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Receptor-Mediated Targeting of Magnetic Nanoparticles Using Insulin as a Surface Ligand to Prevent Endocytosis

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Abstract—Superparamagnetic iron oxide nanoparticles have been used for many years as magnetic resonance imaging contrast agents or in drug delivery applications. Tissue and cell-specific drug targeting by these nanoparticles can be achieved by employing nanoparticle coatings or carrier-drug conjugates that contain a ligand recognized by a receptor on the target cell. In this study, superparamagnetic iron oxide nanoparticles with specific shape and size have been prepared and coupled to insulin for their targeting to cell expressed surface receptors and thereby preventing the endocytosis. The influence of these nanoparticles on human fibroblasts is studied using various techniques to observe cell–nanoparticle interaction that includes light, scanning, and transmission electron microscopy studies. The derivatization of the nanoparticle surface with insulin-induced alterations in cell behavior that were distinct from the underivatized nanoparticles suggests that cell response can be directed via specifically engineered particle surfaces. The results from cell culture studies showed that the uncoated particles were internalized by the fibroblasts due to endocytosis, which resulted in disruption of the cell membrane. In contradiction, insulin-coated nanoparticles attached to the cell membrane, most likely to the cell-expressed surface receptors, and were not endocytosed. The presence of insulin on the surface of the nanoparticles caused an apparent increase in cell proliferation and viability. One major problem with uncoated nanoparticles has been the endocytosis of particles leading to irreversible entry. These results provide a route to prevent this problem. The derivatized nanoparticles show high affinity for cell membrane and opens up new opportunities for magnetic cell separation and recovery that may be of crucial interest for the development of cellular therapies.

Index Terms—Biological cells, drug delivery systems, magnetic materials, nanotechnology.

I. INTRODUCTION

SUPERPARAMAGNETIC iron oxide nanoparticles with tailored surface chemistry have been widely used experimentally for numerous *in vivo* applications such as magnetic resonance imaging (MRI) contrast enhancement, tissue repair, immunoassay, detoxification of biological fluids, hyperthermia, drug delivery, cell separation, etc. [1]–[4]. All these biomedical and bioengineering applications require that these nanoparticles have high magnetization values and size smaller than 20 nm with overall narrow particle size distribution so that the particles have uniform physical and chemical properties. In addition, these applications need special surface coating of the magnetic particles, which has to be not only nontoxic and biocompatible but also allow a targetable delivery with particle localization in a specific area [5].

Cell labeling with ferro/paramagnetic substances is an increasingly common method for *in vivo* cell separation [6], as the labeled cells can be detected by MRI [7]. Most current labeling techniques utilize either of two approaches: 1) attaching magnetic particles to the cell surface [8] or 2) internalizing biocompatible magnetic particles by fluid phase endocytosis [9], receptor-mediated endocytosis [10], or phagocytosis [11]. One strategy to realize efficient and specific cell labeling of magnetic particles is to modify the nanoparticle surface with a ligand that is efficiently taken up by target cells via receptor-mediated endocytosis [10]. A variety of potential ligands have been conjugated to nanoparticle surfaces to facilitate receptor-mediated endocytosis of the particles, including monoclonal antibodies (mabs) [11]. Targeting agents such as transferrin, lactoferrin, albumin, insulin, growth factors, etc., have been demonstrated to preferentially target cell surface, because the receptors for these ligands are frequently overexpressed on the surface of mammalian cells [12], [13]. These receptors are not only cellular markers, but also have been shown to efficiently internalize molecules coupled to these receptors [12]. Furthermore, many of these ligands are stable and generally poorly immunogenic. Despite the well-known ability of these receptors to facilitate internalization of nanoparticles, little effort has been made on delivery of magnetic nanoparticles modified with such ligands.

Superparamagnetic iron oxide nanoparticles of narrow size range can be easily produced and coupled to proteins, thus providing convenient, readily targetable MRI agents. In this study, magnetic iron oxide nanoparticles coated with insulin have been prepared for exact delivery of drugs to target tissues. The influence of these nanoparticles on human dermal fibroblasts *in vitro* has been assessed, as compared to those underivatized particles, in terms of cell adhesion, cytotoxicity, light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). From the results, we observed that insulin-derivatized superparamagnetic iron oxide nanoparticles are targeted at the surface of the fibroblasts and, thus, may serve as a nontoxic and improved way of drug targeting.

