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A Novel Method of using Magnetic Nanoparticles with Congo Red Dye for the Rapid Capture and Detection of Human Pathogens in Food

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ABSTRACT

The detection of pathogenic organisms is an important aspect of the routine monitoring of contaminants in food. In this study, we developed a new concept of measuring bacterial concentration present in food samples using Magnetic Nanoparticles with congo red dye. Magnetic Nanoparticles (MNPs) were synthesized by Chemical co-precipitation method which is often preferred for production of MNPs as it's the best method to improve homogeneity compared to physical or biological synthesis methods. Chitosan from amine group when coated with chemically synthesized MNPs was used to rapidly capture bacteria due to the binding of protein-receptor interactions. Finally, the surface modified chitosan-magnetite nanocomposite particles were recovered from the reaction mixture by using an external permanent magnet. Thus the obtained surface modified MNP's with pathogens were isolated and incubated at 37°C for few minutes and then, conjugated with 10 μL of Red light emitting dye/marker which emit light upon supply of input light source to detect fluorescence and measure the OD (optical density). The assay was measured at absorbance wavelength of 498 nm using fluorescence spectroscopy (Perkin Elmer). Optical Density (OD) or the intensity output gives the measure of bacterial concentration in food. This method of food borne pathogen detection provides a high throughput for the analysis of pathogenic microbes in food samples and also could detect bacterial concentration in few minutes when compared to existing conventional and immunological method of detection.

Key words: Magnetite nanoparticles, Fe_3O_4, iron oxide or magnetite, chitosan coated magnetite nanocomposite particles, transmission electron microscopy, optical density

INTRODUCTION

Magnetic iron oxides, similar to magnetite (Fe_3O_4) are well known materials because of their super paramagnetism when the particles are adequately smaller. This property, along with their low down toxicity and a greater surface/area makes magnetic nanoparticles most impressive for a number of real and promising applications (Chen and Zhang, 2012). For applications in food bacterial detection and environmental fields, magnetic nanoparticles are usually coated with polymers, bounded to the particle through organic linkers. This type of coating is capable to identify specific molecules and ions for their binding onto the surface of cell wall with improved stability (Sanvicens *et al*., 2009).

There is a constant demand for new, rapid and accurate methods to identify and quantify the pathogens in food with high sensitivity to prevent loss of lives. Lack of early detection of the pathogens to prevent and treat infectious diseases is crucial. The identification of intracellular events and protein-receptor interactions are of great importance for food analysis to capture pathogenic bacteria. Optical analysis based on dye doped labeling has been extensively used to study these interactions (Perez-Lopez and Merkoci, 2011). In this study, we describe the synthesis of MNPs, their bioconjugation with chitosan and application in food bacterial detection. The method of ultrasonic coprecipitation was used for the synthesis of MNPs, followed by a bioconjugation of chitosan, a polymer, via the linker, glutaraldehyde to capture the presence of bacteria. Surface functionalized Ch-MNPs possess the phenomenon to bind onto the bacterial surface due to protein-receptor interactions. This was confirmed from our previous work using UV-Visible spectroscopy and TEM measurements (Manonmani *et al*., 2013). Thus the surface modified bacterial solution after incubation at 37°C for 5 min when mixed with congo red dye, could detect the concentration of pathogenic bacteria from the sample under test. This dye doped sample with congo red, a marker, emits light upon excited by input light source would give the measure of fluorescence which was detected using Perkin Elmer Flourescence Spectroscopy (Fig. 3).

MATERIALS AND METHODS

Chitosan is a polymer 150 kDa. Aqueous acetic acid solution used as a solvent for the chitosan polymer and glutaraldehyde used as a cross-linker. All chemicals were of analytical grade and no further purification was required. Ferric chloride, ferrous sulphate, acetic acid, acetone and congo red were obtained commercially.

