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Development of antibiotic selection kit towards veterinary applications using glycine passivated magnetic particles

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ABSTRACT

Glycine functionalized (Gly/Fe_3O_4) and non-functionalized (Fe_3O_4) magnetic particles were synthesized in an autoclave and characterized by transmission electron microscopy (TEM), Fourier transformed infrared spectroscopy (FTIR), vibrating sample magnetometer (VSM), energy-dispersive X-ray spectroscopy (EDX), differential scanning calorimetry (DSC), X-ray diffraction patterns (XRD) and zeta potential. The size of the both these particles were in the range of 220–230 nm but the shape of the Gly/Fe_3O_4 particles was hexagonal in contrast to the spherical shape of Fe_3O_4 particles. The particle characterization tests confirmed that glycine was functionalized on the Gly/Fe_3O_4 particles, they were positively charged and possessed strong magnetic property. These particles possessed the ability to bind to bacteria such as *Escherichia coli*, *Streptococcus* and *Staphylococcus* in the range of 72 – 90%. They were used to entrap bacteria from clinical mastitic milk samples from cows. The entrapped bacteria of the above species from these samples were isolated and used individually in the conventional disc-diffusion method of antibiotic susceptibility determination. The results were compared with that of the bacterial species isolated directly from the mastitic milk samples and were found to be 100% concordant ($n=25$). The developed portable antibiotic selection kit was tested with twenty five samples of mastitic milk. The results indicated that, antibiotic resistant bacteria turned the methylene blue in to white color while the bacteria that were killed (sensitive) retained the blue color of the dye. Thus the right choice of the antibiotic to treat cows with mastitis could be determined based on the naked eye. In conclusion, the kit gave quicker results, was easy to assay and read and can be 'farm-gate' applicable than the presently available conventional method.

1. Introduction

The application of nanotechnology is seen as a performance enhancer for clinical tests (Mahmoudi et al., 2011). In this context, development of new diagnostic assays with the integration of nanoparticles offers better potential in medical and veterinary sciences (Gupta and Gupta, 2005). Application of superparamagnetic iron oxide particles in diagnostic assays offer versatility due to their quick external magnetic responses, easily controllable size preparations and desired surface coating with functional groups (Jain, 2008; Zhu et al., 2011; Yu and Yang, 2010). The design of desirable functionalized magnetic nanoparticles with well-defined surface properties is highly useful for wide range of research applications. Amino acids are easily available and it contains both electrostatic and hydrophobic elements in their structure. The functional groups present on the amino acids enable covalent conjugation with proteins/bio molecules (Park et al., 2009; Yang et al., 2009; Qu et al., 2012). Among all amino acids, glycine possesses more affinity towards metal atoms because of availability of two functional groups (NH_2CH_2–COOH). The terminal amino groups of glycine which remain free on the particle surface ensure their availability for complementary attachment to functional biomolecules or bacterial stabilization in physiological or slightly acidic pH ranges with suitable ionic strength. The glycine shells on the particles are smaller than those based on intermediary conventional polymeric shells; are non-toxic and can be used in biological media. Apart from this, glycine plays an important role during iron oxide nanoparticles growth and changes the particles' shape.

Mastitis is one of the most economically important diseases affecting dairy cattle worldwide (Joshi and Gokhale, 2006). The disease if not treated appropriately, results in partial or complete damage (fibrosis) to udder tissues and thereby decreases the productive life span of the affected animals. Mastitis is classified

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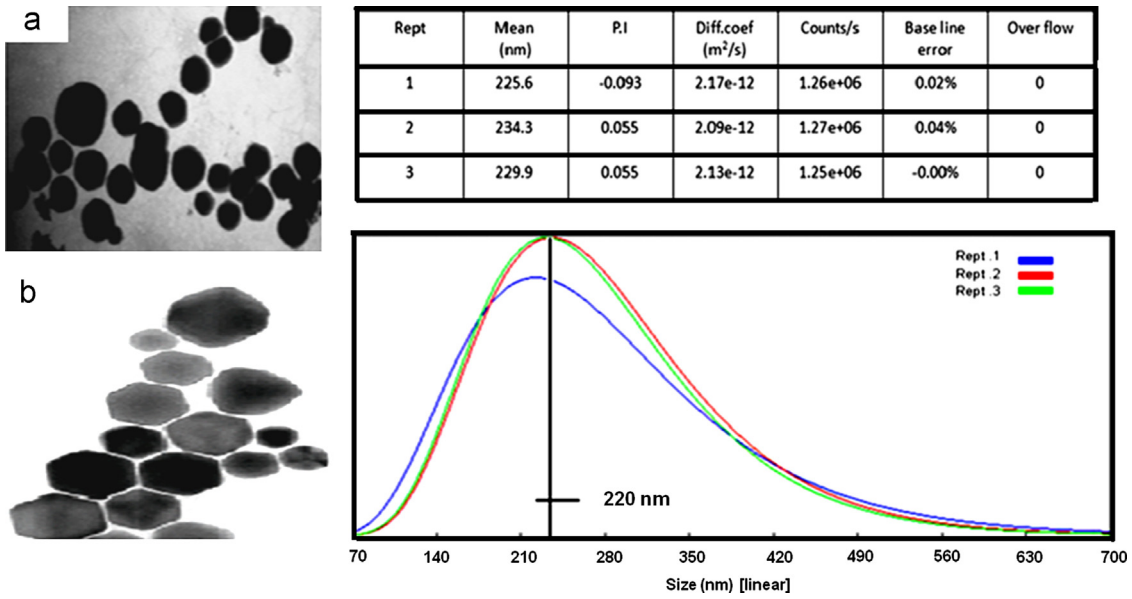

