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The influence of ligand organization on the rate of uptake of gold nanoparticles by colorectal cancer cells

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ABSTRACT

We have explored the uptake of different hydrophilic mono- and dual-ligand gold nanoparticles in colorectal cancer cells *in vitro* and find that the rate of uptake is dependent on the structural organization of the ligands on the surface of the particles rather than their charge or chemical properties. Gold nanoparticles with 50%PEG-NH_2/50% glucose are taken up eighteen fold faster than nanoparticles carrying only PEG-NH_2 or glucose. Glutathione-coated gold particles are by far the most efficiently internalized; however, glucose-glutathione dual-ligand nanoparticles are taken up at a thirty fold reduced rate. We found furthermore that the rates are influenced by the cell density and concentration of glucose in the growth medium. Rather than being internalized through a conventional receptor-mediated mechanism the particles appear to be taken up by the cells via an energy-independent diffusion across the cell membrane through pre-existing pores or openings in the lipid bi-layer created by ligands on the gold nanoparticles.

1. Introduction

Nanoparticles offer important new possibilities in cancer diagnosis and therapy [1–7], and can be used in the location and visualization [8–10] of tumours in their primary and potentially also their secondary locations and as delivery vehicles for anti-cancer drugs and for non-invasive ablation therapies. The transport of the nanoparticles to the tumour is a multistage process. Systemically administrated nanoparticles with tumour-binding ligands can accumulate in the tumours due to more chaotic organized vasculature compared to non-diseased tissue. Since the vessels in the tumours are more leaky and dilated than in normal tissues, nanoparticles can diffuse across the vessel walls into the tumours [11]. However, to be able to access the lumen of the cancers where the most aggressive cancer cells are located, the nanoparticles need to penetrate through the interstitial network of collagen and adhesion molecules. Finally they need to be taken up by the cancer cells and if carrying anti-cancer drugs the nanoparticles need to enter a suitable subcellular compartment where they can deliver their drug for optimal biological effect. Recent data suggest that nanoparticles with a hydrodynamic diameter larger than 10 nm are unable to penetrate the interstitial barrier [12,13] making them less desirable in anticancer therapy.

Small gold nanoparticles (GNPs) of diameters less than 10 nm have completely different physiochemical/thermodynamic properties than metallic gold or larger nanoparticles because of their higher surface-area to volume ratio. The outer atoms form fewer coordinated bonds than the inner atoms and are therefore less stable [14]. Ligands that bind covalently (via thiol groups) to Au atoms during the reduction of the $HAuCl_4$ assemble into this outer layer on the Au crystals. Given the very large surface-to-volume ratio of the small 5 nm nanoparticles with a 2 nm gold core the covalently attached ligands are very densely packed and organized into structures that may resemble lattices in crystals. The choice of ligands and their organization will have a bearing on their biological properties and how the nanoparticles interact with macromolecules in solution and on target cells. Recent studies show that nanoparticles carrying a combination of hydrophobic and hydrophilic ligands can organize them in an orderly fashion into hydrophobic and hydrophilic discs on the surface of the particles [15,16]. The ratio of hydrophilic-to-hydrophobic ligands determines the spacing between these discs thus also the binding enthalpy to soluble proteins [17] and the mode and rate of uptake by the cells

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[15,16]. Whereas mono-ligand nanoparticles with 11-mercapto-undecane sulfonic acid (MUS) without a structured organization will only be endocytosed, dual-ligand combination of MUS and octanethiol (OT) organized in stripe-like structures on the nanoparticles are taken up by the cells through preformed pores in the cell membrane in an energy-independent manner [15,16].

