January 1, 2009

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Journal of Materials Chemistry

www.rsc.org/materials

Volume 19 | Number 35 | 21 September 2009 | Pages 6233–6428

Downloaded by University of Massachusetts - Amherst on 05 October 2012
Published on 08 April 2009 on http://pubs.rsc.org | doi:10.1039/B901616C

Themed Issue: Inorganic nanoparticles for biological sensing, imaging, and therapeutics

ISSN 0959-9428

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Superparamagnetic iron oxide nanoparticles: from preparations to in vivo MRI applications

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Stability, toxicity and differential cellular uptake of protein passivated-Fe₃O₄ nanoparticles†‡

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Received 26th January 2009, Accepted 24th March 2009

First published as an Advance Article on the web 8th April 2009

DOI: 10.1039/b901616c

We have explored the mechanism and differential uptake of BSA coated Fe₃O₄ nanoparticles (NPs) by different cancerous and isogenic cell types.

Introduction

The surface and core properties of nanoparticles (NPs) can be engineered for their use in imaging,¹ drug/gene delivery,² biosensing,³ diagnosis of many diseases⁴ and various other applications.⁵ The delivery of NPs faces challenges associated with toxicity of surface ligands, the range of cell lines they are exposed to, and serum conditions. Cationic NPs interact with cell membranes strongly as compared to anionic and zwitterionic ones,⁶ but are very toxic and immunogenic. These NPs are also prone to interact with serum proteins present in blood, thereby altering delivery profiles of NPs.⁷ Therefore the choice of surface coating of NPs is important to lower toxicity and immunogenicity while increasing transport and delivery efficiency.⁸

To provide an effective passivation strategy for *in vivo* applications, we explored the use of proteins to passivate Fe₃O₄ NPs. Iron oxide

NPs are well known for magnetic resonance imaging (MRI)⁹ and magnetic field induced thermal therapy¹⁰ and various systems like NP-loaded liposomes,¹¹ magnetite-doped microspheres, and magnetic fluids (ferrofluids).^{12,13} Dextran¹⁴ and aminosilane coated iron oxide have been explored for cancer therapy treatments.¹⁵ Proteins provide promising agents for NP functionalization due to their biocompatibility, and low toxicity and immunogenicity. The FDA has recently approved Abraxane™, an albumin-paclitaxel (Taxol™) nanoparticle for treatment of metastatic breast cancer.¹⁶ Recently, Zhang and coworkers have developed BSA coated carbon nano tubes (CNT) for double photodynamic therapy (PDT) and photohyperthermia (PHT) cancer phototherapy system.¹⁷ Samanta *et al.* have reported the synthesis and hyperthermia effect of BSA coated Fe₃O₄ NPs.¹⁸ Until now, however, there has been little systematic study of these protein-functionalized particles. We report here the differential delivery of protein coated NPs into cancerous and isogenic cell types and provide information on the mechanism of their uptake and their intracellular distribution.

Results and discussion

We first explored the interaction of proteins with 12 nm diameter “bare” Fe₃O₄ NPs.¹⁸ Out of 11 proteins studied (Table 1) only five proteins stabilized Fe₃O₄ NPs in water. We next explored the stability of these five protein-coated NPs in Dulbecco's Phosphate Buffered Saline (DPBS) and Dulbecco's Modified Eagle Medium (DMEM) media. Myoglobin, chymotrypsin and fibrinogen-coated NPs precipitated in DPBS and DMEM media; whereas lysozyme and fetal bovine serum albumin (BSA)-coated NPs were stable. Lysozyme NPs, however, were highly toxic to HeLa cells at 4 mg/mL concentration and were therefore not explored further. The effective passivation of nanoparticles with different proteins depends upon the

Fig. 1 Schematic representation of uptake of iron oxide NPs via endocytosis or penetration through cell membranes.

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† This paper is part of a *Journal of Materials Chemistry* theme issue on inorganic nanoparticles for biological sensing, imaging, and therapeutics. Guest editor: Jinwoo Cheon.

‡ Electronic supplementary information (ESI) available: Stability assay data of nanoparticles, uptake in different serum conditions and cell viability assay in different cell types. See DOI: 10.1039/b901616c

Table 1 Stability of protein coated Fe₃O₄ NPs in water, DPBS and DMEM media

Protein	Stability in water	Stability in DPBS	Stability in DMEM
BSA	Yes	Yes	Yes
Hemoglobin	No	—	—
Myoglobin	Stable for some time	—	—
Chymotrypsin	Stable	No	No
Fibrinogen	Stable	No	No
Phosphatase Acid	No	—	—
Alkaline Phosphatase	No	—	—
Amylase	No	—	—
Lysozyme	Yes	Yes	Yes
Protease	No	—	—
Papain	No	—	—

isoelectric point (pI), molecular weight, size and the exposed carboxyl moieties, as the carboxyl groups bind with the iron oxide core. The stability of the passivated nanoparticles in DPBS and DMEM media likewise depends upon these factors and also upon the interactions of the protein passivated nanoparticle components in these solutions.