II. MATERIAL AND METHODS

A. 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 ($FeCl_2 \cdot 4H_2O$), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT, and insulin (human recombinant, pH 8.2, 10mg/ml) were obtained from Sigma (Dorset, U.K.) while sodium hydroxide ($NaOH > 99\%$) and hydrochloric acid ($HCl > 37\%$ v/v)) were obtained from Fluka, Dorset, U.K. Double distilled water was used for all the experiments.

B. Synthesis of Magnetic Nanoparticles

The ferric and ferrous salts (molar ratio $2:1$) were dissolved in deoxygenated water at a concentration of 0.1 M of iron ions. Chemical precipitation was achieved by using a 1-M deoxygenated solution of sodium hydroxide. The reaction was carried out in nitrogen atmosphere at low temperature ($4\,°C$–$6\,°C$) with vigorous stirring. Particles were washed by dialysis using 12-kD cutoff dialysis membrane against double distilled water to remove unreacted water salts. They were then precipitated with acetone and dried in a vacuum oven at $70\,°C$–$80\,°C$. The surface of the particles was neutralized with 0.01-M HCl. A solution of IM sodium oleate was added to form the stable dispersion of the magnetite nanoparticles. To the aqueous suspension of magnetic particles, sodium oleate solution was added dropwise with continuous and vigorous stirring at $60\,°C$–$70\,°C$ under nitrogen atmosphere. Excess sodium oleate was removed through rigorous dialysis using 12-kD cutoff dialysis membrane as above.

C. Derivatization of Magnetic Particles With Insulin

Insulin was coupled covalently at the nanoparticle surface by using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) coupling methods. Magnetic nanoparticles (10 mg/ml) were suspended in 2-ml phosphate buffer ($pH = 4.5$) with vortexing. To this solution a freshly prepared EDCI solution (2% w/v in phosphate buffer, pH-4.5) was added dropwise with shaking. The mixture was allowed to stir at room temperature for 3-4 h. The particles were then washed twice by centrifugation at 10 000 r/min followed by resuspension in phosphate buffer. Finally, particles were centrifuged and resuspended in borate buffer (pH-8.5). About 300–400 μg of the protein insulin [2 mg/ml in phosphate-buffered saline (PBS), pH 7.4] was then added and mixed gently overnight at room temperature on an end-to-end mixer. The solution was then centrifuged for 10 min at 10 000 r/min. The supernatant was used for protein determination. Amount of protein coupled was determined using Lowry's method (BIO-RAD DC protein assay kit, Hercules, CA) by calculating the difference between the total amount of protein added and the amount present in the supernatant. The particles were finally washed with water and kept for future use.

D. Cell Culture

Infinity telomerase-immortalized primary human fibroblasts (hTERT-BJ1, Clonetech Laboratories, Inc., Hampshire, U.K.) were seeded onto 13-mm glass coverslips in a 24-well plate at a density of 1×10^4 cells per 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 with no particles was used. The medium used was 71% Dulbecco's modified Eagle's medium (DMEM) (Sigma, Dorset, U.K.), 17.5% Medium 199 (Sigma, Dorset, U.K.), 9% fetal calf serum (FCS) (Life Technologies, Paisley, U.K.), 1.6% 200 mM L-glutamine (Life Technologies, Paisley, U.K.), and 0.9% 100 mM sodium pyruvate (Life Technologies, Paisley, U.K.). The cells were incubated at $37\,°C$ in a 5% CO_2 atmosphere.

E. Cell Adhesion Assay

The effect of nanoparticles on cell adhesion was determined with cell suspension incubated with or without nanoparticles. Fibroblasts (h-TERT BJ1) were expanded in monolayer tissue culture. The cells were detached using trypsin-EDTA solution and divided into two individual populations. Cells were seeded with or without nanoparticles at concentration 0.1 mg/ml for 24 h onto coverslips (13-mm diameter; in triplicate) at $37\,°C$ in 5% CO_2. The 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 adherent cells were counted in three separate fields under a light microscope using an eyepiece. The stained samples were observed by light microscopy, and digital images of the fibroblasts were captured using a Hamamatsu Argus 20 for image processing.

