Identification of physicochemical factors controlling the capacity of nano-particles to penetrate cells of the respiratory epithelium.

Andrew Thorley and Terry Tetley
Lung Cell Biology, NHLI, Imperial College London, SW3 6LY

Background: The physicochemical properties of nano-sized particles differ from those of equivalent bulk substances and can be unpredictable. The very properties that make nanomaterials so attractive for use in e.g. engineering, electronics, cosmetics, textiles and medicine, may make them hazardous. The increased use of nanomaterials should be matched by parallel research into any potential risks of adverse effects on the environment and/or health. However, as highlighted in numerous recent reports (e.g. Royal Society and the Royal Academy of Engineering, 2004; DEFRA, 2005; Maynard; 1-3) serious and growing concerns have been expressed that research into the unforeseen risks of nanomaterials on health and the environment is insufficient compared to their current, predicted level of production and use. These concerns were also highlighted in the European Commission's Action Plan for Nanotechnology (2006) and the EPA's Nanotechnology White Paper (2005) as well as in reports to many European governments, expressing disquiet over the lack of urgency in identifying any potential risks. It is important to anticipate, and where possible circumvent, any adverse health effects of nanoparticles.

A major concern relating to engineered nanomaterials is that their reactivity could be due to unique physicochemical properties currently not included in standard toxicity screening tests nor understood at a cellular level. Furthermore, many of the commercial uses of such particles might result in exposure in the workplace (although this is unlikely), or there might be release into industrial effluents and into domestic waste streams. The implications of such dispersal are unknown. Neither are the effects of wear and tear of products derived from nanosubstances.

An important area of research is to evaluate the mechanisms of action of engineered nanomaterials and one key aspect of the reactivity of nanosubstances is their interaction with cells and membranes. Previous studies in vivo show that increased surface area is an important component of the pro-inflammatory effects of nanoparticles compared to larger particles (see 4). Other studies have shown that inhaled nanoparticles can access other body compartments (eg cardiovasculature, brain; 5-10), suggesting that they can translocate across the pulmonary epithelium. Ultimately, nanoparticle reactivity is likely to reflect a number of factors, including particle size, charge, chemistry, format and functionalisation. Added to this is the unique environment with which the particles interact. Thus, the characteristics of the particle can in turn dictate the location in the body and duration of presence at that location. In the lung, nano-sized particles will preferentially deposit in the respiratory, alveolar units. This region is coated by pulmonary surfactant, a phospholipid-enriched fluid secreted by the type 2 pneumocytes (alveolar epithelial type 2 cells; AT2). This fluid also contains surfactant proteins A-D and other proteins with unique characteristics that ensure surfactant spreading and reduced surface tension at the air-liquid interface, as well as acting as defence molecules, neutralizing particles deposited in this region. The underlying alveolar epithelium is a major cellular target of these particles. The epithelial barrier consists of the cuboidal AT2 cells and flattened alveolar type 1 (AT1) cells. Although AT2 cells outnumber AT1 cells, 2:1, the large, thinly spread AT1 cells cover over 95% of the alveolar surface and are likely to be critical in controlling particle translocation across the epithelial barrier.

Previous work by the applicant: A unique, immortal AT1-like cell line has recently been established from transformed, primary human AT2 cells (AT2 cells differentiate into AT1 cells in situ, suggesting that immortalization has driven the AT2 cells to differentiate; 11, 12). Techniques have also been developed to routinely isolate primary AT2 cells from normal-appearing pieces of human lung tissue following lobectomy for lung cancer; these primary cells cannot be passaged. The immortalized, human AT1-like cells preferentially internalized 50nm latex particles (80% of cells internalize negatively charged nanoparticles, and 45% of cells internalized positively charged nanoparticles), whereas primary human AT2 cells hardly internalize particles at all. The advantage of using latex particles is that they can be obtained in a wide range of sizes, with different surface charges and they can be fluorescently labeled for microscopical analysis. In collaboration with Professoir Yuri Korchev and Dr Julia Gorelik (Imperial College) these observations have been confirmed using scanning ion conductance microscopy, which allows observation of live cells at the level of the electron microscope. Combined with surface confocal microscopy of the particles, it was possible to watch the particles being translocated from the cell margin to the centre of the cell prior to internalization.

Research plan:
Objective 1: To determine which (combination of) factors influence nanoparticle uptake and/or translocation by human alveolar epithelium - particle size or surface charge? Months 1-3.

Methods
Immortalised AT1 cells were grown to confluency and serum starved for 24 hours prior to experiments. To investigate the effect of size and charge on particle uptake, cells were exposed to fluorescently labeled latex nanoparticles in the size range 30nm-1000nm that were modified to be either charge neutral, negatively charged or positively charged. Uptake was measured using Simple PCI image analysis software over a time course of four hours and again at 24 hours. These studies were also repeated using primary human AT2 cells in order to compare cell differences.

Results
Effect of charge on uptake of 50 and 100nm beads: AT1 cells internalised all nanoparticles to varying degrees (Figure1). Negatively charged 50nm particles were internalised more rapidly and to a greater extent than neutral and positively charged particles (4hr; $P<0.0006$). Furthermore, by four hours, uptake of positive 50nm particles was significantly greater than that of neutral charged particles of the same size (MFI 1526 +ive, 1036 neutral; $P<0.003$). Neutral and negatively charged 50nm particles were internalised significantly more than 100nm particles (4hours; $P<0.002$) whereas 100nm positively charged particles were internalised more than 50nm positively charged particles ($P<0.0001$). The latter particles appeared to exhibit AT1 cell cytotoxicity (see below).
Effect of size on nanoparticle uptake: To investigate the effect of size on uptake of nanoparticles, cells were exposed to increasing sizes of nanoparticles for four hours (Figure 2). Results demonstrated that as particle size increased the degree of uptake significantly decreased ($P<0.009$). As shown in the previous studies negatively charged particles of all sizes were internalised to a greater degree than neutral charged particles of the same size. When these experiments were repeated using AT2 cells, very few particles were internalised over a 24 hour time course (Figure 3). Internalisation was so low that it could not be quantified.

