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Research paper

Assessing safety and protein interactions of surface-modified iron oxide nanoparticles for potential use in biomedical areas

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ABSTRACT

We have investigated the electrostatic interaction between bare iron oxide nanoparticles (IONPs) or low molecular weight chitosan coated iron oxide nanoparticles (LMWC-IONPs) and hen egg white lysozyme (HEWL) at different pH values using protein-nanoparticle *reverse charge parity* model. Physicochemical characterization of both IONPs and LMWC-IONPs were carried out using DLS, TEM, FE-SEM, XRD, TGA, XPS and VSM analysis. DLS, TEM and FE-SEM results indicated that both IONPs were monodispersed, with size ranging from 8 to 20 nm. The coating of LMWC on IONPs was confirmed using zeta potential, TGA, XRD and XPS measurements. The cytotoxicity of both IONPs and LMWC-IONPs was studied *in vitro* in A549 human lung alveolar epithelial cells to assess their use in biomedical applications. Furthermore, the interactions between protein-nanoparticles were investigated by UV-visible, fluorescence and circular dichroism spectroscopic techniques. The present study suggests that water soluble LMWC surface modified IONPs are the promising nanomaterials. The safety and biocompatibility of these nanoparticles render them suitable for biomedical applications.

1. Introduction

In recent years, development of novel nanomaterials and understanding their interaction with cells, membranes, proteins, DNA, phospholipids, endocytic vesicles, biological fluids and other cell organelles has gained attention in various scientific disciplines such as biology, physics, chemistry, and engineering [1]. Many of neurological diseases, such as Alzheimer's disease, Parkinson's disease, Prion diseases, Apolipoprotein amyloidosis, Huntington's disease and Creutzfeld-Jacob disease *etc.*, originate from the misfolding of biologically active proteins [2]. Both organic and inorganic based nanoparticles (NPs) can manipulate the unfolded or misfolded protein and prevent their aggregation, which may be helpful in the treatment of neurodegenerative disorders [3]. The fundamental studies of protein-nanoparticles interaction will provide molecular level information to understand the promotion, inhibition, or depolymerisation in amyloid fibrillation. Therefore, much research has been devoted towards detailed understanding of the precise mechanisms involved in protein-nanoparticles interaction. A variety of NPs have been studied for their interactions with various proteins, including silver NPs [4], magnetic NPs [5], ZnO NPs [6], silica NPs [7], gold NPs [8], carbon nanotubes [9], *N*-isopropylacrylamide: *N*-*tert*-butylacrylamide (NIPAM:BAM) copolymeric NPs [10] and poly (*N*-acryloyl-L-phenylalanyl-L-phenylalanine methyl ester) NPs [11]. Among these, magnetic nanoparticles have been successfully explored in biomedical fields, specifically for the enhancement of contrast in magnetic resonance imaging (MRI), tissue repair, biomedical imaging, immunoassay, drug delivery, cancer hyperthermia, protein separation and purification [12–15]. It is well known that polymer coating on nanomaterials can offer them higher stability and hydrophilicity due to the presence of higher number of diverse functional groups on the NP surface, which also play a major role in binding with proteins [13,16] .

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In recent years, researchers have coated magnetic NPs with different polymers, such as chitosan [17], PLGA [18], polyethyleneimine [19], polyacrylic acid [19] and polydoapmine [20], to improve their biocompatibility and safety in the human body. This is particularly because the coated NPs are less toxic, have greater dispersibility and demonstrate better conjugation/interaction with other bioactive molecules compared to bare NPs. Amongst the various surface modified IONPs, chitosan modified NPs have been considered as promising nanomaterials due to their unique physicochemical properties, high biocompatibility and excellent biodegradability of chitosan. However, biomedical use of chitosan has been limited by its high molecular weight, which results in low water solubility at physiological pH [21]. It is also limited by acid stress on the cells, contributed by the low pH of acetic acid, that is used to dissolve chitosan [22]. Thus, water-soluble, low molecular weight chitosan (LMWC) or chitosan oligosaccharide (COS) have been exploited to modify the surface of NPs to prevent their aggregation and improve their biocompatibility, biodistribution and colloidal stability. LMWCs have been studied as pH-responsive polymers for drug delivery and also as effective materials for modifying the surfaces of liposomes [23], solid lipid NPs [24], gold NPs [25] and IONPs [26]. However, none of these investigations have employed LMWC-surface modified IONPs for protein interaction, to the best of our knowledge.