F. Live–Dead Assay for Cell Viability

Live–dead cell viability assay is a two-color fluorescence assay that is based on the simultaneous determination of the numbers of live and dead cells. Live cells have intracellular esterases that converts nonfluorescent, cell permeable calcein acetoxymethyl (calcein AM) to the intensely fluorescent green calcein which is retained within the cells. On the other hand, ethidium homodimer enters the damaged membranes of dead cells and is fluorescent red when bound to nucleic acids. The fibroblast cells were seeded onto 13-mm glass coverslips at 10 000 cells/ml in a 24-well tissue culture plate. After the cells were attached to the coverslips, cell medium was exchanged with the fresh medium containing nanoparticles and cells were cultured at $37\,°C$. After 24 hours, medium was removed and the cells were washed with PBS followed by viability staining using calcein AM (2 μM, Molecular Probes, Leiden, The Netherlands) and ethidium homodimer (4 μM, Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature. All samples were viewed on a fluorescence microscope.

G. In Vitro Cell Viability/Cytotoxicity Studies

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide) assay is a simple nonradioactive colorimetric assay to measure cell cytotoxicity, proliferation, or viability. MTT is a yellow, water-soluble tetrazolium salt. Metabolically active cells are able to convert this dye into a water-insoluble dark-blue formazan by reductive cleavage

of the tetrazolium ring [14]. Formazan crystals, then, can be dissolved in an organic solvent such as dimethylsulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 550 nm, and the resultant value is related to the number of living cells. To determine cell cytotoxicity/viability, the cells were plated at a density of 1×10^4 cells/well in a 96-well plate at 37 °C in 5% CO_2 atmosphere. After 24 h of culture, the medium in the wells was replaced with the fresh medium containing nanoparticles in varying concentrations. After 24 h, 20 μl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4, MTT Sigma, Dorset, U.K.) was added to each well. After 4 h of incubation at 37 °C and 5% CO_2 for exponentially growing cells and 15 min for steady-state confluent cells, the medium was removed and formazan crystals were solubilized with 200 μl of DMSO, and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader (Dynatech MR7000 instruments) at 550 nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles was calculated by $[A]_{\text{test}}/[A]_{\text{control}} \times 100$.

H. Scanning Electron Microscopy of Cell Morphology

The cells were fixed with 1.5% glutaraldehyde (Sigma, Dorset, U.K.) buffered in 0.1 M sodium cacodylate (Agar, Stansted, U.K.) (4 °C, 1 h) after a 24-h incubation period to allow the viewing of individual cells. The cells were then postfixed in 1% osmium tetroxide (Agar, Stansted, U.K.), and 1% tannic acid (Agar, Stansted, U.K.) was used as a mordanant. Samples were dehydrated through a series of alcohol concentrations (20%, 30%, 40%, 50%, 60%, 70%) followed by further dehydration (90%, 96%, 100%, and dry alcohol). The final dehydration was in hexamethyldisilazane (HMDS) (Sigma, Dorset, U.K.), followed by air-drying. Once dry, the samples were sputter coated with gold before examination with a Phillips SEM 500 field emission SEM at an accelerating voltage of 12 kV.

I. Transmission Electron Microscopy

Cells were incubated with nanoparticle solutions for 24 h as discussed before. 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.

J. Statistical Analysis

The statistical analysis of experimental data utilized the student's t-test, and the results were presented as mean ± standard deviations. Statistical significance was accepted at a level of $p < 0.05$.

