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Surface modified superparamagnetic nanoparticles for drug delivery: Interaction studies with human fibroblasts in culture

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The concept of drug delivery using magnetic nanoparticles greatly benefit from the fact that nanotechnology has developed to a stage that it makes possible not only to produce magnetic nanoparticles in a very narrow size distribution range with superparamagnetic properties but also to engineer particle surfaces to provide site specific delivery of drugs. The size and surface characteristics of the nanoparticles are crucial factors that determine the success of the particles when used *in vivo*. The aim of this study was to modify the surfaces of the magnetic nanoparticles with PEG to improve the biocompatibility of the nanoparticles by resisting protein adsorption and increasing their intracellular uptake. In this study, the poly(ethyleneglycol) (PEG) modified superparamagnetic iron oxide nanoparticles have been prepared and their influence on human dermal fibroblasts is assessed in terms of cell adhesion/viability, morphology, particle uptake and cytoskeletal organisation studies. Various techniques have been used to determine nanoparticle–cell interactions including light, fluorescence, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The modification of nanoparticle surface induced alterations in cell behaviour distinct from the unmodified particles, suggesting that cell response can be directed via specifically engineered particle surfaces.

1. Introduction

Nanometer sized superparamagnetic iron oxide nanoparticles with tailored surface chemistry have been widely used experimentally for numerous biomedical applications such as magnetic resonance imaging, tissue repair, hyperthermia, drug delivery and in cell separation etc. [1–2]. All these applications require that cell efficiently capture the magnetic nanoparticles either *in vitro* or *in vivo*. But, unfortunately the internalisation of nanoparticles into cells is severely limited by the short residence time of these particles in the blood and non-specific targeting for achieving the sustained expression levels required for these applications [3].

Magnetic nanoparticles can accumulate in target tissues based on their natural host cell tropisms and on their biophysical properties [1, 4]. In practice, however, the targeting of bare magnetic nanoparticles is often insufficient for rapid and specific accumulation in target tissues. Engineering surfaces of nanoparticles with polymers and proteins or coupling targeting ligands to these nanoparticles can further improve tissue selectivity [4]. The ability of PEG derivatives to improve uptake of micro- and nanoparticles has been reported and is generally recognised as being the result of solubilisation of the particles in cell membrane lipid bilayer mediated by PEG [5, 6]. It has already been shown that immobilisation of PEG on the nanoparticles increases the amount of nanoparticles uptake into cancer cells in comparison to unmodified nanoparticles [5]. For the past decades, significant effort has been directed towards the surface modification of various polymeric drug carriers with PEG not only to improve their biocompatibility and blood circulation times but also to resist protein adsorption and to increase cell internalisation efficiency [6].

Superparamagnetic iron oxide nanoparticles of narrow size ranges are easily produced and coated with polymers, providing convenient, readily targetable magnetic resonance imaging agents [7]. In this study, PEG-modified iron oxide nanoparticles have been prepared producing stable ferrofluid at physiological pH values to increase their cellular uptake. The influence of these nanoparticles on human fibroblasts *in vitro* has been assessed as compared to those unmodified particles, in terms of cell adhesion/cytotoxicity, light microscopy, SEM, TEM and observation of F-actin and β-tubulin cytoskeleton by fluorescence microscopy.

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2. Materials and methods

2.1. Materials

All the chemicals were of reagent grade and were used without further purification. Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O > 99\%$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), methoxy-PEG ($MW = 5000$ D) and maleic anhydride, *N*,*N*′-methylenebisacrylamide (MBA) were obtained from Sigma, UK. Double distilled water was used for all the experiments.

2.2. Production of PEG-coated magnetic nanoparticles

Magnetic nanoparticles coated with PEG having narrow size distribution were prepared and characterised as reported previously [8]. The nanoparticles were found around 40–50 nm in diameter with outer polymeric shell and inner magnetite core.