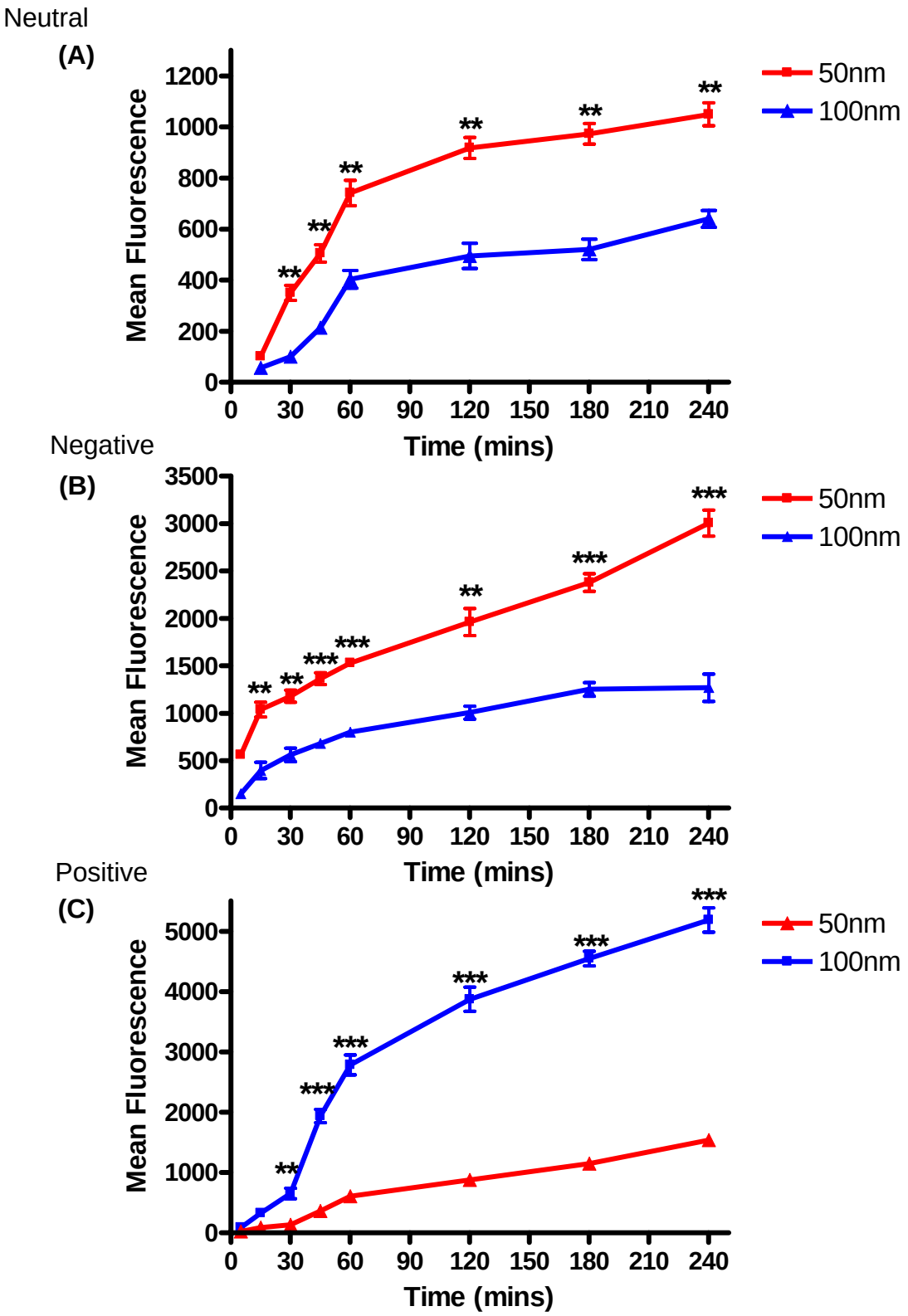
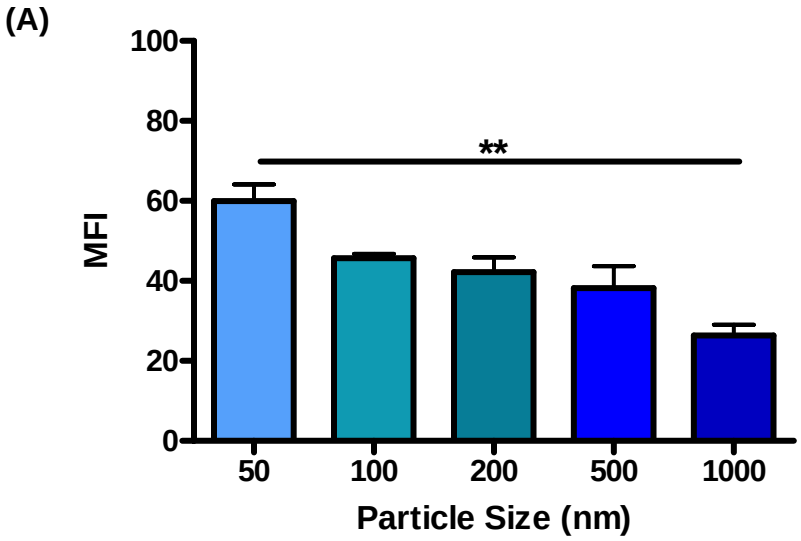


Figure 1. Nanoparticle uptake by immortalised AT1 cells. Cells were exposed to fluorescently labelled 50nm or 100nm latex nanoparticles with either neutral charge (A), negative charge (B) or positive charge (C). n=4 ***P<0.0007 **P<0.005

Neutral



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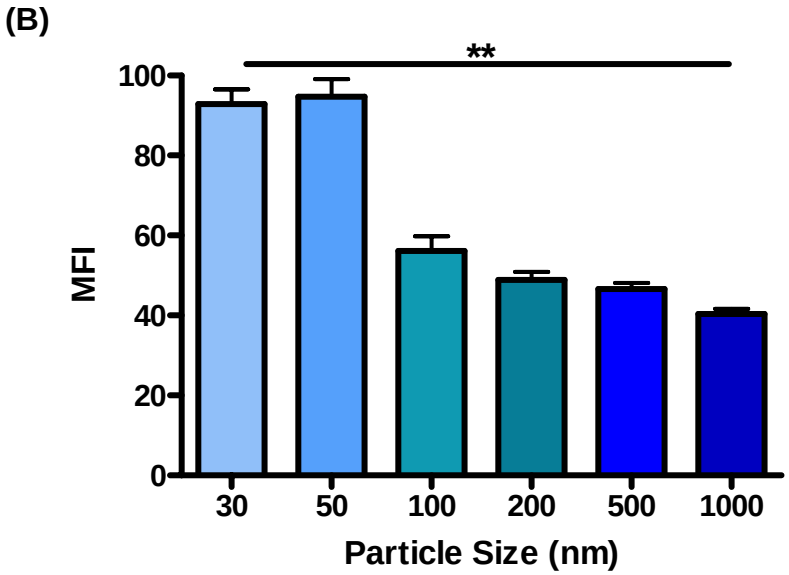

