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1 # Targeted uptake of folic acid-functionalized iron oxide nanoparticles by
2 # ovarian cancer cells in the presence but not in the absence of serum

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9 **Abstract**

10 Targeted delivery of nanoparticles to cells or tissues of interest is arguably the “holy grail” of nanomedicine. Using primary human
11 macrophages and ovarian cancer cells, we evaluated the biocompatibility and specific targeting of folic acid (FA)-conjugated iron oxide
12 nanoparticles with organic [poly(ethylene glycol), PEG] or inorganic (SiO_2) intermediate surface coatings. Reduction of folate receptor-α
13 expression using specific siRNA resulted in a significant decrease in cellular uptake of the SiO_2-coated nanoparticles, but did not affect
14 uptake of PEG-coated nanoparticles. Notably, specific (i.e. FA-dependent) uptake was observed only in the presence of serum proteins. The
15 strategy presented here for receptor-mediated uptake of nanoparticles with pre-defined surface chemistry may enable targeting of
16 nanoparticles for therapeutic and imaging applications.
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18 *Key words:* Superparamagnetic nanoparticles; Biocompatibility; Folate receptor; Ovarian cancer; Targeting

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Background 20

Nanoparticles are potentially very useful for targeted drug 21
delivery, not least in the field of cancer therapy, as this 22
approach could increase the efficacy of the drug through an 23
increase of the dose at the target site and a reduction of the 24
dose in bystander tissues.[1] Recent studies support the concept 25
of active targeting of nanoparticles, not only in pre-clinical 26
animal models, but also in human cancer patients, using, e.g. 27
transferrin or prostate-specific antigen to target cancer cells 28
overexpressing the corresponding receptors.[2,3] 29

Nanoparticles that interact with biological systems are 30
likely to acquire a surface ”corona” of biomolecules that may 31
dictate their biological behavior.[4] Indeed, the combination of 32
material intrinsic properties (i.e. the ‘synthetic identity’) and 33
context-dependent properties determined, in part, by the bio- 34
corona of a given biological compartment (i.e. the ‘biological 35
identity’) is likely to dictate the interactions of nanoparticles 36
with cells and tissues[5] and the propensity of particles to cross 37
biological barriers.[6] This has important implications for 38
targeted nanoparticles with surface-bound recognition ligands. 39
Indeed, it has been suggested that the corona of proteins may 40

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obscure specific recognition of targeting ligands.[7] In support of this contention, Salvati *et al.* recently reported that transferrin-functionalized nanoparticles lose their targeting capabilities in the presence of serum.[8] On the other hand, Simberg *et al.*[9], who studied the full repertoire of super-paramagnetic nanoparticle (SPION)-binding proteins, found that both the dextran coating and the iron oxide core remained accessible to specific probes after incubation of SPIONs in plasma, suggesting that the nanoparticle surface could be "seen" by cells, despite the formation of a protein corona. Several possible scenarios may be invoked to account for these differences. First, as implied by the latter study on SPIONs, the corona coverage may not be complete. Alternatively, there may be variations within a population of nanoparticles such that some particles display complete corona coverage while some nanoparticles display accessible targeting ligands; in fact, recent work suggests that cells may perceive different populations of nanoparticles with different 'biological identities'.[10] Furthermore, the adsorption of biomolecules may be reversible, as shown recently for small nanoparticles (similar in size to proteins),[11] implying that the formation of a long-lived 'hard corona'[12] may not always occur. More studies are required to understand the potential impact of the bio-corona on targeting of nanoparticles.

Therapeutic applications of nanotechnologies are frequently compromised by the rapid elimination of nanoparticles from the blood stream due to their recognition and elimination by macrophages.[13] Modification of particle surfaces with polymers, such as poly(ethylene glycol) (PEG), is a popular strategy in nanomedicine and this results in a reduction of non-specific protein adsorption[14] and particles display longer circulation time in blood.[15] It is known that nanoparticles passively accumulate in tumor tissue as a consequence of the enhanced permeability and retention effect (EPR). However, cellular internalization of such nanoparticles may be improved through the attachment of a targeting ligand such as transferrin or folic acid (FA) to trigger receptor-mediated uptake.[16]

Using a publically available *in silico* transcriptomics database,[17] we previously noted that the folate receptor (FR)-α or FOLR1 is significantly overexpressed in ovarian cancer,[18] the leading cause of death from gynaecological malignancies in the Western world.[19] Here, we decided to test whether FA functionalization of nanoparticles would lead to specific uptake by ovarian cancer cells. To this end, nanoparticles consisting of a crystalline iron oxide core were functionalized with FA through intermediate layers of inorganic (SiO_2) or organic (PEG) compounds to produce two nanoparticles designated Fe_3O_4-SiO_2-FA and Fe_3O_4-PEG-FA, respectively, and tested for biocompatibility and internalization using primary human monocyte-derived macrophages and the human ovarian cancer cell line, SKOV-3. Excellent *in vitro* biocompatibility was noted for both nanoparticles. Moreover, Fe_3O_4-SiO_2-FA and Fe_3O_4-PEG-FA were both internalized by SKOV-3 cells. However, specific, FR-α-mediated cellular uptake was observed only for Fe_3O_4-SiO_2-FA nanoparticles, and only in the presence of serum in cell culture medium. Our data suggest that Fe_3O_4-SiO_2-FA nanoparticles could be useful for biomedical applications.

Methods

Synthesis of folic acid-conjugated Fe_3O_4 nanoparticles

Fe_3O_4-PEG nanoparticles were synthesized following a slightly modified solvothermal process previously reported by Yan *et al.*[20] Experimental details are provided in the Appendix. Core shell nanostructures were synthesized using a variation of the well-known Stöber process. The synthetic pathway for Fe_3O_4-SiO_2-FA particles and Fe_3O_4-PEG-FA particles is shown schematically in Figure 1, *A* and *B*, and experimental details are provided in the Appendix. The Fe_3O_4-PEG-FA and Fe_3O_4-SiO_2-FA nanoparticles were characterized as described below in Results; experimental details are provided in the Appendix. Endotoxin content was assessed prior to cell experiments as previously described.[21]

Primary human monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMC) were prepared from buffy coats obtained from healthy blood donors (Karolinska University Hospital, Stockholm, Sweden) as previously described.[22] CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for positive selection of monocytes and $CD14^+$ monocytes were then cultured in the presence of 50 ng/mL recombinant macrophage-colony-stimulating factor (M-CSF) (Novakemi, Handen, Sweden) for 3 days to obtain human monocyte-derived macrophages (HMDM). For further details on cell culture procedures, see Appendix.

Human ovarian carcinoma cells

Human ovarian carcinoma cells SKOV-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2. For some experiments, FBS was omitted, as detailed in Results.

Cell viability assessment

Release of lactate dehydrogenase (LDH), a marker of loss of plasma membrane integrity, was determined by using CytoTox 96 non-radioactive cytotoxicity assay (Promega G1780, Madison, WI). Mitochondrial function, reflective of cell viability, was determined using the 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich). ZnO nanoparticles (100 μg/ml) (IBU-tec, Weimar, Germany) were included as a positive control. The experimental details are provided in the Appendix.

siRNA transfection

Transfection of SKOV-3 cells using specific siRNA against *FOLR1* (ON-TARGETplus, SMARTpool FOLR1, Gene ID 2348) was performed with Transfection Reagent DharmaFECT1 (Dharmacon, Lafayette, CO) according to the manufacturer's protocol. A scrambled sequence was used as a negative control (ON-TARGETplus Non-Targeting siRNA #1).

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