Magnetic ZnFe$_2$O$_4$@polyhydroxybenzoic acid nanostructure for efficient B.subtilis capturing

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Objective(s): This work focuses on preparing an efficient bacterial capture system based on the magnetic polyphenolic nanostructure. For a reason, a one-step hydrothermally route was employed to prepare ZnFe$_2$O$_4$@hydroxybenzoic acid-resorcinol nanohybrid.

Methods: The nanostructure was characterized by X–ray diffraction (XRD), field emission scanning electron microscopy (FE–SEM), transmission electron microscopy (TEM) vibration sample magnetometry (VSM) and zeta potential measurement. Bacillus subtilis was employed as a sample pathogen to evaluate bacterial capture efficiency of the nanohybrid.

Results: Characterization results confirmed that the hybrid material is in nano scale. Moreover, it has a magnetic saturation of 6.7 emu g$^{-1}$ which is in right level to be employed for magnetic separation. Effect of relevant variables on capturing efficiency including pH, contact time and adsorbent dosage was investigated, and optimum levels were obtained.

Conclusions: It found that the capturing efficiency is independent of solution pH. Moreover, capturing experiments showed fast equilibrium time of 20 min with the effectiveness more than 99%.

KEYWORDS:
Subtilis
Polymer
Magnetic nanohybrid
ZnFe$_2$O$_4$

INTRODUCTION
Pathogenic microorganisms are in the spotlight of health care field. In other words, infections by harmful bacteria are of great concern in surgery equipment and water purification systems [1–3]. Therefore there are growing demands for antimicrobial materials for controlling bacteria growth as well as eliminate the need for disinfection process [4,5]. Recent advances in nanotechnology fulfilled the escalating request of artificial material to cope with bacterial infections [6,7]. Up to now various bioactive nanomaterials have been developed for the treatment of microorganisms in water including metal oxides[8,9] and polymer nanocomposites[10,11]. The mentioned antimicrobial agents possess superior efficacy and reduced toxicity [12] hence antimicrobial modification of them attracted great interest. The main strategy to prepare antimicrobial polymeric nanostructure includes covalent attachment of antibacterial functional groups on polymer structure [13,14]. Amine, carboxylic acid, quaternized amino groups and phenolic compounds are main polymeric functional groups as the latest groups are unique natural distributed materials in plants, which serves as a defense against plant pathogens [15]. According to the merits mentioned above, this work focuses on preparing an efficient antimicrobial system based on polymer nanocomposite. The mentioned polymer is a combination of phenolic and carboxylic acid functional groups. Polyphenolic
compounds have classified as smart hydrogels with a three-dimensional cross-linked structure that is able to retain water and solute molecules [16,17]. However, low adsorption rate is a major limitation of some of these materials. This limitation can eliminate by preparing organic–inorganic nanocomposites through using cavitation during the synthesis [18]. Among various types of inorganic reinforcing materials, metal oxides especially magnetic ferrite nanoparticles, facilitate handling of the antimicrobial compound from the reaction vessel. As a result, magnetic ZnFe₂O₄ polymer nanohybrid was synthesized by one step hydrothermally reaction of 4-hydroxy benzoic acid and resorcinol through condensation Michel-reaction using p–formaldehyde as a linker. Bacillus subtilis (B. subtilis ATCC6633) was selected as sample pathogen. B. subtilis with endospore-forming abilities may proliferate in water and foods hence efficient elimination of it is vital for human health and environmental safety [19]. Effective parameters on the capturing efficiency including; solution pH, contact time and nanocomposite dosage optimized and results discussed.

MATERIALS AND METHODS

Reagents and instruments

4-Hydroxybenzoic acid, resorcinol, para -formaldehyde, NaOH, Zn(NO₃)₂·6H₂O, FeCl₃·6H₂O and ammonia (25% w/w) were supplied from Merck (Darmstadt, Germany). Bacteria were supplied from Rasoule - Akram hospital, Tehran, Iran. The crystallinity and surface morphology of prepared composite were studied by X-ray diffraction (XRD), field emission scanning electron microscope (FE-SEM) and transmission electron microscopy (TEM) using a Phillips powder diffractometer, X’ Pert MPD, SIGMA VP ZEISS and JEM-2010 instruments. The magnetization measurement has performed on a vibration sample magnetometer (VSM) with MDKFD instrument, Kashan, Iran.