Figure 2. Effect of particle size on uptake by AT1 cells. Cells were exposed to neutral charge (A) and negatively charged (B) particles of increasing size for four hours and the degree of uptake measured using Simple PCI image analysis software. n=3 **P<0.009

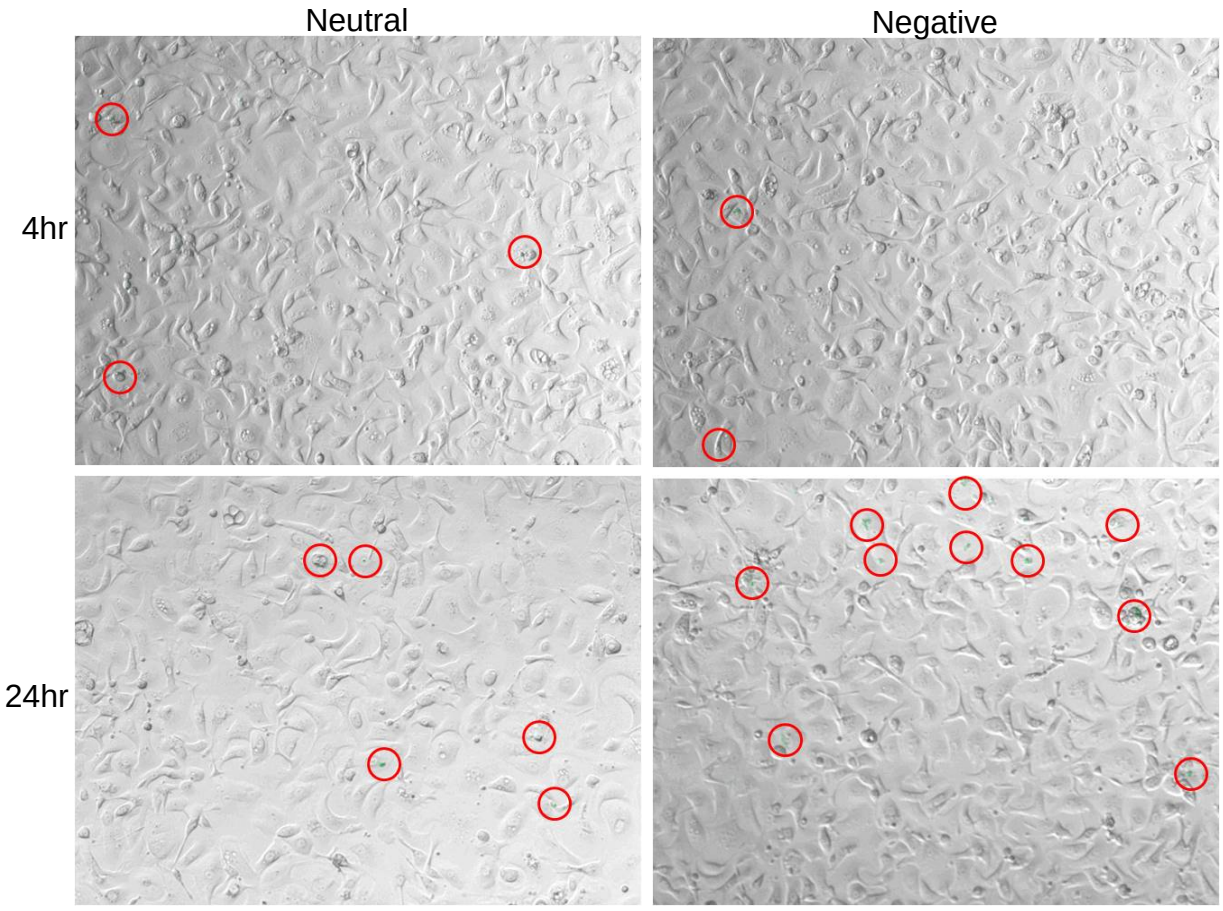

Figure 3. Uptake of nanoparticles by primary human AT2 cells. Primary human AT2 cells were exposed to 50nm neutral and negatively charged particles for 4 and 24 hours. Internalisation of particles could be seen in a limited number of cells (red circles) and did appear to increase with time but the amount internalised was below the limit of detection of image analysis software. Cells did not internalise positively charged particles, neither were the positively charged particles toxic.

Objective 2: To determine the fate/cellular location of internalized nanoparticles and whether particle uptake is active or passive. Months 2-5.

Methods

To investigate the cellular fate of nanoparticles, ATI cells were exposed to neutral, negative and positive charged beads of 50nm and 100nm diameter. Following exposure to the beads, cells were washed, fixed and stained with fluorescent antibodies raised towards proteins and structures important in cell trafficking. The proteins were: Clathrin-HC, caveolin-1, golgin, tubulin and actin. Using confocal microscopy the particles and fluorescently labelled proteins could be visualised to determine whether they co-localise. To assess the contribution of passive diffusion to the uptake of nanoparticles, AT1 cells were grown to confluency and serum starved for 24 hours prior to experiments. Cells were then incubated at 4°C for 2 hours in order to inhibit active processes. Cells were then exposed to 50nm nanoparticles for up to 24 hours and the degree of particle uptake measured as described above. To investigate the active mechanisms by which particles may be internalised, cells were treated with inhibitors of the endocytic pathway. Prior to addition of nanoparticles cells were pre-incubated with: Filipin (an inhibitor of caveolae), chlorpromazine (an inhibitor of clathrin-mediated endocytosis), nocodazole (a microtubule disruptor) or Cytochalasin (an actin disruptor) for 30 minutes. Following pre-treatment, cells were exposed to particles as described above for 24 hours and the level of particle uptake measured.

Results

Using confocal microscopy uptake of all particles was detectable after four hours.