2.3. Cell culture

Infinity™ telomerase-immortalised primary human fibroblasts (hTERT-BJ1, Clonetech Laboratories, Inc., USA) were chosen for cell culture studies as these fibroblasts are highly stable, fast growing and represent well defined internal cytoskeleton components. The fibroblasts were seeded onto 13 mm glass coverslips in a 24-well plate at a density of 1×10^4 cells/well in 1 ml of complete medium for 24 h after which the growth medium was removed and replaced with the medium containing nanoparticles. For control experiments, medium without particles was used. The medium used was 71% Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK), 17.5% Medium 199 (Sigma, UK), 9% fetal calf serum (FCS) (Life Technologies, UK), 1.6% 200 mM L-glutamine (Life Technologies, UK), and 0.9% 100 mM sodium pyruvate (Life Technologies, UK). The cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.4. Cell adhesion assay

Fibroblasts were seeded with/without nanoparticles at concentration 0.1 mg/ml for 24 h at 37 °C in 5% CO_2. The adhered cells were washed twice with PBS, fixed in 4% formaldehyde/PBS (15 min, 37 °C), washed with PBS again and finally stained for 2 min in 1.0% Coomassie blue in acetic acid/methanol mixture at room temperature. The stained cells were counted in three separate fields under a light microscope.

2.5. Scanning electron microscopy of cell morphology

The fibroblasts were seeded with nanoparticles as before and fixed with 1.5% glutaraldehyde (Sigma, UK) (4 °C, 1 h). The cells were then post-fixed in 1% osmium tetroxide (Agar, UK), and 1% tannic acid (Agar, UK) was used as a mordanant. Samples were dehydrated through a series of alcohol concentrations (20, 40, 50, 60, 70, 90, 96, 100% and dry alcohol) followed by final dehydration in hexamethyldisilazane (Sigma, UK). Once dry, the samples were sputter coated with gold before examination with a Philips SEM 500 field emission scanning electron microscope at an accelerating voltage of 12 kV.

2.6. Immunofluorescence and cytoskeletal observation

The cells were fixed in 4% formaldehyde/PBS at 37 °C for 15 min, washed with PBS and a permeabilising buffer (10.3 g of sucrose, 0.292 g of NaCl, 0.06 g of $MgCl_2$, 0.476 g of Hepes buffer, 0.5 ml of Triton X, in 100 ml of water, pH 7.2) was added at 4 °C for 5 min. The samples were then incubated at 37 °C for 5 min in 1% BSA/PBS. This was followed by the addition of anti-β tubulin primary antibody (1 : 100 in 1% BSA/PBS Sigma, Poole, UK) for 1 h (37 °C). Simultaneously, rhodamine-conjugated phalloidin was added for the duration of this incubation (1 : 100 in 1% BSA/PBS, Molecular Probes, Eugene, OR). The samples were next washed in 0.5% tween 20/PBS (5 min × 3). A secondary, biotin-conjugated antibody (1 : 50 in 1% BSA/PBS, monoclonal antimouse (IgG), Vector Laboratories, UK) was added for 1 h (37 °C) followed by washing. A FITC conjugated streptavidin tertiary antibody was added (1 : 50 in 1% BSA/PBS, Vector Laboratories, UK) at 4 °C for 30 min, and given a final wash. Samples were mounted in Vectorshield fluorescent mountant (Vector Laboratories, UK), then viewed by fluorescence microscope (Vickers M17). The imaging system used was a Hamamatsu Argus 20 with an × 7 Hamamatsu CCD camera.

2.7. Transmission electron microscopy

Cells were fixed as for SEM studies, stained for 60 min with 1% osmium tetroxide and then taken directly through the alcohol steps up to dried absolute alcohol. The cells were finally treated with propylene oxide followed by 1 : 1 propylene oxide : resin for overnight to evaporate the propylene oxide. The cells were subsequently embedded in Spur's resin, and ultrathin sections were cut and stained with lead nitrate and viewed under a Zeiss 902 electron microscope at 80 kV.

2.8. Statistical analysis

The statistical analysis of experimental data utilised the Student's *t*-test and the results were presented as mean ± SD Statistical significance was accepted at a level of $p < 0.05$.

3. Results and discussion

Nanoparticle–cell interaction depends on the surface aspects of materials, which may be described according to their chemistry or hydrophilic/hydrophobic characteristics. These surface characteristics determine how the nanoparticles will adsorb to the cell surface and more particularly determine the cell behaviour on contact. Cells in the presence of nanoparticles first attach, adhere and spread on the surfaces. Thereafter, the quality of cell adhesion will influence their morphology, cytoskeleton organisation and their capacity for proliferation and differentiation [9]. It is known that cell adhesion is mediated by the interaction of surface proteins such as

