.5 dendrimer. G2.5 dendrimers were treated with 5ml of EDA to form G3 dendrimers. G3 dendrimers were treated with 6ml of MM to form G3.5 dendrimers. G3.5 dendrimers were treated with 6 ml of EDA to form G4 dendrimers. This formulation is abbreviated as F0.
2.2.2. Preparation of nanoparticles and dendrimericbiosensors.
Nanoparticles were prepared using concanavalin A, glucose oxide and calcium hydrogen phosphate. In this synthesis, 1.38 mg of concanavalin A and 0.115 mg of glucose oxidase were

dissolved in 5ml phosphate buffer saline (pH 6.8). To the above mixture 1.5mg of calcium hydrogen phosphate and 1mg calcium chloride were added and allowed to react for 8h. Finally, the mixture was filtered and dried at room temperature. This formulation is abbreviated as F1. Ten milligrams of nanoparticles were accurately weighed and added to 1g of (G4) dendrimers and allowed to react at room temperature for 12h. This formulation is abbreviated as F2.

2.2. Characterization.

2.3.1. Size and surface charge.

The particle size and surface charge of formulations F0, F1 and F2 were examined by Malvern zetasizer (UK) using Millipore water as a medium at ambient temperature.

2.3.2. Copper sulphate test.

Copper sulphate test was done to confirm the formation of half and full generations of dendrimers. Ten percentage of 3ml copper sulphate solution was added to dendrimers and colour change was observed.

2.3.3. FTIR spectroscopy.

FTIR spectrometer (Perkin Elmer, USA) was used to study the chemical characteristics of formulations F0, F1 and F2 at wavelength 400-4000 cm^{-1} by using KBr pellet method.

2.3.4. SEM (Scanning electron microscopy).

Morphology of formulations F0, F1 and F2 were assessed using FEG-SEM (S48001, Type II, and Hitachi, Japan). The steps for SEM studies include preparation of sample, surface cleaning, sample stabilization, rinsing and drying the sample, mounting sample on metal holder and coating the sample with an electrically conductive material.

2.3.5. Immobilization efficiency.

Immobilization efficiency of glucose oxidase onto the surface of dendrimers was determined using fluorometer (Elico, India).

2.3.6. pH measurement.

pH of the samples like masala rice, schezwan rice, bread, bhel, soya vegetable, upma, ice cream, chana masala, curd, usal, mix vegetable, coconut chutney, green chutney, schezwan chutney, misal, pavbhaji, cold coffee, sambar, dal, raw milk, boiled milk, tap water, distilled water, purified water were measured using (Labindia, India) pH meter.

2.3.7. Method for estimation of E.coli O157:H7.

200 µL of food sample solution was added to antibody plate and incubated at 37ºC for 60 mins and then washed with buffer 3 times. 150µL of dendrimer solution was added to the plate and incubated at 37ºC for 30 mins and then washed with buffer for 3 times. 200 µL of 0.5M glucose solution was added to the plate and incubated at 37ºC for 30 mins. Finally, pH of the solution was measured using pH meter (PICO+ LABINDIA, India).

Twenty-four different samples were taken in a sterile test tube and diluted with sterile distilled water. These samples were added to the antibody plate and incubated for 1h and washed with buffer for 3 times. The prepared dendrimeric biosensors were diluted 10 times with distilled water and added to the plate. Finally, 0.5 M of glucose solution was added to the plate and after 30 mins incubation pH of samples was measured. All the fresh samples showed neutral pH in range of 6.6-7.1 which indicated that the samples were not contaminated with *E. coli O157:H7* and are safe to consume. One ml of 10^2 CFU/mL samples were added to all the samples and kept for 8h. All the samples were again added to plate and incubated for 1h and washed 3 times with buffer. Then dendrimeric biosensors were added to plates and incubated for 30 mins and washed with buffer for 3 times. Finally the prepared glucose solution was added to the plates and incubated for 30 mins and pH of all samples was measured using pH meter. The same samples were kept for 24h and again added to the plate and incubated for 1h and washed with buffer for three times. Dendrimeric biosensors were added to the plates and incubated for 30 mins and washed with buffer for 3times. Then, glucose solution was added to the plate incubated for 30 mins and pH of all samples was measured as shown in table 2.