Actin: Figures 4, 5. When cells were stained for actin, expression was detected throughout the cell but was most concentrated at the cell membrane. There was very little co-localisation of actin with 50nm negatively charged beads, whereas there was a small amount of co-localisation with neutral charge beads and more obviously with positively charged 50nm beads. Interestingly, when this experiment was repeated with 100nm beads, it all three types of bead co-localised with actin.

Clathrin: Figures 6, 7.Cells that were stained for clathrin demonstrated that particles of all charges and both sizes co-localised with clathrin.

Golgin: Figures 8, 9. When cells were stained for golgin all particles co-localised with Golgi although this was very low for the 50nm, negatively charged beads.

Tubulin and caveolin: Due to poor staining for the proteins tubulin and caveolin-1, we were unable to determine whether beads co-localised with microtubules and caveolae.

Passive uptake: Both neutral charge and negatively charged 50nm particles were internalised at 4°C (Figure 10). However, as with previous studies negatively charged particles were internalised significantly more than neutral charge particles (24hour; $P<0.0018$). The degree of uptake at 4°C however, was significantly less than that at 37°C for both type of particles (Figure 11, 24 hour; $P<0.018$), but nevertheless accounted for a high proportion (~50% neutral; ~70% negative) of the total at 37°C.

Pharmacological inhibition of endocytic pathways: Inhibition of key points in the endocytic pathway demonstrated varied results depending on the size and charge of the

particle (Figure 12). These data did not coincide with the confocal microscopy data obtained for clathrin and actin.

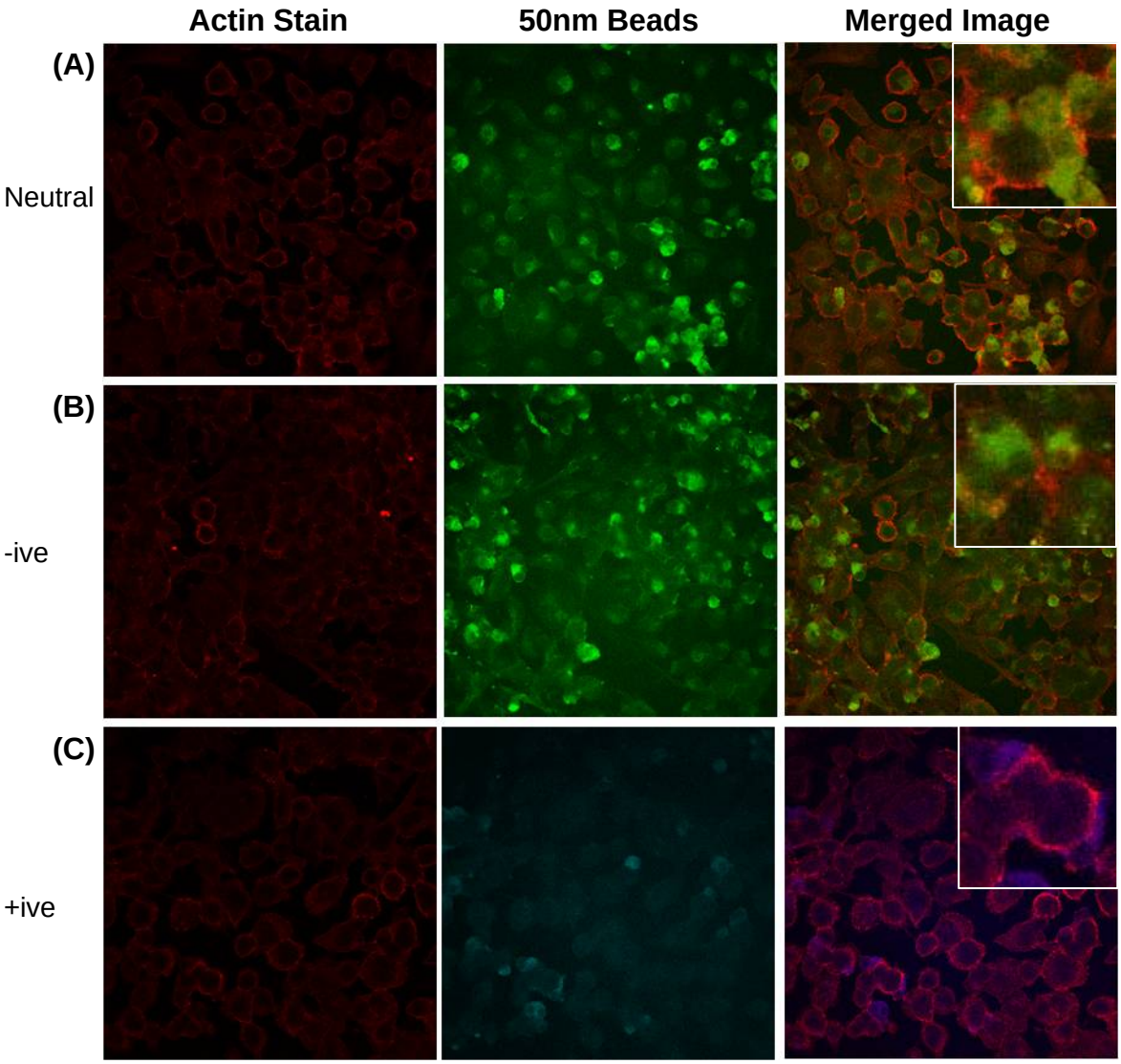

Figure 4. Confocal images of 50nm particle uptake and actin. ATI cells were exposed to neutral charge (A) negatively charged (B) and positively charged (C) 50nm fluorescent nanoparticles for 4 hours and subsequently stained with a fluorescently-labelled anti-actin antibody. Using confocal microscopy, particle uptake and subsequent association with actin could be visualised. Co-localisation can be seen as yellow in the merged (A) and (B) images and pink in the (C) image.