Rept	Mean (nm)	P.I	Diff.coef (m^2/s)	Counts/s	Base line error	Over flow
1	225.6	-0.093	2.17e-12	1.26e+06	0.02%	0
2	234.3	0.055	2.09e-12	1.27e+06	0.04%	0
3	229.9	0.055	2.13e-12	1.25e+06	-0.00%	0

Fig. 1. Transmission electron microscopic (TEM) images of circular magnetic (a) and hexagonal glycine passivated magnetic particles (b). The DLS analyses of glycine passivated magnetic particles performed in aqueous buffer revealing an average size of ~220 nm.

into three major classes (i) clinical (ii) subclinical and (iii) chronic mastitis. Mastitis can be caused by a large number of bacterial species (Pyorola, 2003), but the most prevalent pathogens isolated from clinical cases include *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae* (Cheng et al., 2010; Keefe, 1997). The treatment of bacterial mastitis is mainly based on the results of conventional antibiotic susceptibility tests.

In this work, initially we developed glycine passivated magnetic particles (Gly/Fe_3O_4) using an autoclave-based approach. These particles strongly interacted with bacteria through electrostatic forces. We studied their bacterial entrapment capacity using the common mastitis-causing bacteria. These particles were applied to entrap bacteria from clinical mastitic milk samples that were used in antibiotic susceptibility tests.

2. Materials and methods

2.1. Synthesis and characterization of iron oxide particles and its glycine modification

For particle synthesis, 0.7 g of Iron (II) chloride, 1.8 g of iron (III) chloride were mixed with a molar ratio of 1:2, and then 10 mL of 0.1 N sodium hydroxide solution was added and it was stirred for 10 min. Finally 20 mL of (75 mg/mL) glycine was added and the solution was heated at 121 °C/15 psi for 15 min in an autoclave. The synthesized Gly/Fe_3O_4 particles were collected by magnetic separations and washed repeatedly. For the unmodified particle synthesis, after adding sodium hydroxide the final solution was heated at 121 °C/15 psi for 15 min in an autoclave.

Both the synthesized particles were dried at 55 °C and stored at room temperature before use. For the bacterial entrapment, magnetic separators were designed by using the permanent magnetic field. The magnet holder with the sample phase was made by using acrylic sheet. The total magnetic field strength was around 14 T and the magnet size is $4 \times 0.55 \times 0.55\ cm^3$. For bacteria separations, the vial containing mastitic milk samples were mixed with the magnetic particles and exposed to the magnetic field.

The Fe_3O_4 or Gly/Fe_3O_4 particle morphologies, chemical composition, crystalline structure and thermal properties were examined by using transmission electron microscopy (TEM), Fourier transformed infrared spectroscopy (FTIR), vibrating sample magnetometer (VSM), energy-dispersive X-ray spectroscopy (EDX), zeta potential, Differential scanning calorimetry (DSC), Dynamic light scattering (DLS) based particle size analyzer and X-ray diffraction patterns (XRD) at the Sophisticated Analytical Instrumentation Facility (SAIF), Indian Institute of Technology, Chennai using established methods.

2.2. Bacterial capture efficiency by the magnetic particles

Pure stock cultures of *Staphylococcus aureus (S. aureus)*, *Streptococcus agalactiae* (*S. agalactiae*) and *Escherichia coli* (*E. coli*) available in the Department of Animal Biotechnology, Madras Veterinary College were used.

The impact of solution pH and ionic strength on the binding was also investigated. To study the pH effect, phosphate buffered saline (PBS) solution pH was adjusted from 2.0 to 11.0 by adding dilute NaOH or HCl. The bacterial samples were mixed with different pH solution, and incubated with Fe_3O_4 or Gly/Fe_3O_4 particles and bacterial entrapment efficiencies assessed.

The bacteria were initially mixed in PBS buffer in a concentration range of 5–200 mM. After that, the samples were incubated with Fe_3O_4 or Gly/Fe_3O_4 particles and bacterial entrapment efficiencies assessed as follows. The bacterial concentrations were adjusted to 5×10^6 cfu/mL based on plate counts and mixed with 1 mL of 1.5 mg/mL Fe_3O_4 or Gly/Fe_3O_4 particles, followed by incubation for 30 min with mild shaking at 100 rpm. Then the Fe_3O_4 or Gly/Fe_3O_4 particles were separated using magnetic separators and the concentrations of bacteria present in the supernatant estimated by plate counts. Three trials were performed and the mean entrapment efficiencies for each bacterial species were determined. Their means were compared statistically using the Student "*t*" test. The binding of bacteria by the synthesized Gly/Fe_3O_4 particles was also assessed qualitatively by transmission electron microscopy (TEM).

2.3. Isolation and identification of bacteria in mastitis milk samples

Mastitis milk samples were made into 2 aliquots of 1 mL each and to one aliquot Gly/Fe_3O_4 particles were added and incubated