3. RESULTS

3.1. Particle size and surface charge.

Average particle size of formulations F0, F1 and F2 was found to be in nanometer range. The surface charge of all the formulation indicated that they are stable. The surface charge was found to be ≥+25 mV or ≤-25 mV more stable. (Table 1).

Table 1. Particle size and surface charge.

Formulation	Particle size (nm)	Surface charge (mV)
F_0	301±3.4	-15.5 ±1.4
F_1	201± 1.8	-21.4 ±0.6
F_2	520 ± 2.5	-23.7 ±1.9

3.2. FTIR spectroscopy.

FTIR spectroscopy of G0, G1, G2, G3, G4, showed peaks at 1643 cm^{-1}, 3400.34 cm^{-1}, 1735 cm^{-1}, 1645.7 cm^{-1}, 15.79 cm^{-1}, 1029.22 cm^{-1}, 2669 cm^{-1} of the respective functional groups N-H bending, N-H stretching of primary amine, C=O stretching of ester, NH-CO stretching of amide, C-O stretching, CH bending peak. This indicated the formation of all the generation of dendrimers and is shown in fig.1.

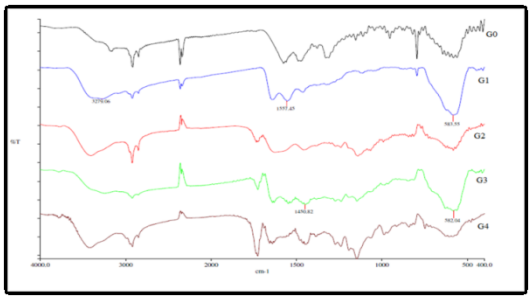

Figure 1. FTIR spectroscopy of G0, G1, G2, G3 and G4 of dendrimers.

3.3. Copper sulphate test.

Full generation of dendrimers showed purple colour after addition of copper sulphate due to the presence of amine group on the surface and half generation showed blue colour.

3.4. SEM of formulation F0.

Particle size of formulations F0, F1 and F2 were found in order of F1<F0<F2. Particle size of formulation F0 using SEM analysis was found to be 27.9± 3.3 nm and branch like structure was observed. Average particle size of formulation F1 was found to be 10.2±1.8 nm. The particles of formulation F1 was found to be spherical shaped and size less than F0 formulation. After

immobilizing glucose oxidase on the surface of formulation F1 particle size of formulation F0 was increased cause of hyper branched structure of G4 dendrimers. The particle size of F2 was found to be 30.5± 2.5nm (Fig. 2).

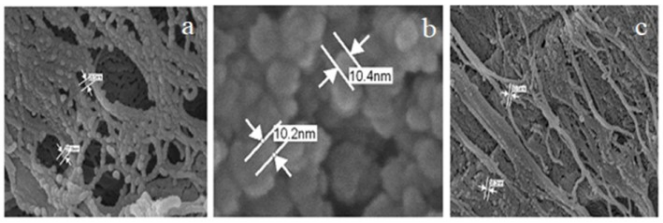

Figure 2. SEM images of formulation a. F0, b. F1 and c. F2.

3.5. Immobilization efficiency.

Immobilization of glucose oxidase onto the surface of dendrimers was determined using flurometer. Formulation F2 was added in a known concentration of glucose solution to which Tris (1, 10-phenanthroline) ruthenium (II) chloride hydrate dye was added. Glucose oxidase is an oxidising agent that catalyses glucose to gluconic acid as the oxygen gets consumed the fluorescence intensity of the dye decreases due to quenching effect. Relative intensity of formulation F2 decreased with time which confirmed immobilization of glucose oxidase on dendrimer surface (Fig. 3).