The behaviour of the MUS/OT dual-ligands gold nanoparticles suggest therefore that mixing hydrophobic and hydrophilic ligands on the surface of small gold nanoparticles might resemble the properties of alloys in giving the nanoparticles altered biophysical properties. Little is known about how dual hydrophilic ligands on the small gold nanoparticles affect their biological properties. Li et al. suggested that dual-ligand gold nanoparticles of folic acids and glucose show enhanced binding to the folate receptor on cancer cells resulting in more efficient internalization than particles with folic acid alone [18]; however the mechanism for this needs to be elucidated. Since small gold nanoparticles have such potential in nano-oncology, we have explored the uptake of single- and dual-ligand gold nanoparticles in colorectal cancer cells further. We find that the rate of uptake is not determined by the charge of the ligands but rather by the way the ligands are organized on the surface on the particles. Our data furthermore suggest that the mono- and dual-ligand gold nanoparticles of less than 10 nm diameter are not being internalized by the cells by an energy-dependent active process.

2. Materials and methods

2.1. Materials

The uptake inhibitors cytochalasin B, cytochalasin D, wortmannin, MK571, nystatin, amyloid hydrochloride hydrate and methyl-β–cyclodextrin as well as bovine insulin and puromycin dihydrochloride were obtained from Sigma–Aldrich (Poole, UK). Mouse anti-human Glut-1 monoclonal antibodies (clone 202915) were purchased from R&D Systems Inc. and goat anti-mouse $F(ab)_2$ conjugated to AlexaFluor647 and Hoechst 33342 were obtained from Invitrogen Inc. (Paisley, UK). Medium, foetal calf serum and trypsin-EDTA were purchased from Invitrogen Inc. (Paisley, UK), and Dulbecco's minimum essential GlutaMax medium (DMEM) and phosphate-buffered saline (PBS) from Sigma–Aldrich (Poole, UK).

2.2. Nanoparticle production

Table 1 lists the different gold nanoparticles used in this study together with a schematic structure of the particles. The following general method was used to produce nanoparticles with gold metal cores of ~1.6 nm diameter, noting that the ligand coronas increase the hydrodynamic diameters to ~5 nm. Oxidized ligand, either glutathione (Fluka 49741) or β2 mercaptoethoxy-glucose (synthesized in house), were dissolved in 9:1 methanol:water and gold III chloride (Sigma–Aldrich, Poole, UK) added. The organic ligands were used at a fourfold molar excess relative to the gold. The solution was then mixed for 5 min gently on a flat bed shaker. The nanoparticles were produced by reduction following the rapid addition of a 20 fold molar excess relative to the gold, of freshly made 1 M sodium borohydride (Sigma–Aldrich, Poole, UK) under vigorous vortexing. The samples were vortexed for a total of 30 s followed by a further 1 h gentle mixing on the flat bed shaker. As the nanoparticles are not soluble in methanol/water solvent, initial purification was by bench centrifugation, supernatant removal and dispersion of the nanoparticle pellet in water. Further purification was achieved by 4 water washes in 10 kDa vivaspin centrifugation devices (GE Healthcare). The gold concentration of all nanoparticle preparations was determined by a simple colorimetric assay. Briefly 10 μl of nanoparticle sample or 12 mg/ml gold standard (Fluka (Sigma–Aldrich, Poole, UK)) and blanks were digested with 30 μl of 50:50 water:aqua regia in an ELISA plate for 1 min, this was followed by addition of 150 μl of 2 M NaBr, the 405 nm absorbance was then measured immediately, the assay having excellent linearity over the 0–10 μg range.

2.3. Cell culture

The human colorectal cell lines HCT-116 (CCL-247), HT 29 (HTB-38), LS154T (CL-188) and SW640 (CCL-227) were purchased from ATCC (American Tissue Culture Collection) and grown in pyruvate-free DMEM-GlutaMax containing 0.45% glucose and 1% penicillin-streptomycin.

HCT-116 cells were transfected with hPKM2-shRNA (sc-62820-sh) plasmid (Santa Cruz Biotech Inc.) using transfection (Invitrogen). After 24 h the cultures were trypsinized and diluted 3× before being replated. 2 ng/ml puromycin (Sigma–Aldrich) was added 24 h later. Individual transfectants were selected 14 days later and maintained in 2 ng/ml puromycin-containing medium.