Synthesis of magnetite nanoparticles (MNPs): Synthesis of MNPs in a solution occurs by chemical reactions forming stable nuclei with subsequent particle growth. This phenomenon of precipitation of solids in solution has been well studied. Chemical reaction occurs upon addition of precipitating, reducing, or oxidizing agents to the solution and becomes super saturated. Super saturation drives the chemical system to deviate from the minimum free energy configuration for the precipitating species in solution (Manonmani *et al*., 2013).

The chemical reaction of Fe_3O_4 precipitation is expected as follows:

$$FeCl_2.4H_2O+2FeCl_3.6H_2O+8NaOH \Rightarrow Fe_3O_4+8NaCl+20H_2O \quad (1)$$

From Eq. 1, Fe_3O_4 nanoparticles were prepared by co-precipitation method with a ferrous complex in presence of sodium hydroxide (NaOH). Firstly, $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ [Fe^{2+}: Fe^{3+}= 1:2] were dissolved in 50 mL millipore water with constant stirring at 70°C using magnetic stirrer. Next, this iron solution source was added drop-wise into NaOH solution under agitating magnetic stirrer for 30 min and bubbling N_2 gas. Black Fe_3O_4 particles were decanted by permanent magnet and cleaned by millipore water several times. The magnetic nanoparticles are determined by attraction force between magnets. By virtue of magnetic property, the synthesized MNPs get attracted to the external magnetic field. The attraction of magnetite nanoparticles towards the applied magnetic field confirms their presence in the colloidal solution. As shown in Fig. 1, the black precipitate formation confirms the synthesized MNPs.

Preparation of chitosan magnetite nanocomposites: The suspension cross-linking technique was used for the preparation of magnetic chitosan nanoparticles. In this specific procedure, a 5% chitosan solution was prepared using 2% aqueous acetic acid solution containing 0.2 g Fe_3O_4 dry

Fig. 1: Preparation of MNPs by magnetic separation

Fig. 2: Pathogen isolation using Ch-MNPs by placing external magnet near test tube

magnetic nanoparticles. Further, this solution was poured, drop-wise, into the dispersion medium which was composed of 30 mL paraffin and 0.5 mL span-80. During this process, the dispersion medium was stirred with a magnetic stirrer at room temperature. Finally, 3 mL of glutaraldehyde solution was added and the solution was allowed for 5 h stirring. From Fig. 2, the MNPs with chitosan were recovered from the reaction mixture by using an external magnet; the mixture collected in pellet was washed with ethanol and dried overnight with acetone (Manonmani *et al*., 2013).

Dye preparation: 5 mM congo red dye was prepared by adding 2 mg in 1 mL methanol. 20 μL of 10^5 cells of each bacterial strain (*Salmonella typhimurium, proteus vulgaris* and *Staphylococcus aureus*) was to 180 μL of LB (Luria-Bertani) media in a 96 well plates. A blank was also set up which contains only LB medium. Then the 96 well plates were incubated for 7 h at 37°C

and the growth of bacterial strain was measured at absorbance 560 nm wavelength. Then 10 μL of 5 mM dye was added to each blank and sample well. The assay was measured at absorbance 498 nm wavelength using fluorescence spectroscopy.

RESULTS

A pinch of food sample was isolated from contaminated food and was grinded to make powder. It was dissolved in distilled water of particular mL. Bacteria suspended on the liquid and the food particles deposited at the bottom (or) centrifuge at 3000 rpm for 5 min. The supernatent was collected and pellet was discarded. The supernatent contains many types of bacteria. To isolate pathogenic bacteria from the solution, the powder form of 0.1 g of chitosan coated MNPs was dissolved in the solution. Iron coated film slide is dipped on the solution. The iron coated slide attracts MNPs due to magnetic attraction.