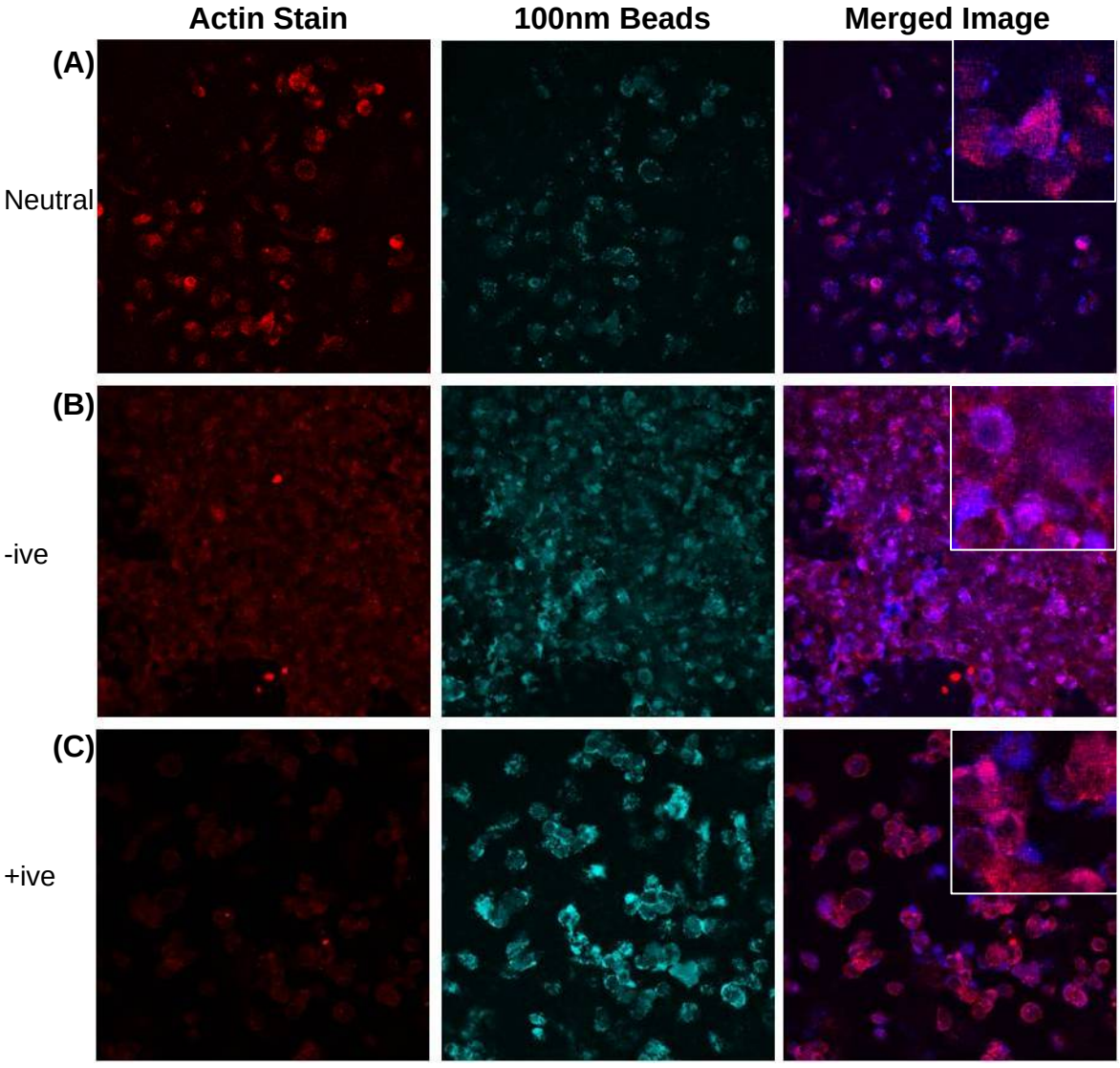

Figure 5. Confocal images of 100nm particle uptake and actin. ATI cells were exposed to neutral charge (A) negatively charged (B) and positively charged (C) 100nm fluorescent nanoparticles for 4 hours and subsequently stained with a fluorescently-labelled anti-actin antibody. Using confocal microscopy, particle uptake and subsequent association with actin could be visualised. Co-localisation can be seen as pink in the merged images.

