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Attachment, uptake and transport of nanoparticles coated with an internalin A fragment in Caco-2 cell monolayers

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Four phases of the interaction with cells in monolayer culture of polystyrene nanoparticles – with and without a putative receptor specific surface protein – have been explored. Attachment of the particles and their adhesion to the surface of the cells, uptake, followed by translocation within the complex milieu of the cell interior and finally the release of the particles from the basolateral aspect of the cell monolayer have been studied. One-micrometre polystyrene particles were observed, uncoated and coated with a fragment of internalin A, the surface protein of Listeria monocytogenes *that facilitates the uptake and translocation of the organism from the gut through epithelial tissues through its interaction with E-cadherin receptors. Cell monolayer attachment is facilitated by the decoration of the surface of the nanoparticles, adhesion being reduced by incubation of the monolayer with anti-E-cadherin antibody, or with the internalin A protein. However, uptake into the cells and escape from the cell into the basolateral chamber is not enhanced at all cell loadings: at very low loadings there is some evidence of enhanced uptake, but not at 1:100 ratios. No significant difference was found between basolateral efflux of plain particles and internalin A fragment coated systems, suggesting that the limiting step may be the movement of the nanoparticles in the cytoplasm.*

Key words: Nanoparticles – Adhesion – Translocation – E-cadherin – Internalin A – Polystyrene latex.

Understanding the uptake and fate of nanoparticles and microparticles is a task of growing importance, not only from the perspective of drug delivery by a variety of routes but also from a general toxicological viewpoint. The utilisation of nanoparticles decorated with specific ligands chosen to interact with cell surface receptors is a means to enhance uptake and to better understand the absorption and translocation processes. Many surface ligands have been used to explore nanoparticle behaviour *in vitro* and *in vivo* [1-3]. Koch *et al.* [4] have used Tat peptides, a polyarginyl peptide to observe the effect on nanoparticle trans-membrane passage. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* use invasin to gain access to the liver, spleen and blood via the gut wall [5]. Dawson and Halbert [6] found the effect of invasin on cell uptake of PLGA nanoparticles to be size dependent. In our laboratories tomato lectin [7] and also invasin [8] have been adsorbed to the surfaces of polystyrene latex particles. Enhanced uptake from the gastro-intestinal tract was observed after oral administration [9].

L. monocytogenes interacts with the gut epithelial cells through the protein internalin, targeting the M-cells of Peyer's patches [10]. Here we employ a protein fragment from the surface protein internalin A, expressed by *L. monocytogenes*. Internalin A allows the organism access to host cell interiors through interaction with the E-cadherin receptor. The protein was adsorbed onto 1-μm polystyrene latex particles and the adsorption to, uptake into and translocation of the nanoparticles through a Caco-2 cell monolayer investigated.

I. MATERIALS AND METHODS

Polystyrene nanoparticles of 500 nm and 1 μm in diameter were purchased from Polysciences (UK).

1. The internalin A fragment

L. monocytogenes isolates 4885, 7973, 9862, 11994, 12426 and 12480 were obtained from the UK National Culture Collection and stored at - 70°C. Genomic DNA from each strain was obtained using the Wizard genomic isolation kit (Promega, Southampton, UK). The sequence that encodes Internalin A (inlA) known to contain the E-cadherin binding motifs were amplified by PCR: amplification was performed using a Mastercycler (Eppendorf, Cambridge, UK) with Pfu DNA polymerase (Stratagene, Cambridge, UK) and an annealing temperature of 40°C. Primers used for amplification of internalin fragments that bind E-cadherin (inlAf) were based on GenBank accession number AJ012346 data for the *L. monocytogenes* internalin operon: InlA1 (5'-GCAGGATCCATTACACAAGATACTC-3') [positions 1635-1659] and InlA2 (5'-TTCGGATCCTAAATTCCCAGCTTC-3') [positions 2848-2871]. BamH1 restriction endonuclease sites (underlined) were introduced into the primers to facilitate cloning of the PCR reaction products. PCR products were subjected to electrophoresis on 1% (w/v) agarose gels, bands of the predicted size were purified using a QIAquick gel extraction kit (Promega, Southampton, UK) and the sequences cloned into the BamH1 site of pQE-16 (Qiagen Ltd., Crawley, UK). Plasmid DNA was introduced into competent *Escherichia coli* One Shot TOP10F' (Invitrogen Ltd., Paisley, UK). PCR was used to confirm the orientation of InlAf in the vector and only clones with the correct insert orientation were used; the identity of InlAf sequences was confirmed by DNA sequencing. Colonies carrying inframe InlAf sequences were grown in batch culture at 37°C to exponential phase (OD_{600} = 0.6) and InlAf synthesis induced with 1 M isopropanyl-β-D-thiogalactopyranoside; after a further 4 h incubation at 37°C, cells were harvested, lysed and His_6-tagged recombinant InlAf purified by immobilised metal (Ni) affinity chromatography. Predicted molecular weights of recombinant InlAf were confirmed by SDS-polyacrylamide gel electrophoresis. Five InlAf proteins were obtained using sequences from *L. monocytogenes* strains 11994 and 12426 and were designated 11994-1, 11994-2, 11994-3, 11994-4 and 12426-2.