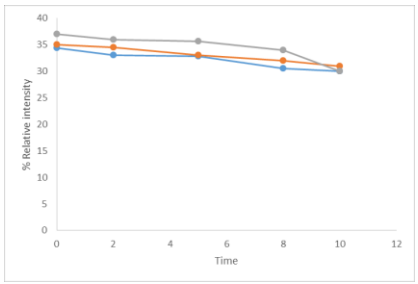

Figure 3. Time vs % relative intensity graph of a (Batch 1), b (Batch 2), c (Batch 3).

3.6. Samples tested and pH.

All the fresh samples i.e. solid, semisolid and liquid showed pH in neutral range i.e. 6.9-7.1 that indicated that the samples were not contaminated and are safe to consume. pH of all samples after 8 and 24 h was found to be slightly acidic and highly acidic respectively. This indicated growth of *E. coli O157:H7* in samples. Concentration of *E. coli O157:H7* in each sample was calculated and are shown in tables 4.

3.7. Estimation of *E. coli O157:H7* in samples after 8 and 24h.

Estimation of *E. coli O157:H7* in all the samples was calculated by measuring pH of all samples after 8h. The concentration was found in range of 10^1-10^2 as shown in table 3. After 8h, pH of solid samples like bread> masala rice > schezwan rice > upma > ice cream > soya vegetable > bhel, the concentration of *E. coli* O157:H7 in bread was found to be maximum as compared to other food samples. After 8h, pH of semisolid samples like green chutney > chana masala> curd > pavbhaji > misal > usal > schezwan chutney > mix vegetable and coconut chutney, the concentration of *E. coli* O157:H7 in green chutney was found to be maximum as compared to other food samples. After 8h pH of liquid samples like sambar > boiled milk > cold coffee > distilled water > tap water > dal > raw milk > purified water, the concentration of *E. coli* O157:H7 in sambar was found to be maximum as compared to other food samples. Concentration of *E.coli O157:H7* in all the samples was calculated by measuring pH of all samples after 24h. The concentration was found in range of 10^5-10^8 as shown in table 4. After 24h pH of solid samples like bread> upma > ice cream > bhel > masala rice > schezwan rice > soya vegetable, the concentration of *E. coli* O157:H7 in bread was found to be maximum as compared to other food samples. After 24h pH of semisolid samples like green chutney and misal > mix vegetable > usal > pavbhaji > curd > schezwan chutney > chana masala > and coconut chutney, the concentration of *E. coli* O157:H7 in green chutney and misal was found to be maximum as compared to other food samples. After 24h pH of liquid samples like sambar > cold coffee > tap water > boiled milk > purified water > dal > raw milk > boiled milk, the concentration of *E. coli* O157:H7 in sambar was found to be maximum as compared to other food samples. Bread is a fermented product that is rich in carbohydrate and contains added sugar that contributes to contamination. Sambar is prepared using vegetables, water and sugar, due to which it gets contaminated. Water used for preparing must be purified and vegetables used must be fresh. Sometimes used oil is reused to prepare sambar that may also cause contamination. Green chutney is prepared using green chili, onion paste, mint, coriander and water. This chutney is not cooked hence it gets contaminated if not stored in refrigerator.

Table 2. pH of samples tested at different time points.

Sr.no.	Samples	pH of fresh samples	pH of samples after 8h	pH of samples after 24h
	Solid samples			
1	Masala rice	6.8	4.4	2
2	Schezwan rice	6.9	4.6	2.1
3	Bread	6.8	3.6	0.8
4	Bhel	7	6.5	1.6
5	Soya vegetable	6.8	6	2.2
6	Upma	7	5.4	1.2
7	Ice cream	6.6	5.5	1.3
	Semisolid samples			
1	Chana masala	6.7	4.8	1.9
2	Curd	6.9	4.9	1.7
3	Usal	7.1	5.9	0.9
4	Mix vegetable	6.8	6.5	0.7
5	Coconut chutney	6.6	6.5	2.2
6	Green chutney	6.6	4.5	0.4
7	Schezwan chutney	6.8	6	1.5
8	Misal	7	5.8	0.4
9	Pavbhaji	7	5.4	1.2
	Liquid samples			
1	Cold coffee	6.8	4.9	0.7
2	Sambar	6.7	4.6	1.7
3	Dal	7	6	0.4
4	Raw milk	7.1	6.2	1.8
5	Boiled milk	7	4.7	2.1
6	Tap water	6.7	5.9	0.8
7	Distilled water	6.8	5.6	1.6
8	Purified water	6.9	6.6	1.3