Synthesis of magnetic nanocomposite

For preparing ZnFe₂O₄@polymeric nanohybrid, 0.5g of resorcinol and 0.5g of hydroxybenzoic acid was dissolved in 25 mL of distilled water containing 3mL of concentrated NH₃ (25% W/W). Thereinafter, 1.12g of FeCl₃·6H₂O and 0.6g of Zn(NO₃)₂·6H₂O in 10mL of distilled water has been added to the above solution and stirred for 5min. In a subsequent step, 0.5g of p–formaldehyde added to the mixture, and the pH of them adjusted to 12 with NaOH. After stirring for 30min, the reactants were transferred into a Teflon lined autoclave and kept at 150°C for 24h. The resulted gray products were collected by filtration and washed with distilled water then dried at 70°C for 6 h.

Bacterial capturing

The bacterial capture efficiency of the nanohybrid was evaluated using gram – positive strain, B. subtilis, as a model pathogen. The bacteria cultivate growth medium include: 100mL of Luria Broth (LB) consisting 5g L⁻¹ bacto-yeast extract, 15 g L⁻¹ tryptone and 5 g L⁻¹ NaCl. The strains were shacked in an incubator at 30°C for 32 h then were separated by centrifugation (4000g for 10 min at 4°C). The bacterial pellets were washed with 0.9% of NaCl at pH 7.0 and re-suspended in the physiological saline to obtain a cell density of 1.5 ×10⁸ Colony Forming Unit per milliliter (CFU mL⁻¹). 20mg of the nanocomposite was added in 10mL bacteria solution with a concentration of 1.5 ×10⁸ CFU mL⁻¹ to perform bacterial capture experiments. The mixture incubated for 20min then the particles were separated from the suspension. Bacterial capture efficiency was determined after culturing 1.0mL of diluted stock sample solution or supernatant in LB agar plates, and removal percentages (%R) were obtained by counting of colonies before and after capturing experiment using following equation:

\[
% R = 100 \times \frac{CFU_0 - CFU_t}{CFU_0} \tag{1}
\]

where CFU₀ is initial colony numbers and CFUₜ is the number of unabsorbed colonies in the supernatant [20].

RESULTS AND DISCUSSIONS

Characterization of the nanohybrid

The XRD pattern of nanohybrid shown in Fig.1a. The pattern contain typical main peaks of zinc - ferrite nanoparticles at 2θ = 29.64, 35.48, 35.7, 44.2, 52.72, 56.2, 61.76 and 75.1 corresponding to (220), (311), (222), (400), (422), (511), (440) and (535) planes [21]. Moreover, the pattern showed a deep scattering with a maximum height at 17° which can be assigned to the polyphenolic derivative.
The VSM graph of the magnetic nanocomposite shown in Fig. 1b. It can be seen that the saturation magnetization (Ms) value of nanohybrid is 6.7 emu g\(^{-1}\). Low Ms value can be attributed to the presence of the polymer onto the composite structure. The value of magnetic remnant (Mr) for the nanocomposite was 0.01 emu g\(^{-1}\), which indicate that it possesses paramagnetic properties because of the Mr/Ms ratio of 0.0014 lies in the range for paramagnetic behavior (0 < Mr/Ms < 1)\(^{[22]}\).

The FE-SEM and TEM image at Fig. 2 showed fine regular uniform spheres with a diameter less than 100 nm. Spherical morphology can be explained by the concept of an isoelectric point (IEP). In general, the formation of the sphere-like structure occurs at pH higher than IEP. In the synthetic protocol owing to the addition of NaOH, the pH of the solution was around 12. However, magnetic nanoparticles show IEP of 6 - 7 \(^{[23]}\) which means that the surface of nanoparticles have a negative charge at the synthetic period. Under such conditions, there is a net repulsion force between primary ZnFe\(_2\)O\(_4\) particles and deprotonated phenolic monomers as the growth along various directions was approximately same leading to the formation of sphere–like structure \(^{[24–26]}\).