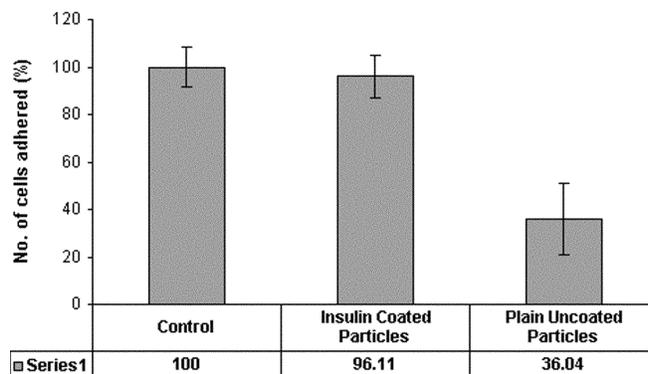| ■ Series1	Control	Insulin Coated Particles	Plain Uncoated Particles
	100	96.11	36.04

Fig. 1. Graphical representation of number of cells adhered, when incubated with uncoated plain, and insulin-coated particles onto glass coverslips, after 24-h culture as compared to controls (results are represented as mean ± standard deviations; $n = 3$, counted in triplicate in individual microscope fields).

III. RESULTS

The magnetic nanoparticles were synthesized by coprecipitation of ferrous and ferric salts solution by concentrated sodium hydroxide solution and characterized as reported previously [15]. Amount of protein bound to the nanoparticles was determined by Lowry's method and calculated by the difference between the total amount of protein added and the amount present in the supernatant. The percentage of protein binding to nanoparticles by two-step EDCI coupling process was found to be around 60% of the total protein added initially for binding.

The effect of incubating cells with nanoparticles on cell adhesion to glass coverslips, as compared to control cells (without particles), was determined, and the results are shown in Fig. 1. It was observed that insulin coating on nanoparticle surfaces gives rise to changes in adhesion capacity of the fibroblasts on glass. The figure shows that the number of attached cells was decreased significantly, up to 53% in the case of uncoated plain nanoparticles compared to the corresponding control cell number (no particle). Growing the cells with insulin-coated samples produced no significant difference compared to that of control cell population.

The general morphology of the fibroblasts incubated with nanoparticles after staining with Coomassie blue is shown in Fig. 2. The figure shows that the cells were well spread, and there was no distinct change in morphology after 24-h incubation with insulin-coated particles relative to control cells. But the cells grown in the presence of uncoated plain nanoparticles were found to be less spread with altered cell morphology possibly due to endocytosis of particles. Cell viability staining using calcein AM/ethidium homodimer showed that the cells exposed to insulin-coated nanoparticles were more than 99% viable.