BSA is a predominantly anionic protein (pI of 4.8), so adsorption is expected to proceed through the aspartate and glutamate functionality of BSA, as previous studies¹⁹ showed that carboxylate groups provide excellent ligation for iron oxide NPs. To determine the stability of BSA coated NPs in serum conditions, we dispersed NPs in DMEM media supplemented with different percentages of fetal bovine serum (FBS). No change in absorption of NPs at 350 nm (Fig. S1, ESI†) clearly precludes the possibilities of NP aggregation or precipitation. The stability of NPs in DMEM media supplemented with 50% of serum makes these NPs suitable for exploring their *in vivo* applications.

We next explored NP uptake experiments in different cancer cell types in no serum and 50% serum (*i.e.* biomimetic) conditions. NP uptake was quantified using a Prussian blue assay (Fig. 2).²⁰ In no

Fig. 2 Uptake of BSA-coated Fe_3O_4 NPs in different cell types in the absence and presence of serum ($p < 0.05$).

Fig. 3 Uptake of BSA-coated Fe_3O_4 NPs in isogenic cell types in the absence and presence of serum ($p < 0.05$).

serum conditions we observed differential uptake of iron oxide NPs between cell types. Maximal uptake was observed with GH3 (rat placental cancer cell line) that is nearly two-fold higher than the other cell types studied.

The presence of serum plays an important role for *in vivo* applications of NPs. In our studies, serum reduces cellular uptake of NPs as compared to serum-free conditions. Marked differences were observed between cell lines. Similar uptake was observed with MEF, GH3 and HeLa cells, with HepG2 roughly 50% of the other cell. Surprisingly, there was no uptake of NPs in the case of MCF-7 cell line (breast cancer cell) (Fig. 2 and Fig. S2, ESI†).

Isogenic cell types possess the same genetic background but differ in their growth characteristics and cellular behavior.²¹ These cells provide a very stringent test bed for differential uptake. For our studies we chose three cell lines: CDBgeo (normal), TD (cancerous) and V14 (metastatic) from BALB/c mice (see Materials and methods section for details). As before, we observed statistically-significant differences between the cell lines both with and without serum (Fig. 3). This differential uptake provides a possible mechanism for selective administration of therapeutics.

We next explored the mechanism of cell uptake with the BSA-coated particles. The mechanism for internalization of nanomaterials into cells depends upon the size and surface chemistry of NPs. NPs can be internalized into cells *via* endocytosis or by diffusion/penetration across the plasma membrane. Endocytosis is an energy dependent process that is inhibited at low temperature and/or in presence of a metabolic inhibitor. Small molecules such as NaN_3 and deoxyribose are known to disrupt specifically the production of ATP in cells and inhibit endocytosis.^{22,23} To probe the mechanism for cellular uptake of the NPs, we incubated different cell types with BSA-coated NPs at 4 °C and in the presence of NaN_3 (0.05% w/v). We observed a 4-fold decrease in uptake of NPs at 4 °C as compared to 37 °C in all cell lines tested (Fig. 4). Similarly in the presence of NaN_3 , there was a two-fold decrease in uptake of BSA-coated NPs (Fig. 4). These results clearly indicate that uptake of NPs occurs through energy dependent endocytosis, consistent with prior studies where it was established that cells uptake the protein coated carbon nanotubes and gold nanoparticles by endocytosis.^{22,23}

For biomedical applications, NPs should ideally be distributed evenly inside the cytoplasm and nucleus rather than localized in

Fig. 4 Cellular uptake of Fe_3O_4 NPs at 4 °C and in the presence of NaN_3 as compared to uptake at 37 °C in different cell types.

Fig. 5 Imaging of HeLa cells after 48h upon incubation with BSA-FITC coated Fe_3O_4 NPs, nuclei are stained with DAPI.

Fig. 6 Cell viabilities of CDBgeo cells upon incubation with BSA coated Fe_3O_4 NPs at different concentrations after 6h.

endosomal vesicles. To explore localization of NPs, we synthesized fluorescein isothiocyanate (FITC) labelled BSA coated NPs and incubated with HeLa cells. Nuclei of cells were stained with 4',6-diamidino-2-phenylindole (DAPI). We observed distribution of NPs throughout the cytoplasm and inside the nucleus as well (Fig. 5). These results shows that BSA helps in the release of NPs from the endosome as well and in distribution throughout the cytoplasm and nucleus.

Cell viability studies (Fig. 6) indicate IC_{50} values greater than 4 mg/mL for all cell lines. More than 80% cell viability was found at 4 mg/mL and nearly 100% viability was found at 2 mg/mL except for HepG2 cell line where 80% cell viability was found at 2 mg/mL (ESI†).