**Optimizing effective variables on bacterial capturing**

The influence of pH on bacterial capture efficiency was studied at three pH levels of 2, 5 and 8. The composite dosage was 20 mg and time was 30 min. The results shown in Fig.3a revealed that capturing efficiency is 98% at the pH of 8 that reached to 99 and 100% at the pH of 5 and 2, respectively. The surface charge of the nanocomposite and bacteria at various workings pH is helpful to interpret the observed results. The curve for zeta potential measurements of the nanocomposite depicted at Fig.3b. It can be seen that the nanocomposite possess positive charge at the pH below 4.5 which changes to negative charge at a higher value of pH. Besides the zeta potential of B. subtilis used in this study was estimated to be around pH of 1.6 which means that cell surface of bacteria have negative charge upper these pH values \(^{[27]}\). Based on this situation bacteria capturing by the nanocomposite is approximately independent of solution pH.

The cell walls of bacteria contain methylene, oxy/hydroxy as well as COOH functional groups which can react with the sorbent surface hydrophobic interaction, electrostatic attraction, and hydrogen bonding. Hydrophobic interaction is the most operating mechanism to describe bacteria/solid surface interaction. In other words, nanocomposite contains benzenoid rings which attract bacterium through interaction with methylene groups in lectin of bacteria. Electrostatic attraction can be considered as another mechanism for bacterial capturing. In other words, the cell walls of B. subtilis have negative charge based on an isoelectric point which means the functional groups on the cell surface is in deprotonated form hence it estimated that repulsion force should decrease capturing efficiency at alkali situations. However, according to results for effect of pH, bacterial capture efficiency
is approximately constant at the studied pH ranges which mean that electrostatic attraction is not the main factor for bacterial capturing by this sorbent. However, this mechanism cannot be discarded completely since at the alkaline solutions the highly ionized carboxylate groups on the bacteria cell surface electrostatically bind with positive Fe and Zn ions on the ferrite structure which induce the large adhesion force [28]. The high efficiency of the nanocomposite for bacteria capturing at alkaline media can be further described with combining of hydrogen bonding and hydrophobic interaction. Since nanocomposite structure reached with H-bonding donor–acceptor sites as OH groups along with aromatic backbone which efficiently interacts with bacteria cell surface [29].

The effect of time on capturing of $1.5 \times 10^8$ CFU mL$^{-1}$ of bacterial solutions (10 mL) was investigated with a dosage of 20 mg at pH=8, and the results were given in Fig.3c. It was found that capture of bacterium occurs efficiently after 10 min with efficiencies more than 98%. The capture efficiencies slowly increased with increasing time and reached to 100% after 20 min. Fast capturing is owed to low energy barrier at bacteria-composite interaction as well as high diffusivity which decrease kinetic of adsorption [30]. In other words, the low size of the polymer nanocomposite, as well as ease accessibility of various functional groups on the composite structure (carboxyl and aromatic backbone), promote capturing reaction with lower equilibrium time in a low energy barrier way.

The effects of nanocomposite dosage on bacteria capture ($1.5 \times 10^8$ CFU mL$^{-1}$) were examined at three dosage range from 10 – 20 mg and time of 20 min using bacillus as model cells. According to results at Fig.3d, with 10 mg nanocomposite, the capture efficiency was 98% as the capture efficiencies increase to more than 99% by using 20 mg of the nanocomposite. This could be attributed to the fact that nanocomposite could provide a high value of accessible active binding points to adhere to bacteria cell surface.

CONCLUSIONS
An efficient B.subtilis capturing system was developed based on magnetic polymer nanocomposite. Results showed that the magnetic materials have efficiency more than 99% for bacterial removal. Effect of pH showed that capturing is independent of this variable. Moreover capturing followed fast equilibrium time as more than 98% of bacteria can be removed by first 10 min reaction. According to results, the system has real potential for bacterium capturing from aqueous solutions.

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CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

REFERENCES