The MTT assay for cell viability evaluation has been described as a very suitable method for detection of biomaterial toxicity [14]. The MTT assay relies on the mitochondrial activity of fibroblasts and represents a parameter for their metabolic activity. The proliferation/viability of fibroblasts was measured by MTT assay after culturing for 24 h, and it showed that cell proliferation was more favorable in case of protein-coated particles than with uncoated ones. Insulin-coated nanoparticles revealed no cytotoxic effects to cells, and
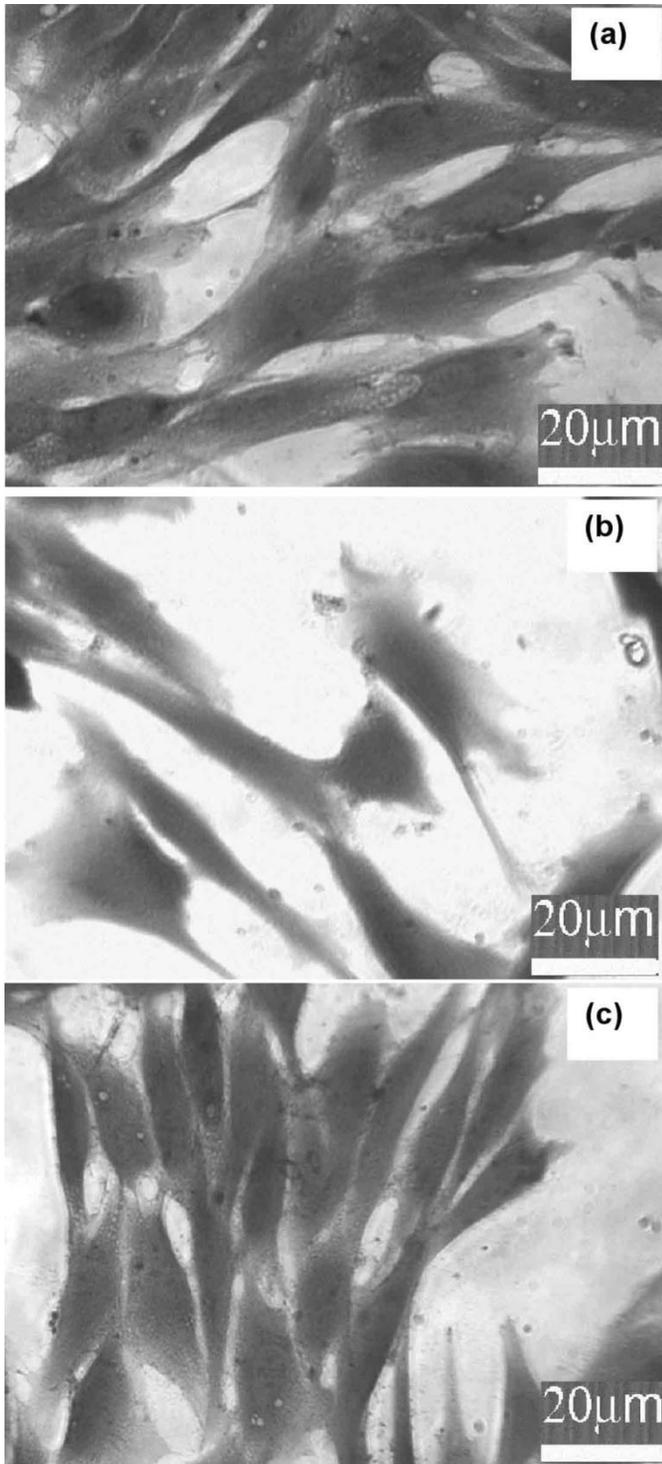
Fig. 2. Coomassie blue stained cells incubated with different magnetic particles for 24 h at 37 °C. (a) Control. (b) Uncoated. (c) Insulin-coated nanoparticles; $(n = 3)$.
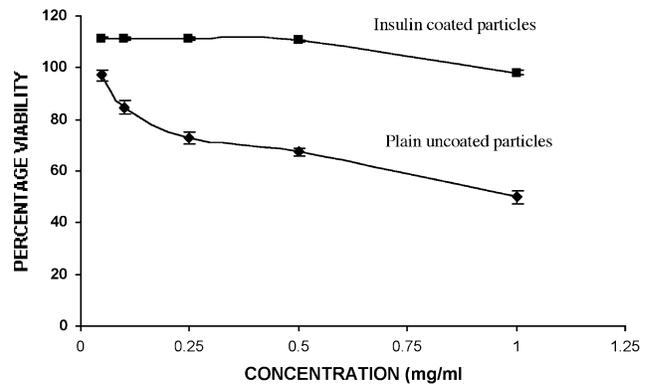

Fig. 3. Cytotoxicity profiles of magnetic nanoparticles when incubated with human fibroblasts as determined by MTT assay. Percentage of viability of fibroblasts was expressed relative to control cells $(n = 6)$. Results are represented as mean $\pm$ standard deviations.

centration. Incubation with uncoated plain magnetic particles, the fibroblasts showed significant loss in viability of about 25%-50% observed at concentrations ≥ 250 μg/ml. Below this concentration, cellular metabolic activity did not change much in comparison with control cells.

SEM images taken at 24 h provided further information on cell morphology in response to particle incubation. It was observed from the SEM results (Fig. 4) that the control cells are flat and well spread with small lamellapodia, suggesting cell motility. Insulin-coated nanoparticles appeared to localize and adhere to the cell surface as demonstrated in the figure. Plain uncoated particles were found to be endocytosed by the cells. The fibroblasts exhibited vacuoles in the cell body with cell membrane abnormalities. In addition, cells were less spread, small in shape, and stimulated the formation of many lamellapodia and filopodia, observed projecting from the cell membranes over the glass surface.

The results obtained from light microscopy and SEM studies were confirmed with TEM studies, as shown in Fig. 5. The pictures showed that the uncoated plain magnetic nanoparticles are internalized within the fibroblast as a result of endocytosis. Several electron lucent voids containing nanoparticles can be seen in the cytoplasm of the fibroblasts forming the vacuoles. The cellular burden of the particles was often so great that much of the cell area was compromised of nanoparticles. It is apparent from the pictures that surface derivatization with insulin made the nanoparticles strongly cell surface adhesive, and the particles could be seen on the surface of the fibroblasts with no particle internalization.