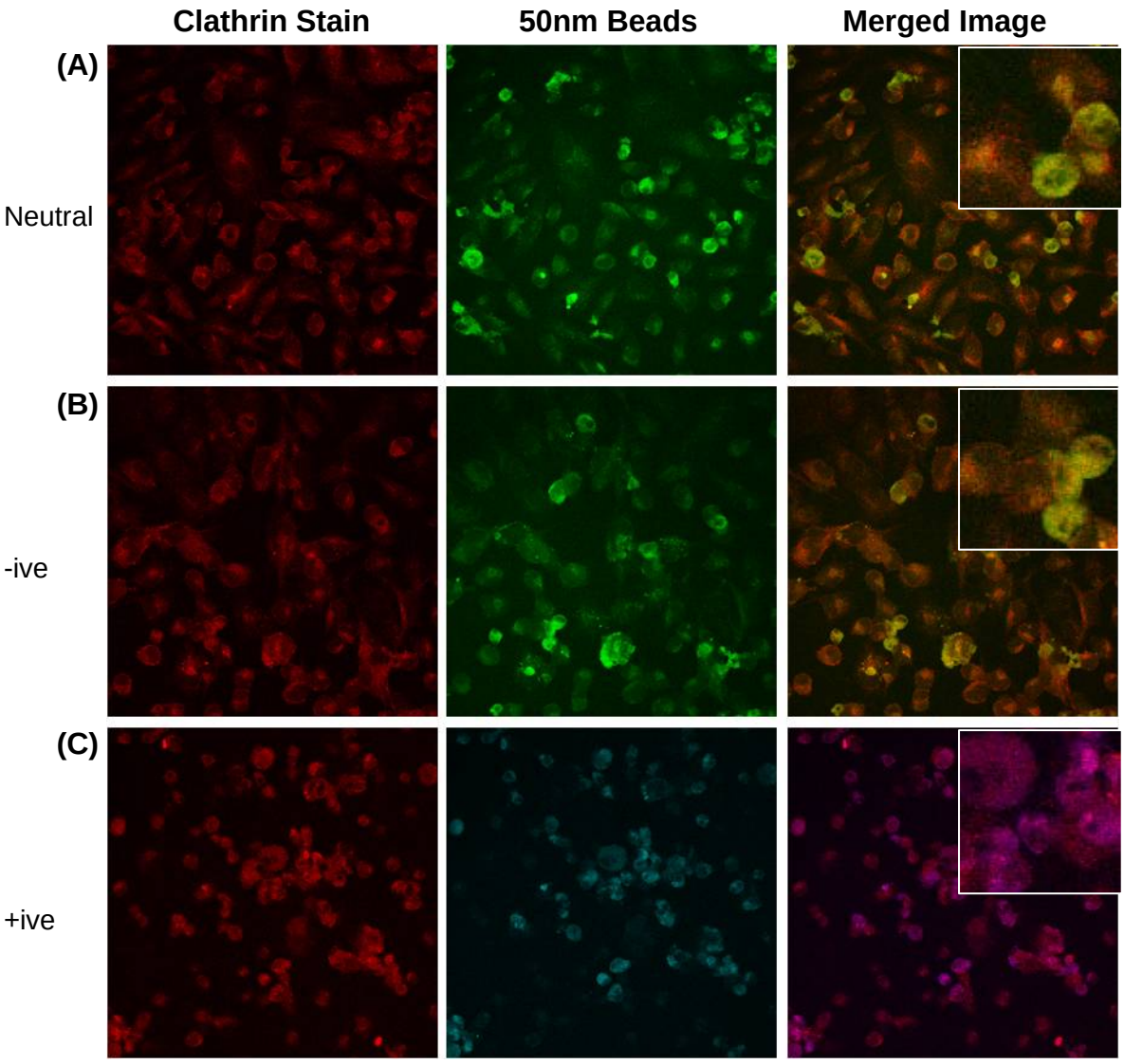

Figure 6. Confocal images of 50nm particle uptake and clathrin. ATI cells were exposed to neutral charge (A) negatively charged (B) and positively charged (C) 50nm fluorescent nanoparticles for 4 hours and subsequently stained with a fluorescently-labelled anti-clathrin antibody. Using confocal microscopy, particle uptake and subsequent association with clathrin could be visualised. Co-localisation can be seen as yellow in the merged (A) and (B) images and pink in the (C) image.

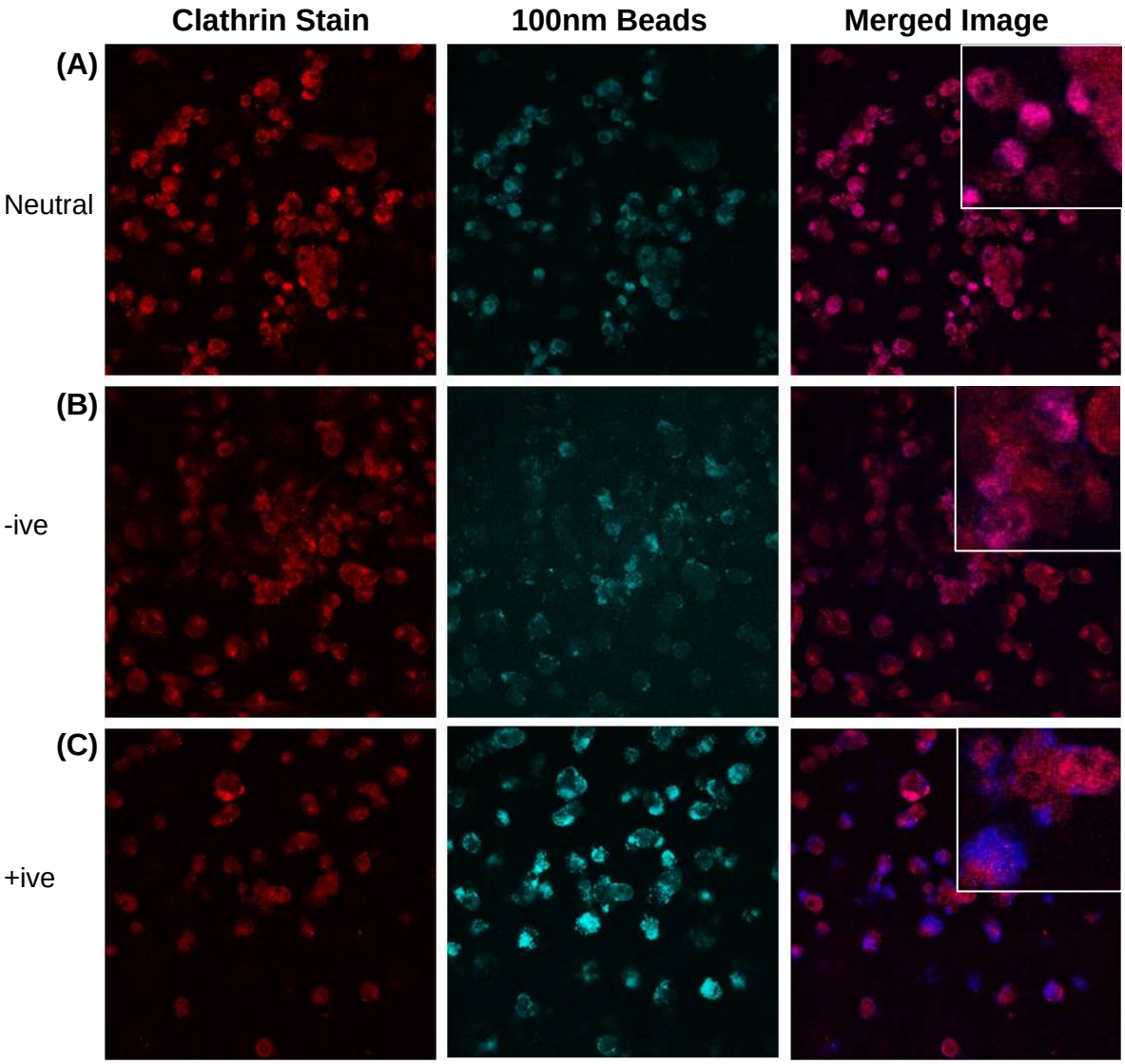

Figure 7. Confocal images of 100nm particle uptake and clathrin. ATI cells were exposed to neutral charge (A) negatively charged (B) and positively charged (C) 100nm fluorescent nanoparticles for 4 hours and subsequently stained with a fluorescently-labelled anti-clathrin antibody. Using confocal microscopy, particle uptake and subsequent association with clathrin could be visualised. Co-localisation can be seen as pink in the merged images.