Hence, pathogenic bacteria are isolated from the solution to slide. Further, the slide is dipped in solution to transfer bacteria to testing solution. Then, the sample solution is added with dye to visualize the adhered pathogens and further tested with flourescence spectroscopy (Fig. 3) to measure fluorescence. Initially reading from blank was taken and then sample (S1-S4 and T1) reading was taken. Difference in observance of fluorescence reading confirms that bacteria present in the sample:

$$\text{Bacterial presence} = \text{Blank-Sample}$$

From Table 1, the OD (optical density) at 560 nm with the test sample T1 (50 μL mg^{-1} of surface modified bacteria with bioconjugated MNPs) was found to be 1.271 which was more or less nearer

Table 1: Flourescence readings obtained from blank and test samples with Congo red dye

NO	Broth (μL mg^{-1})	Distilled water (μL mg^{-1})	Dye (μL)	OD (mV)
B		50	10	0.507
S1	50		10	1.478
S2	40		10	1.619
S3	30		10	1.07
S4	20		10	1.226
T1	50		10	1.271

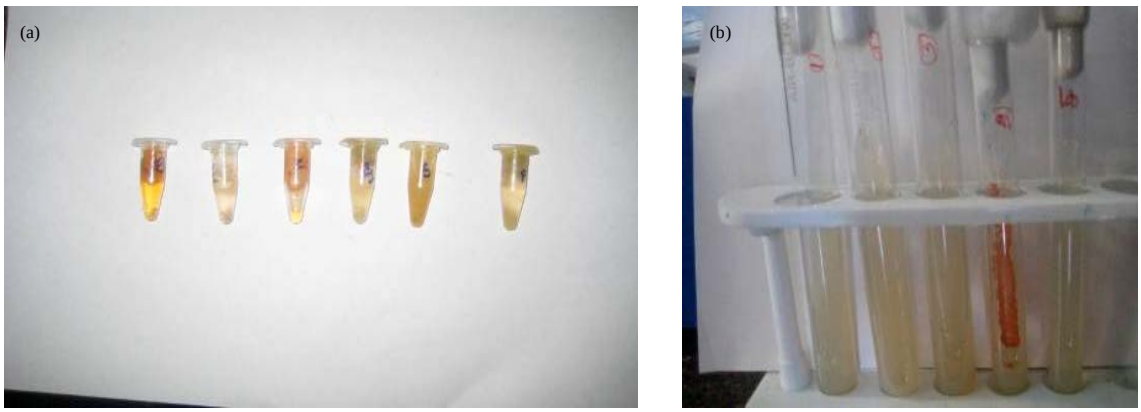

Fig. 3(a-b): Test samples from various contaminated food samples and their extract, (a) Test samples and (b) Extract

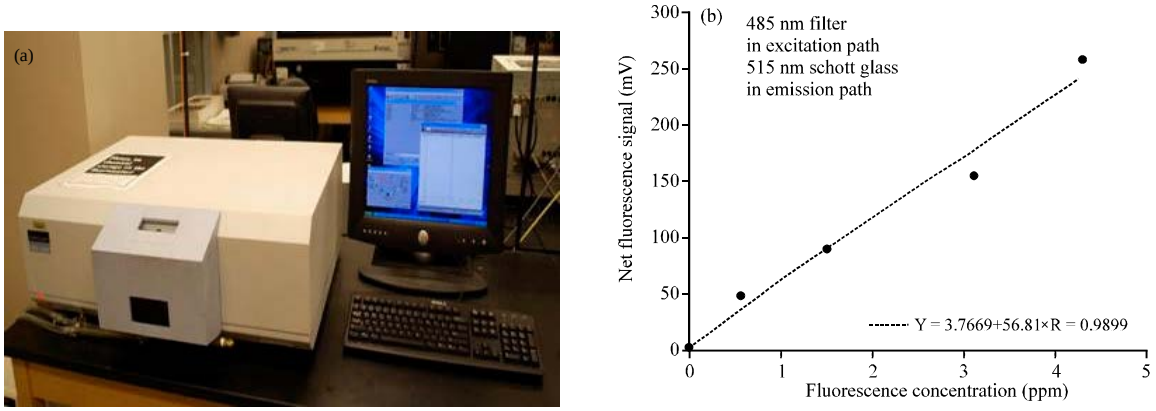

Fig. 4(a-b): Perkin Elmer flourescence spectroscopy and its expected output, (a) Perkin Elmer flourescence spectroscopy and (b) Output

to the OD of 1.478 with 50 μL mg^{-1} broth (pure culture of *Salmonella aureus*) S1. Thus the obtained bacteria's are in purest form was confirmed and also about 90% of the bacteria was isolated and detected using fluorescence spectroscopy. Figure 4 gives the graphical representation between observed voltage in mV and series of volume gives the number of cells present in the sample. The blank value is initially marked and the stock solution readings are filtered with blank values.