IV. DISSCUSSION

Insulin was coupled to the nanoparticle surfaces to preferentially target the human fibroblasts that have the receptors for these proteins expressed on their surface. The derivatization of insulin on the nanoparticles was carried out using a two-step EDCI coupling procedure without affecting the colloidal stability of the ferrofluids.

Nanoparticle–cell interaction depends on the surface aspects of materials, which may be described according to their chemistry, hydrophilic/hydrophobic characteristics or surface energy.

they remained more than 100% viable relative to control at concentration as high as 1mg/ml, as shown in Fig. 3. The increased cell viability can be explained by nutrient effect [16]. Plain uncoated nanoparticles affected the metabolic activity in concentration dependent manner when they were added in the concentration range of 0–1000 μg/ml to the cells. Cytotoxicity of the nanoparticles increased in relation to increasing con-
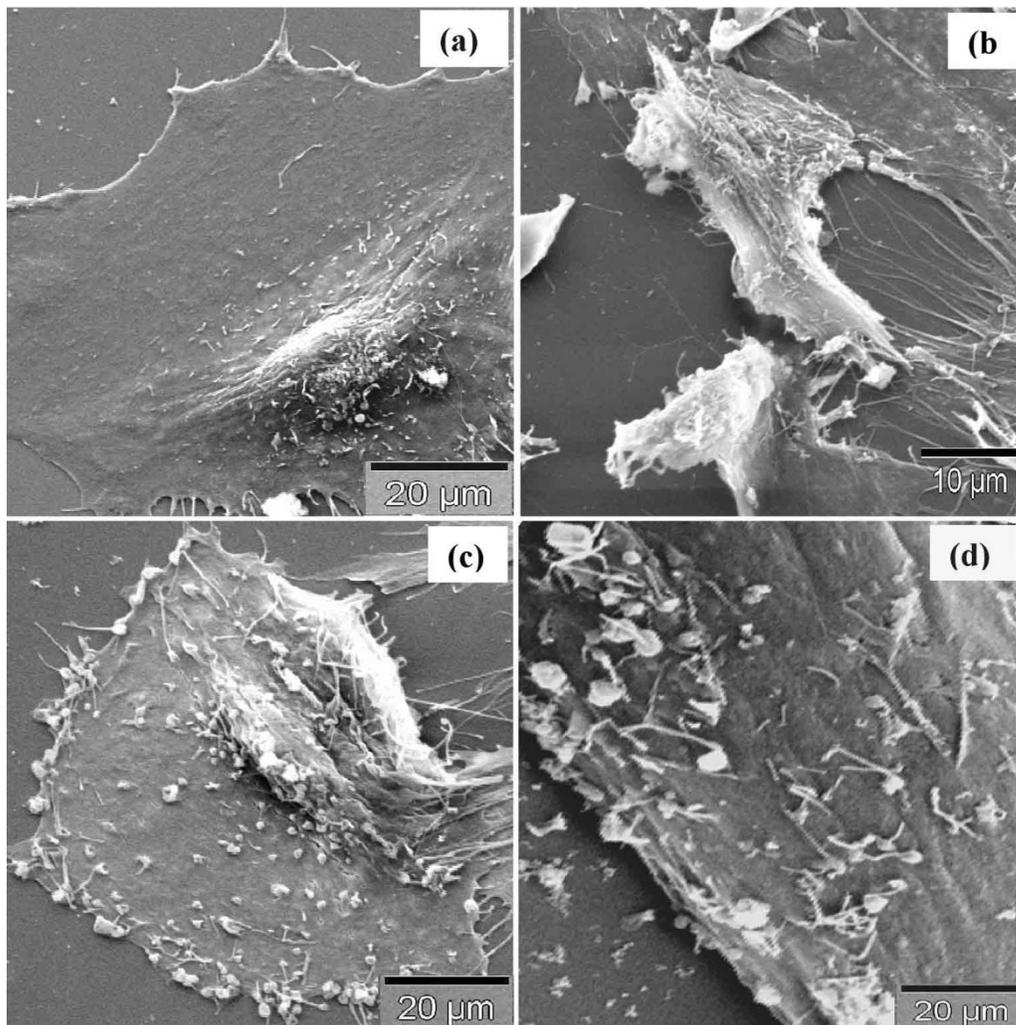
Fig. 4.   SEM pictures of human fibroblasts incubated with magnetic nanoparticles. (a) Control cells. (b) Plain uncoatd particles. (c) and (d) Insulin-coated nanoparticles. The pictures shows that insulin-coated nanoparticles adhere to the cell surface whereas plain uncoated particles were found to be endocytosed by the cells.