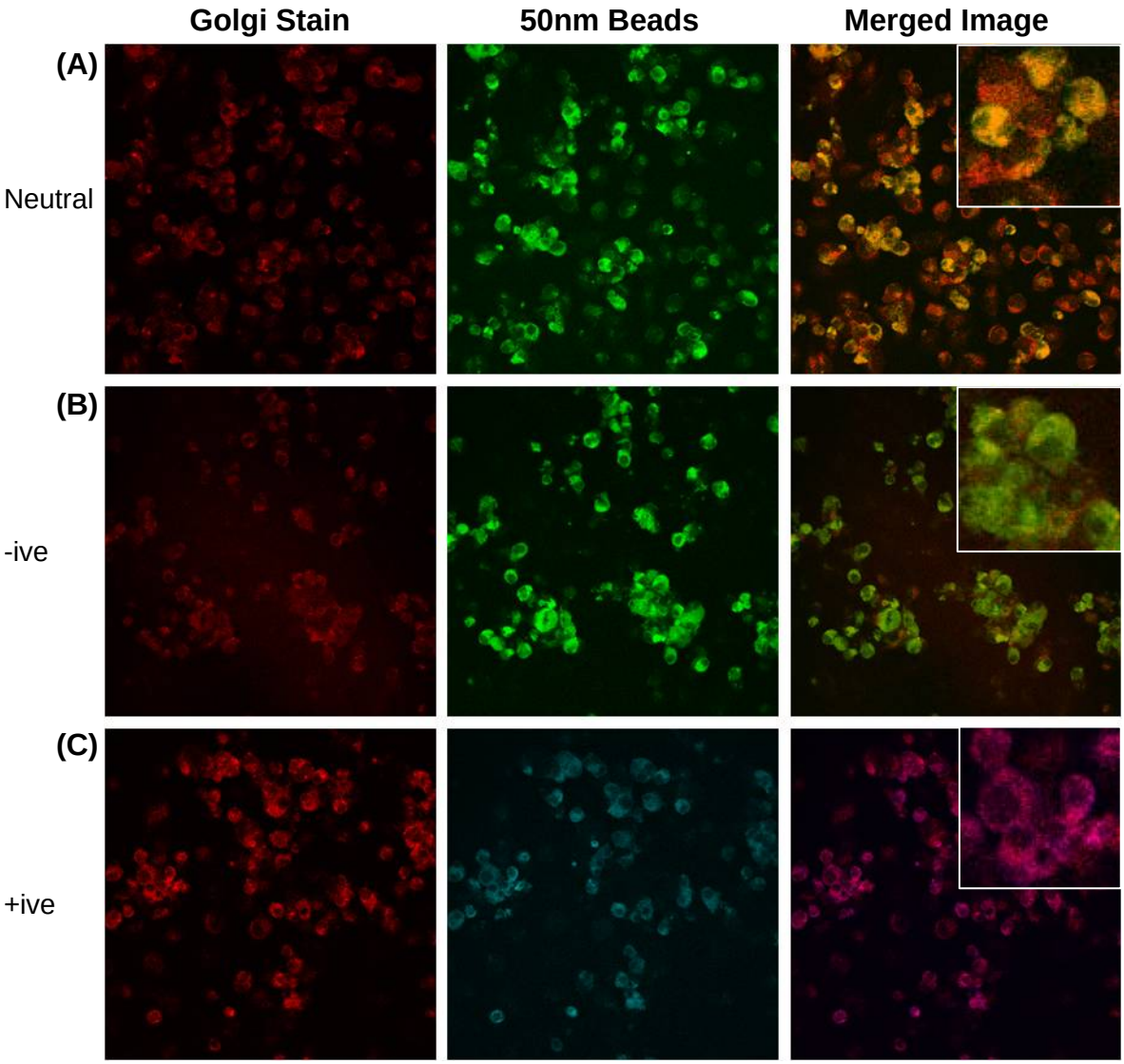

Figure 8. Confocal images of 50nm particle uptake and golgi. ATI cells were exposed to neutral charge (A) negatively charged (B) and positively charged (C) 50nm fluorescent nanoparticles for 4 hours and subsequently stained with a fluorescently-labelled anti-golgi antibody. Using confocal microscopy, particle uptake and subsequent association with golgi could be visualised. Co-localisation can be seen as yellow in the merged (A) and (B) images and pink in the (C) image.