DISCUSSION

Magnetic nanoparticles for isolation of food borne pathogens: The magnetic nanoparticles are used in the isolation of food borne pathogens due to its specificity and selectivity in targeting the pathogens. The magnetic nanoparticles are treated with amine functionalized group to increase its specific targeting of bacteria (Velusamy *et al*., 2010). The bacterial surface is negatively charged due to the accumulation of techoic acid on its surface. The negatively charged bacteria are attracted by the positively charged amine group due to its electrostatic interaction between charges. Hence, the amine functionalized magnetic nanoparticles helps in targeting the bacteria present in food (Heo and Hua, 2009).

Dye conjugation and sample testing: The bacterial pathogen detection system used to detect bacteria from contaminated food sample such as Egg, Beef, Chicken, Vegetable, Rice and Fruit (Fig. 5). Food spoilage refers to microorganisms present on food that cause it to become inedible. In order to isolate the microorganism from the food serial dilution procedure is followed, the quantification process reveals the number of bacteria present in per unit volume of original sample. Blanks contain a specified amount of distilled water. The contaminated food sample is taken and using a sterile spatula and sterile petri dish, weigh out 10 g of sample. Add the sample to a sterile blender filled with one bottle of the sterile water. Blend for several minutes to disperse the bacteria. This is a 1:10 dilution. Using clean gloves weigh out 10 g of sample and put it into wide-mouthed sterile 99 mL water blank. The blank contains only distilled water and the series of test tubes containing S1-S4 and T1. S1 is the bacterial extract from pure culture; S2, S3 and S4 are the samples of decrease in concentration of bacterial extract from pure culture. T1 is the test sample of surface modified MNPs and bacterial extract from spoiled food.

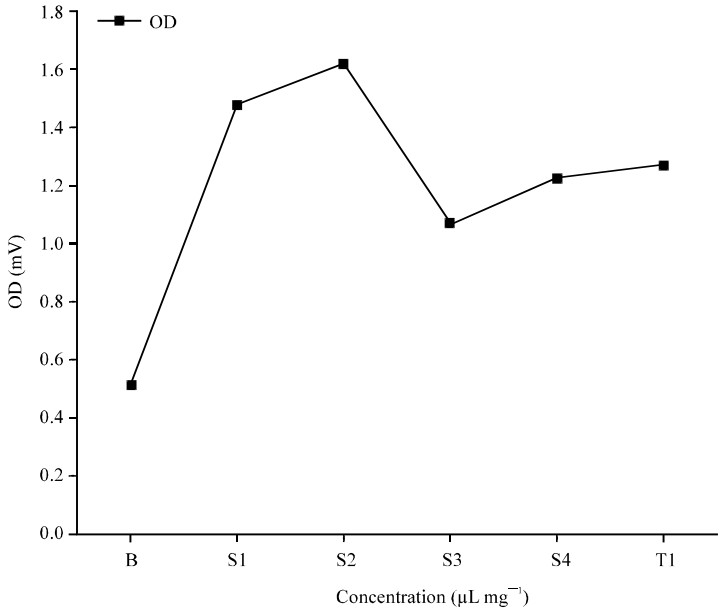

Fig. 5: Graph between concentration vs. net flouresence signal (mV)

Dye marker conjugation: The congo red coated nanoparticles powder is dispersed in every test tube from Blank to S1-S4 and T1. The sample is allowed to incubation for 5 min. 1 mL of sample is then analyzed with fluorescence spectroscopy. The graph was plotted between bacterial concentrations vs. net fluorescence signal.

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