These surface characteristics determine how the nanoparticles will adsorb to the cell surface and more particularly determine the cell behavior 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 and their capacity for proliferation and differentiation [17]. It is known that cell adhesion is mediated by the interaction of surface proteins such as integrins with proteins in the extracellular matrix or on the surface of other cells or particles. The phenomenon of cell adhesion is of crucial importance in governing a range of cellular functions including cell growth, migration, differentiation, survival, and tissue organization [17].

It was observed from cell culture studies that the plain uncoated nanoparticles reduced cell adhesion and viability significantly as compared to the cells that were not exposed to the nanoparticles. One possible explanation for this large decrease in cell adhesion and viability is that these nanoparticles are taken up by the cells as a result of endocytosis or are promoting apoptosis (programmed cell death) due to weak cell adhesive interactions with the nanoparticles. The low toxicity of nanoparticles derivatized with insulin may be attributed to the fact that these ligands act as cellular markers that are targeted at the surface receptors expressed on the cell surface without being internalized. Receptors are highly regulated cell surface proteins, which mediate specific interactions between the cells and their extracellular milieu and they are generally localized on the plasma membrane.

The SEM studies also verified the above results. These studies showed that the each nanoparticle type with different surface characteristics caused a distinct cell response. The uncoated particles were endocytosed by the fibroblasts during the 24-h incubation, thereby causing cell death possibly through apoptosis due to the internalization [18]. Endocytosis of the particles resulted in disruption of the cell membrane. Cells were found to be less spread, small in size, and stimulated the formation of many lamellipodia and filopodia, observed projecting from cell membranes over the glass surface. From SEM studies, it could be seen that insulin-derivatized particles are highly adhesive to the cell surface receptors.