2. Adsorption of Internalin A fragment (InlAf) protein to nanoparticles

Five hundred-nanometre and 1-μm particles were suspended in sterile filtered water. The particles were centrifuged (10 min, 14.5 Krpm) and resuspended in sterile filtered water to a final concentration of 5 mg/ml. The particles were dispersed in InlAf protein solution and 1 M boric acid to a final concentration of 1 mg/ml (particles) and ~ 300 μg/ml (protein). The suspensions were left shaking overnight and were then centrifuged (10 min, 14.5 Krpm) to harvest the particles. The particles were washed in an equal volume of sterile filtered water

and finally resuspended in sterile filtered water. The supernatants were retained and combined for protein quantification. The particles were stored at 4ºC and sonicated for 15 min prior to use, after which a sample of supernatant was analysed for protein to check if sonication disturbed the adsorbed protein.

3. InlAf protein coated and uncoated particles

The coated and plain (undecorated) particles were harvested, washed once in sterile filtered water, resuspended in Optimem-1 media to the required concentration and sonicated for 15 min. The particle suspensions were applied to the apical chamber in ratios to the number of Caco-2 cells seeded onto the membrane.The transepithelial resistance (TEER) was measured. After 30, 60, 90 and 120 min, the monolayers and their supports were placed in fresh 12 well plates containing 1.5 ml of pre-warmed Optimem-1 and the basolateral media retained. After 120 min the apical suspension was removed and discarded. The monolayers were washed twice with 500 μl PBS. The monolayers were then treated with an acid wash buffer (pH 3) (500 μl) and agitated gently. This was repeated and this second acid wash was retained. The monolayers were then lysed with 500 μl of ice-cold 1% Triton X-100 in sterile filtered water and agitated. These cell lysates were retained. All retained fractions were stored at 4ºC and the particle concentration estimated using a haemocytometer.

4. Characterization of particles

Particles were characterized with respect to their loading with protein, size distribution and surface charge (zeta potential).

4.1. Size distribution of particles

The size distribution and the average diameter of the particles were determined by laser diffraction using a Malvern MasterSizer 3 (Malvern Instruments, Malvern, UK). Suspensions (3 ml) of coated 500-nm non-fluorescent particles (0.25% (w/v)) in sterile filtered water were placed in cuvettes and inserted into the sample holder of the spectrophotometer. The mean diameter size and distribution of the coated sample was determined, and compared with uncoated particles. Uncoated particles had a mean diameter of 444 ± 68 nm, whereas protein coated nanoparticles had larger diameters ranging from 588 to 651 nm. This is most likely due to some association between particle rather than significant enlargement of the individual particles due to adsorption.

4.2. Protein quantification

The supernatant fractions were combined and their protein content quantified using the standard Bio-Rad protein assay, adapted from the Bradford assay. Supernatant (10 μl) was mixed with 200 μl of a 1 in 4 dilution of the protein assay dye reagent in a flat bottomed 96 well plate, stirred on a rocking platform for 20 min and the absorbance determined at 570 nm. Samples were also run on a 12% SDS-PAGE gel to visualise the protein. The percentage of protein adsorbed ranged from 43 to 61%, there being no significant differences between the samples.