Table 3. Estimation of *E.coli O157:H7* in samples after 8h.

Sr.no.	Samples	Concentration
	Solid samples	
1	Masala rice	3.7×10^2
2	Schezwan rice	2.7×10^2
3	Bread	3.5×10^2
4	Bhel	5.0×10^2
5	Soya vegetable	1.1×10^1
6	Upma	1.1×10^2
7	Ice cream	2.6×10^1
	Semisolid samples	
1	Chana masala	2.7×10^2

Sr.no.	Samples	Concentration
2	Curd	3.7×10^{1}
3	Usal	3.5×10^{1}
4	Mix vegetable	2.5×10^{1}
5	Coconut chutney	2.5×10^{1}
6	Green chutney	4.5×10^{2}
7	Schezwan chutney	5.0×10^{2}
8	Misal	1.1×10^{1}
9	Pavbhaji	1.1×10^{2}
	Liquid samples	
1	Cold coffee	2.7×10^{2}
2	Sambar	1.4×10^{3}
3	Dal	1.9×10^{1}
4	Raw milk	1.4×10^{2}
5	Boiled milk	9.0×10^{2}
6	Tap water	1.1×10^{1}
7	Distilled water	3.5×10^{1}
8	Purified water	2.5×10^{1}

Table 4. Estimation of *E. coli* O157:H7 in samples after 24h

Sr.no.	Samples	Concentration
	Solid samples	
1	Masala rice	4.2×10^{5}
2	Schezwan rice	4.2×10^{5}
3	Bread	4.6×10^{8}
4	Bhel	8.0×10^{7}
5	Soya vegetable	1.3×10^{6}
6	Upma	2.6×10^{7}
7	Ice cream	6.0×10^{6}
	Semisolid samples	
1	Chana masala	1.3×10^{6}
2	Curd	4.4×10^{6}
3	Usal	8.4×10^{7}
4	Mix vegetable	6.2×10^{5}
5	Coconut chutney	4.2×10^{6}
6	Green chutney	8.4×10^{6}
7	Schezwan chutney	6.0×10^{7}
8	Misal	2.7×10^{8}
9	Pavbhaji	2.6×10^{7}
	Liquid samples	
1	Cold coffee	6.2×10^{7}
2	Sambar	2.4×10^{8}
3	Dal	2.7×10^{6}
4	Raw milk	2.4×10^{6}
5	Boiled milk	4.6×10^{7}
6	Tap water	3.3×10^{6}
7	Distilled water	3.3×10^{6}
8	Purified water	6.2×10^{7}

4. CONCLUSIONS

Biosensors are miniature devices that can be used for sensitive and specific detection of pathogens in diet. Dendrimers as biosensors provide large surface area for immobilizing enzymes and proteins. The study from this research revealed that initially pH of fresh samples was found to be neutral which indicated that the samples were not contaminated with *E. coli* O157:H7. After 8h, the pH of all samples was found to be slightly acidic and the concentration of *E.coli O157:H7* was found to be in range 10^{1}- 10^{2} CFU/mL. After 24h, pH of all samples was found to be highly acidic and the concentration of *E. coli O157:H7* was found to be in range 10^{3}- 10^{8} CFU/mL. Sensitive and specific method for estimating *E. coli O157:H7* was developed with detection time 2h.

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