Conclusion

In summary, we have explored the mechanism and differential uptake of biocompatible BSA-coated iron oxide NPs in different cancerous and isogenic cell types. This differential uptake of NPs can be extended for *in vivo* delivery applications of BSA-passivated NPs that can further provide the selective thermal ablation agent for cancer therapy. The protein coating NP can provide a platform for incorporating targeting moieties for targeted tumor thermal therapy.

Materials and methods

All the proteins, DMEM, epidermal growth factor (EGF), insulin, gentamycin were purchased from Sigma-Aldrich. Serum was purchased from HyClone. All other chemicals were purchased from Fisher Scientific and were used without further purification unless otherwise specified. Aquasonic (Model 150 T) ultrasonic cleaner was purchased from VWR scientific products. Fluorescence imaging was done using an Axiovert microscope.

Synthesis of protein coated Fe_3O_4 NPs

Fe_3O_4 NPs were synthesized under alkaline conditions as reported previously.¹⁹ Briefly, 1.72g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 4.67g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (molar ratio of $\text{Fe}^{2+}:\text{Fe}^{3+} = 1:2$) were dissolved under an argon atmosphere in 60 mL of deionized water with vigorous stirring. The solution was purged with argon for 5–10 min to remove any dissolved oxygen present in the deionized water. After the addition of NH_4OH (15 mL) to the reaction mixture, a black precipitate was formed. The reaction mixture was maintained at pH 10–12. The black precipitate was heated at 90 °C for 20 min. The reaction mixture was cooled to room temperature and washed with deionized water three times to remove unreacted chemicals and to decrease the pH of the solution. For the protein coating of NPs, 1 g of protein was dissolved in 20 mL deionized water and NP precursor was added under argon. Then the reaction mixture was sonicated for 2.5 h using an Aquasonic ultrasonic cleaner. A clear reddish-brown solution was formed. Excess protein was removed by ultracentrifugation at 50 000 rpm for 30 min. The process was repeated twice, and the pellet of protein coated NPs was dissolved in milliQ water.

Cell culture

All cell lines except CDBgeo, TD and V14 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS) in T75 culture flasks and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 . CDBgeo, TD and V14 cells were maintained in DMEM-F12 media supplemented with 2% ABS (Adult bovine serum), 25mM HEPES, 10 $\mu\text{g}/\text{mL}$ insulin, 5ng/mL EGF, 15 $\mu\text{g}/\text{mL}$ gentamycin. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in PBS (pH 7.2). CDBgeo cells were prepared by retroviral infection with a marker gene encoding fusion of β -galactosidase and neomycin resistant.²⁴ These cells exhibit normal outgrowths when transplanted into the mammary fat pads. TD cells are a tumorigenic derivative of CDBgeo. The TD cells were prepared by treatment of 10ng/mL TGF- β for 14 days followed by withdrawal for 5 passages. This resulted in a persisted epithelial to mesenchymal transformation of cells and acquisition of tumorigenic growth when transplanted. The V14 cell line was established from a primary mammary tumor arising in BALB/c-*Trp53*± mice. The cells lack p53 protein and form aggressive tumors that are locally invasive in mice.²⁵

Cellular uptake studies

For a typical uptake experiment, nearly 1×10^6 cells per well were plated in a 6-well plate. After 24h of cell plating, medium was removed and cells were washed with DMEM medium. Cells were then treated with BSA- Fe_3O_4 NPs at a concentration of 2mg/mL in

medium. After 6h of incubation, NPs were removed and cells were washed with DPBS three times to remove excess NPs. Cells were then lysed using 1mL/well of lysis buffer (Promega). Cell lysate was centrifuged at 4000rpm for 5 min. and the pellet was dissolved in 200 μ L of 2% HCl solution with bath sonication. For Prussian blue assay, 50 μ L of cell solution, 50 μ L of 2% HCl and 100 μ L of 2% potassium ferrocyanide (in HCl) were mixed and absorption at 704 was observed. A standard curve was prepared using BSA-coated NPs. For uptake studies in the presence of serum, cells were treated with NPs dispersed in media having different percentages of serum.

Cell viability assay

Cytotoxicity assay on different cell lines was performed using alamar blue assay. Typically 10 000 cells/well were plated in a 96-well plate. After 24h, the cells were treated with different concentrations of NPs. After 6h of incubation, nanoparticles were removed and were treated with 10% alamar blue solution and kept at 37 °C for another 2 h. Red fluorescence, resulting from the reduction of alamar blue, was monitored (excitation/emission: 535/590) on a SpectroMax M5 microplate reader (Molecular Device).

Cell imaging

BSA was labelled with FITC using published protocols²⁶ and FITC-BSA coated nanoparticles were prepared as mentioned previously. HeLa cells were incubated with the Fe₃O₄ NPs. After the incubation, cell nuclei were stained with DAPI and the images were taken from an Axiovert microscope.

Acknowledgements

This research was funded by the UMass Center of Excellence in Apoptosis Research (CEAR), the NSF Center for Hierarchical Manufacturing at the University of Massachusetts (NSEC, DMI-0531171), MRSEC facilities, and the NIH (GM077173).

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