4.3. Stability

The particles were stored at 4ºC for up to 48 h. After 2, 4, 6, 24 and 48 h, the particles were harvested and resuspended in an equal volume of sterile filtered water. The supernatant was retained and its protein content quantified. The particles retained 90-95% of the adsorbed protein over a 48 h period.

4.4. Surface charge of particles (zeta potential)

The zeta potentials of the coated particles and uncoated particles were determined using a MasterSizer 3000 (Malvern Instruments, Malvern, UK). Five hundred-nanometre fluorescent particles were coated in InlAf protein and diluted in sterile filtered water (0.25% w/v). Plain latex particles had a zeta potential of - 25 mV; the adsorbed protein reduced the zeta potential by an average of 3.0 ± 0.6 mV.

5. Caco-2 cell propagation

Harvested Caco-2 cells were gently resuspended in non-antibiotic containing supplemented DMEM to a concentration of 1 x 10^6 cells/ml; numbers were estimated using the trypan blue method. The cell suspension (450 μl) was placed in the apical well of a prepared Costar Transwell. Twelve well Transwells were prepared by addition of 1.5 ml of supplemented DMEM to the basolateral chamber and 200 μl to the apical well. The plates were then incubated (37°C, 5% CO_2) for 20 min prior to seeding. Cells were seeded onto Transwells with a pore size of 3 μm in diameter, incubated (37°C, 5% CO_2) for 21 days, fed every second day with the TEER monitored from day 14.

To investigate the toxicity of the particles towards the Caco-2 cells, cells were seeded onto prepared flat-bottomed 96 well plates. Plates were prepared by addition of 100 μl of supplemented DMEM and incubated (37°C, 5% CO_2) for 20 min. Plates were seeded with 100 μl of a cell suspension containing 1 x 10^6 cells/ml, and the monolayers were then confirmed to be complete by observation under a light microscope (Olympus CK40-F200) with a x10 objective

6. Cell viability using the MTT assay

Supplemented DMEM was removed from the 96 well plates seeded with Caco-2 cells after visual confirmation that the monolayer was intact. The monolayers were then rinsed with PBS and 200 μl of Optimem-1 medium added. The cells were left to equilibrate for 30 min. The Optimem-1 was removed from the apical chamber and replaced with 200 μl of the required concentration of particles suspended in Optimem-1. Particles were added at concentrations of 1000, 100 and 10 μg/ml to achieve Caco-2 cell to particle ratios of 1:100, 1:10 and 1:1. The plates were incubated for the required time. The particle suspension was removed and the monolayers gently rinsed with phosphate buffered saline (PBS). Two hundred microlitres of DMEM containing 10 μg/ml MTT was applied and incubated for 3 h 30 min. The medium was then removed, 200 μl of DMSO added to each well and the plates agitated for 30 min. The absorbance of each well at 570 nm was measured.

7. Transport and uptake

Caco-2 cells with a stable TEER of between 600-800 $\Omega.cm^2$ were rinsed with PBS and left to equilibrate in Optimem-1 for 30 min. The media from the apical chamber was removed, the monolayer rinsed with PBS and replaced with 450 μl of particles suspended in Optimem-1. Over time the inserts were placed in fresh wells with 1.5 ml of pre-warmed Optimem-1 in the basolateral chamber and the TEER measured. The medium from the basolateral chamber was stored at 4ºC until analysis. After the incubation period the monolayers were rinsed with PBS to remove unattached particles and then treated with acid wash buffer to remove attached particles. The monolayers were then lysed as described to release particles taken up by the monolayers. All data are the result of three experiments on one day, replicated on three separate occasions.

8. Antibody treatment of monolayers

Monolayers were treated with anti-E-cadherin antibody designed to block the function of human E-cadherin (Zymed, Cambridge Bioscience, Cambridge, UK). Prior to application of the particle suspension, the monolayers were treated for 10 min with a serum blocking solution, rinsed in PBS followed by a 25-min incubation at room temperature with a 1:100 dilution (diluent from Cambridge Bioscience, Cambridge, UK) of the antibody. Control wells were treated in the same manner, up to the point of application of the antibody; pure diluent was applied to the control wells and the experiment continued as above.