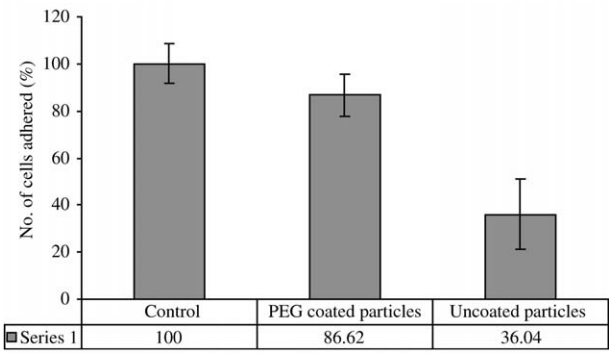

Figure 1 Graphical representation of number of cells adhered, when incubated with uncoated and PEG-coated particles onto glass coverslips, after 24-h culture as compared to controls (results are represented as mean $\pm$ SD; $n = 3$).

integrins with proteins in the extracellular matrix or on the surface of particles [10]. The phenomenon of cell adhesion is of crucial importance in governing a range of cellular functions including cell growth, migration, differentiation and survival [10].

The effect of incubating cells with PEG coated particles on cell adhesion to glass coverslips, as compared to control cells (without particles) was determined. Fig. 1 shows that the number of attached cells was decreased significantly upto 64% in case of uncoated nanoparticles compared to the corresponding control cell number. Growing the cells with PEG coated samples produced no significant difference compared to that of control cells. The low toxicity of the PEG coated nanoparticles may be attributed to high solubility of PEG in cell membranes [11].

SEM images taken at 24 h provided further information on cell morphology in response to particle incubation. It was observed from SEM results (Fig. 2) that the control cells are flat and well spread with small filopodia, suggesting cell motility. Uncoated particles

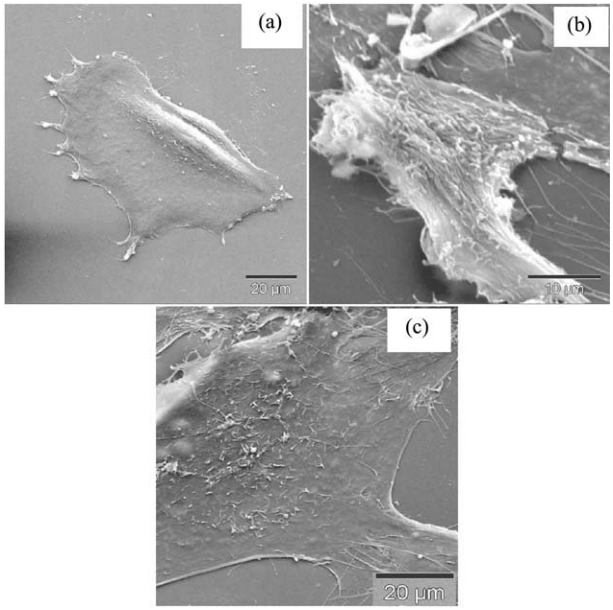

Figure 2 SEM pictures of human fibroblasts incubated with magnetic nanoparticles (a) control cells, (b) uncoated and (c) PEG-coated nanoparticles.

were found to exhibit vacuoles in the cell cytoplasm with cell membrane abnormalities. In addition, cells were less spread and stimulated the formation of many lamellapodia and filopodia. The PEG-modified particles showed an increase in cell number after 24 h with bumped cell morphology and many protrusions extending from the cell body as a result of particle uptake.

Upon endocytosis the particles may effect the overall cytoskeleton of the cells by forming the vacuoles in the cell body which may result in disrupted cytoskeleton and cell membrane [12]. Immunofluoroscent images (Fig. 3) were taken by staining for F-actin using rhodamine–phalloidin and for tubulin using anti-tubulin antibodies.