Much of the published literature has focused on the synthesis of surface modified magnetic nanoparticles and their interaction with different proteins include α-lactalbumin, bovine serum albumin, β-lactoglobulin, ovalbumin, bovine serum albumin and hen egg white lysozyme (HEWL) [27,28]. Among these, lysozyme is a natural and abundantly available proteins that bears structural resemblance to the human lysozyme [29] . Therefore, in the present paper we have used lysozyme as a model protein for studying protein-nanoparticle interaction. Lysozyme is a small globular protein, with a molecular weight of 14.4 kDa, that is naturally present in various biological fluids and tissues including avian egg and animal secretions, human milk, tears, saliva, mucus, airway secretions, and secreted by polymorphonuclear leukocytes [30]. It is composed of 129 amino acid residues and contains an α-helical structure with two short β-strands, six tryptophan (Trp) (28, 62, 63, 108, 111, and 123) and three tyrosine (Tyr) residues, with four disulfide linkages, contributing to the stabilization of this biomolecule [2]. Among the amino acid residues, Trp62 and Trp108 are the most dominant fluorophores that are majorly involved in binding with substrates and inhibitors [31]. These residues thus help in analyzing the binding location of a ligand on the protein by intrinsic fluorescence quenching study. It is worth to note that pH 9.0 favors strong electrostatic interactions between positively charged lysozyme and negatively charged NPs, than hydrophobic forces. At this pH, both IONPs and LMWC-IONPs have negative charge and lysozyme protein have an positive charges [32].

In an earlier paper, Ghosh et al. [14] have reported the electrostatic binding between positively charged lysozyme and negatively charged IONPs in water at neutral pH. These scientists have proposed a *reverse charge parity* model for electrostatic binding of proteins with charged nanoparticles. Based on this earlier publication, in the present study we have used *reverse charge parity* model to assess the electrostatic interactions between LMWC-surface modified IONPs and lysozyme at three different pH values 3.0, 9.0, and 12.0. The interactions were assessed using UV-visible, fluorescence and circular dichroism spectroscopic techniques. Physicochemical characterization of IONPs and LMWC-IONPs were carried out using DLS, TEM, FE-SEM, XRD, TGA, XPS and VSM. *In vitro* cytotoxicity of both IONPs and LMWC-IONPs was investigated in A549 human lung alveolar epithelial cells by MTT assay. Thus, surface modification of nanoparticle and subsequent knowledge of protein-nanoparticle interaction is important on the ground of the *in vivo* safety of nanomaterials for various biomedical applications.

2. Materials and methods

2.1. Materials

We have used HEWL (lysozyme from chicken egg white, L 6876, lyophilized powder, protein ≥90%, ≥40,000 units/mg protein, M_W = 14.3 kDa), $FeCl_3 \cdot 6H_2O$ (97.0% purity), $FeCl_2 \cdot 4H_2O$ (99.0% purity), 30% NH_4OH, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide was purchased from Sigma chemical Co. (St. Louis, MO, USA). Low molecular weight chitosan (20 KDa) was obtained as a gift sample from Amicogen Co., Ltd, Korea. Deionized water (electrical resistivity is 18.2 MΩ cm, Milli-Q Plus system, Millipore, Bedford, MA, USA) was used in all the experiments. All materials were used without further purification. For all measurements, lysozyme solutions were prepared in 10 mM Tris buffer and used immediately after preparation. The pH of buffers was adjusted with 0.1 M KOH and 0.1 M HCl (S.D. Fine Chem. Ltd., Mumbai, India).

2.2. Methods

2.2.1. Synthesis of IONPs by co-precipitation method

IONPs were synthesized by co-precipitation method [14]. Briefly, 12.16 gm $FeCl_3 \cdot 6H_2O$ and 5.99 gm $FeCl_2 \cdot 4H_2O$ were transferred in a round bottom flask and dissolved in 25 mL deionized water (DIW). The temperature of the reaction solution was maintained at 60 °C, with continuous stirring (400 rpm). After 30 min of heating, 20 mL of 30% NH_4OH was added quickly and a black precipitation was instantly obtained. The precipitate was collected using a strong permanent magnet placed underneath the reaction flask and washed 4–6 times with DIW, until the pH of the supernatant was neutral. The supernatant was thereafter discarded. The precipitate was dried at 25 °C for 24 h.

2.2.2. Surface modification of IONPs by LMWC

Water soluble low molecular weight chitosan (LMWC) was used to modify the surface of IONPs, using a method reported earlier [14]. Briefly, 500 mg of LMWC dissolved in 5 mL of DIW and dried bare IONPs were dispersed sonicated for 2 h. Afterwards, LMWC surface functionalized IONPs were separated from free LMWC, using a strong magnet, and then washed several times with DIW. The final precipitate was dried at 25 °C for further use. The protonated amino groups on LMWC are responsible for electrostatic interaction with IONPs. Schematic representation of LMWC surface modified IONPs shown in Fig. 1.

2.2.3. Dynamic light scattering and zeta potential measurements

Particle size distributions and zeta potential of IONPs was analyzed using Zetasizer Nano ZSP (Malvern Instruments Ltd., UK) instrument. Zeta potential of bare IONPs, LMWC-IONPs and lysozyme were measured at different pH values ranging from pH 3.0 to 12.0. All measurements were recorded in triplicate (n = 3) at 25 °C.

2.2.4. Transmission electron microscopy

Surface morphology of both IONPs and LMWC-IONPs was studied using transmission electron microscope (JEM 2100 JEOL Ltd., Tokyo, Japan). Samples were prepared by dropping 10 μL of nanoparticle dispersions on Formvar® carbon film-coated copper grids (200 meshes, Electron Microscopy Services, Ft. Washington, PA), followed by air-drying at 25 °C. All samples were analyzed at an acceleration voltage of 200 kV.