2.4. Quantification of cell-associated GNPs

Cells were grown in 6-well plates (Invitrogen) and at 80% confluence the medium was changed to pyruvate-free DMEM-GlutaMax containing 0.45% glucose and 1% penicillin-streptomycin or pyruvate-free DMEM-GlutaMax containing 0.45% D-xylose, 170 nM bovine insulin and 1% penicillin-streptomycin. After 2 h incubation, the medium was replaced with the same medium containing GNP (10 μg/ml Au) and the cells incubated for 22 h. The cells were then rinsed three times with PBS, trypsinized and washed twice more with PBS before being lysed in 0.5 ml 2 M NaOH and diluted to 5 ml with PBS. The concentration of gold was quantified using a Thermo-Scientific iCAP 6000 ICP spectrometer calibrated with 2, 20, 200 and 2000 ng/ml solutions of gold standard (Fluka 08269 Au std). The cell numbers were estimated by measuring absorption at 280 nm and correlated with a standard curve of similarly lysed HCT-116 cells.

2.5. Microscopy

For electron microscopy HCT 116 cells were grown overnight on coverslips. After a rinse with PBS the medium was changed to pyruvate-free DMEM-GlutaMax containing 0.45% D-xylose, 170 nM bovine insulin and 1% penicillin-streptomycin. GNPs were added after 4 h or 13 h. After a further 1.5 h incubation, the cells were rinsed three times in PBS and fixed in 2% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3. They were then osmicated in 1% OsO_4/0.1 M phosphate buffer, dehydrated in a graded ethanol-water series, cleared in propylene oxide and infiltrated with Araldite resin. Ultra thin sections were cut using a diamond knife, collected on 300 mesh grids, and stained with uranyl acetate and lead citrate. For detection of the 2 nm gold cores the cells were permeabilized either by a freeze thaw method where the cover slips were immersed in a cryoprotectant 25% sucrose, 10% glycerol in 50 mM phosphate buffer pH7.4 for 30 min then lowered into a liquid nitrogen slush, frozen and then thawed twice or alternatively, the cells were subjected to a brief exposure to 0.01% Triton in phosphate buffer (<1 min). The cells were then subjected to silver enhancement using a nano probe Kit LI Silver Enhancement Kit (Molecular Probe, Invitrogen Ltd., Paisley, UK). The silver enhancement was performed as described by the manufacturer's protocol at a temperature of 5 °C. Enhancement was carried out for 12 min each time with the osmication kept to 5 min at 5 °C in the dark and processed as described above. The cells were viewed in a Jeol 1010 transmission electron microscope (Jeol, Herts, UK) and the images recorded using a Gatan Orius CCD camera (Gatan, Abingdon, UK).

3. Results

3.1. Uptake rates for single-ligand gold nanoparticles

When colon cancer cells are grown in the presence of 10 μg Au/ml glutathione-coated gold nanoparticles, the amount taken up by the cells increase linearly with time over a period of at least 80 h (Fig. 1A). We therefore selected 22 h as a convenient incubation time for the remaining experiments described below. Fig. 1B shows that glucose-GNPs are internalized with a rate that increases linearly with the dose (cm^2/ml) reaching a saturation level at 46.6 cm^2/ml with a LC50 of 23.15 cm^2/ml equivalent to 1.6×10^{12} particles taken up per cell in a 22 h period. We observed an experiment-to-experiment variation in the amount of GNPs engulfed by the cells suggesting that the cell density might affect the rate of uptake. Indeed exposing HCT-116 cells at different density to GNPs revealed that the rate of uptake of glutathione-GNPs increases linear with cell density (Fig. 1C). However, such a cell density dependent increase in the rate of uptake was not seen for glucose-GNP showing a constant rate of uptake for the cells irrespective of the growth medium (Fig. 1C).

3.2. Rate of uptake is controlled by the structure of ligands on the surface of the gold nanoparticles

To examine how composition of ligands on the small gold particles affects the rate of uptake in the HCT-116 cells, we formulated gold nanoparticles with single ligands of different charges (glucose^0, $-NH_3^+$ and glutathione$^-$) and with dual-ligands