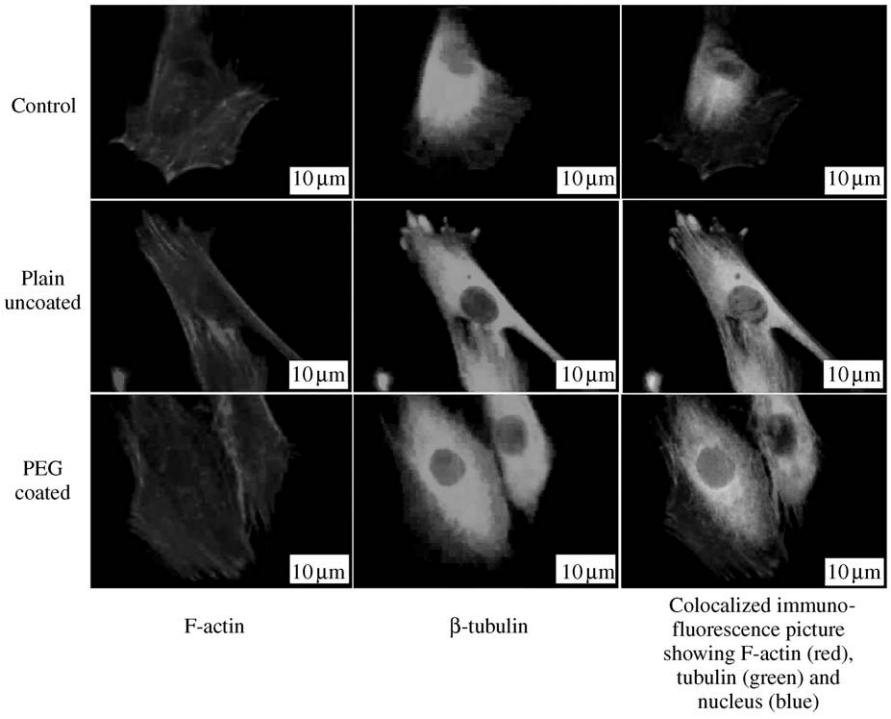

Figure 3 Immunofluorescence pictures of cells incubated with different particles and stained for tubulin (green), F-actin (red) and nucleus (blue) ($n = 2$).

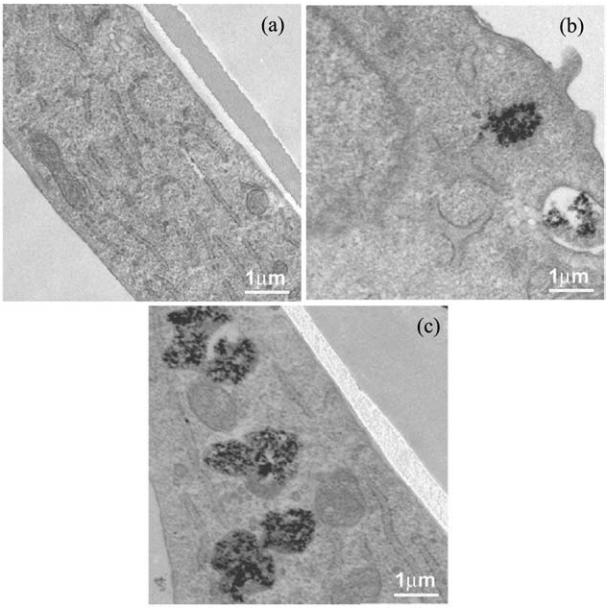

Figure 4 TEM pictures of Human fibroblasts incubated with magnetic nanoparticles (a) control, (b) uncoated and (c) PEG-coated magnetic nanoparticles.

In the control cells, the microfilaments were well organised in thick bundles forming stress fibres. The microtubules also form a dense network equally distributed around the nucleus in the whole cell volume. In the case of cells incubated with PEG-coated nanoparticles, there were prominent stress fibres reflecting from the cell periphery as observed in the control cell. Endocytosis of the uncoated particles resulted in disruption of the cell membrane and disorganised cell cytoskeleton. It was observed that PEG-coated particles are hugely internalised probably due to fluid phase endocytosis mechanism without affecting the cytoskeleton of the fibroblasts.

The above results were confirmed with TEM studies as shown in Fig. 4. The pictures showed that the uncoated magnetic nanoparticles are internalised within the fibroblast cytoplasm forming the vacuoles. After PEG modification, the nanoparticles uptake by human fibroblasts increased greatly in comparison to that of unmodified nanoparticles. Once inside the cells, magnetite particles are clustered within the lysosomes. Degradation of iron oxide nanoparticles into iron ions is presumed to occur in intracellular lysosomes under the influence of a variety of hydrolytic enzymes, low pH of lysosomes and proteins participating in iron metabolism and utilisation according to natural iron pathways [13].

4. Conclusions

In this study, we have modified the surfaces of the superparamagentic nanoparticles with PEG and studied their influence on human fibroblasts. The PEG coated nanoparticles did not affect the cell adhesion behaviour and morphology and their internalisation into endosomes offer the opportunity to label a variety of cells with high efficiency. Combined with existing advanced concepts of particulate drug delivery and magnetic therapy, the PEG-coated magnetic particles may provide additional specificity and efficacy, which are required in many drug and gene therapy approaches.

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