Phagocytosis involves uptake of extracellular cargo that is generally larger than 500 nm, otherwise, uptake is due to the endocytosis or pinocytosis. Upon endocytosis the particles may
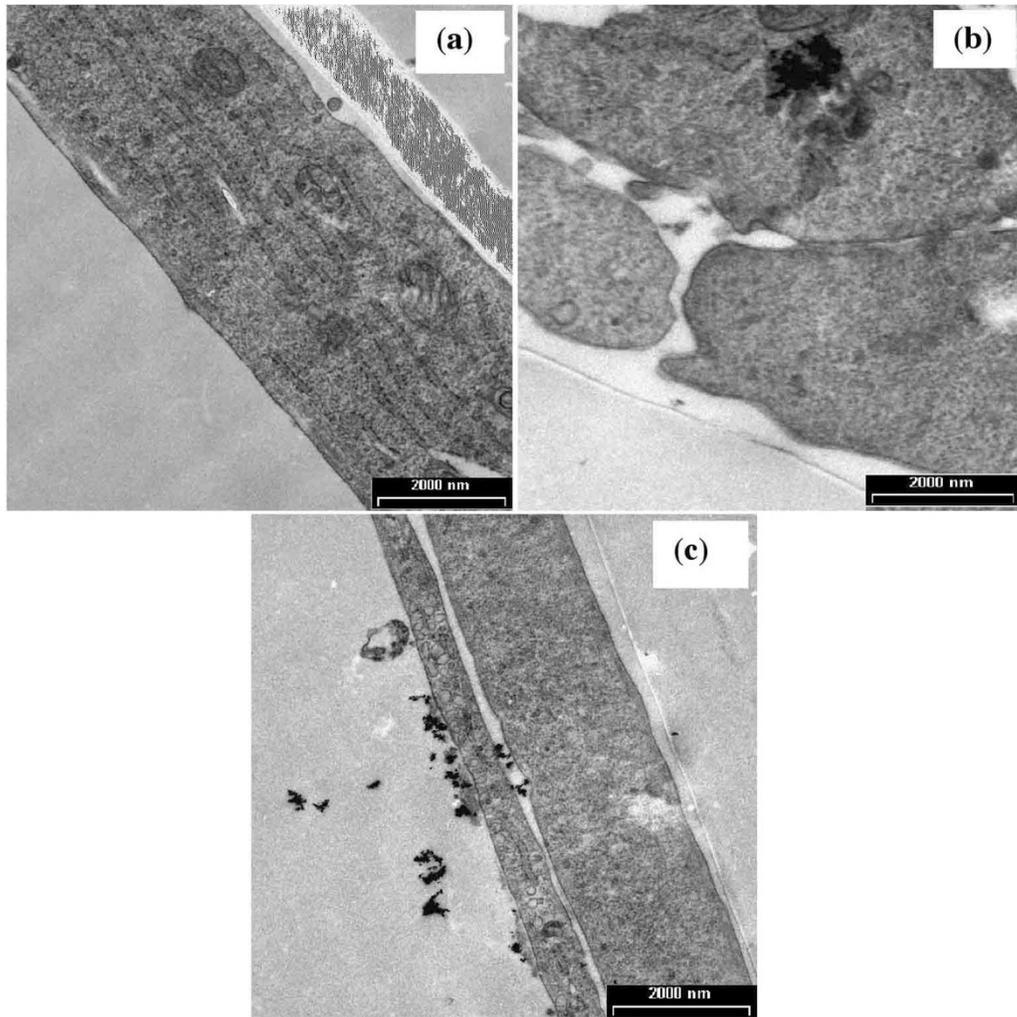
Fig. 5.   TEM prictures of human fibroblasts incubated with nagnetic nanoparticles. (a) Control. (b) Plain uncoated. (c) Insulin-coated magnetic nanoparticles.

result in the formation of the vacuoles in the cell body and extended cell membrane protrusions [19]. From SEM and light microscopy results, we observed that the surface functionalized particles did not change the cell morphology to a greater extent as compared to uncoated ones. The TEM studies indicated that a substantial number of uncoated particles were internalized by the cells, confirming the above SEM and light microscopy studies. It was also concluded from the TEM pictures that the insulin-derivatized particles are not internalized but found to adhere at the cell surface.

In the absence of any system to inhibit endocytosis, most underivatized nanoparticles are endocytosed by cells and eventually sequestered in digestive vacuoles in the cell. Once the particles are endocytosed, they are probably removed from contact with specific cell surface receptors and become effectively ineffective. As a result of these events, the cells are at high risk of apoptosis from overload with particles. If the particles can be prevented from leaving the cell surface, they will remain in contact with their specific receptors and would be expected to leave the cell in a state of prolonged stimulation while protecting the cells from side effects due to endocytosis. In the present study,

we have discovered a route to derivatising superparamagnetic nanoparticles with various proteins that bind strongly to surface receptors that phagocytosis is inhibited. Confinement to the cell surface would provide a route that might allow removal of the particles from the cells after an appropriate residence time.

V. CONCLUSION

Superparamagnetic nanoparticles with distinct surface characteristics induces either endocytosis or adhesion to the cell membrane. Both types of response could provide routes of investigation with regards to drug delivery techniques. Surface functionalized nanoparticles with insulin showed high affinity for cell surface receptor mainly due to ligand–receptor interactions. Their specific attachment to cell surface offers the opportunity to label the cells with magnetic particles while reducing nonspecific phagocytosis. This type of directed action would prove useful in drug delivery to specific cell types without causing any harmful effects to healthy cells. For drug delivery systems where cell death is required, as in the case of cancer cells, the uncoated particles may prove useful.

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Catherine Berry, photograph and biography not available at the time of publication.

Mona Gupta, photograph and biography not available at the time of publication.

Adam Curtis, photograph and biography not available at the time of publication.