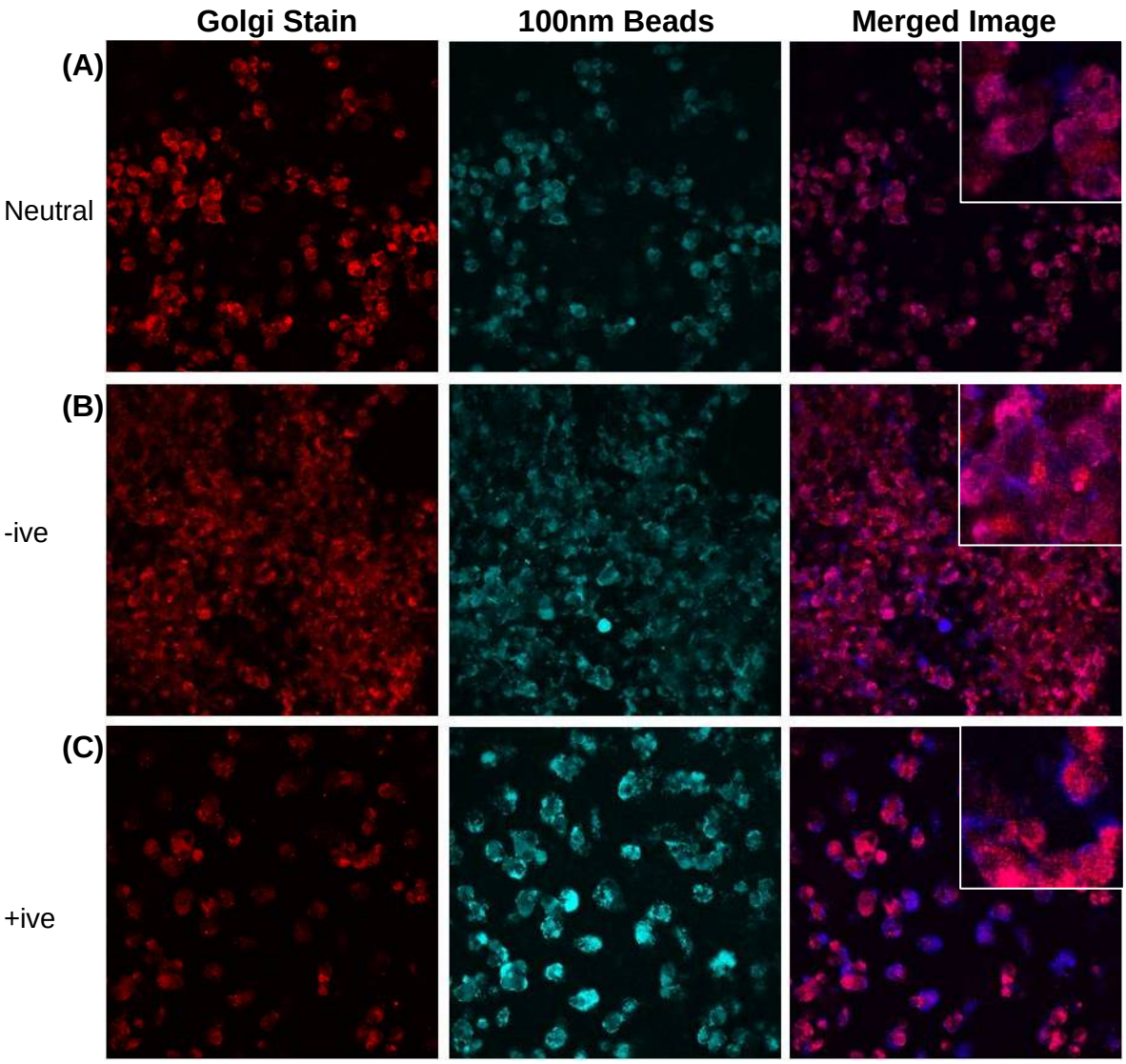

Figure 9. Confocal images of 100nm particle uptake and golgi. ATI cells were exposed to neutral charge (A) negatively charged (B) and positively charged (C) 100nm fluorescent nanoparticles for 4 hours and subsequently stained with a fluorescently-labelled anti-golgi antibody. Using confocal microscopy, particle uptake and subsequent association with golgi could be visualised. Co-localisation can be seen as pink in the merged images.

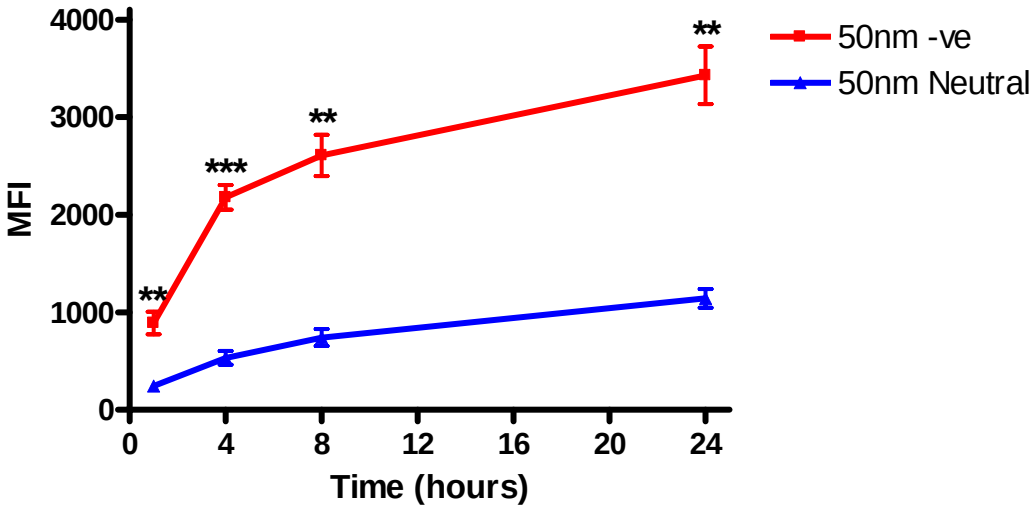

Figure 10. Uptake of nanoparticles by AT1 cells at 4°C. In order to assess the contribution of passive diffusion to nanoparticle uptake cells were chilled to 4°C to inhibit active processes prior to being exposed to 50nm neutral charge and negatively charged nanoparticles. Uptake was measured over a time course of 24 hours. n=3 ***P<0.0003 **P<0.006

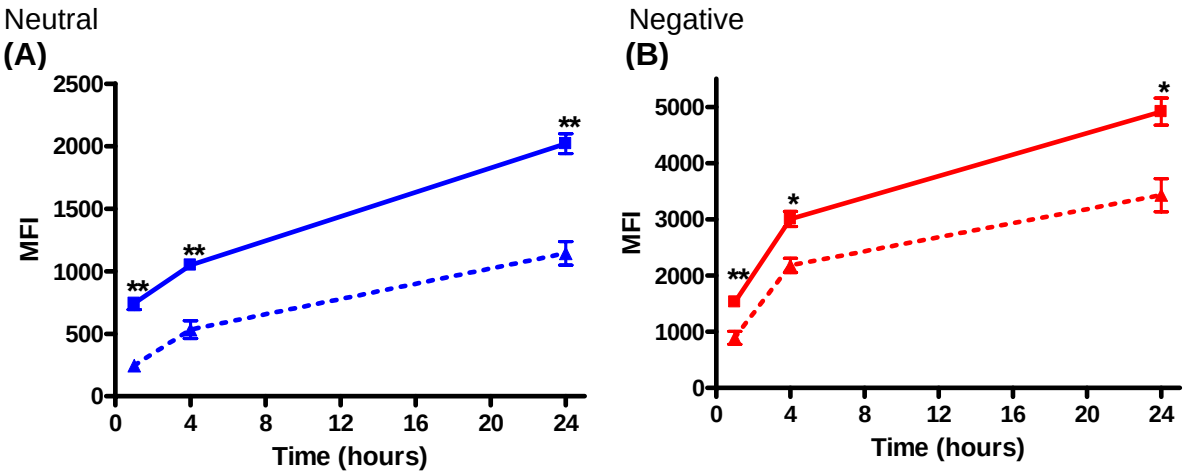

Figure 11. Comparison of nanoparticle uptake at 4 and 37°C. Cells were exposed to 50nm neutral charge (A) or negative charge (B) nanoparticles for up to 24 hours at 4 (dotted line) and 37°C (solid line) in order to assess the degree of particle uptake that may be due to passive diffusion. For both type of particle significantly more particles were internalised at 37°C compared to 4°C. n=3 **P<0.0055 *P<0.018

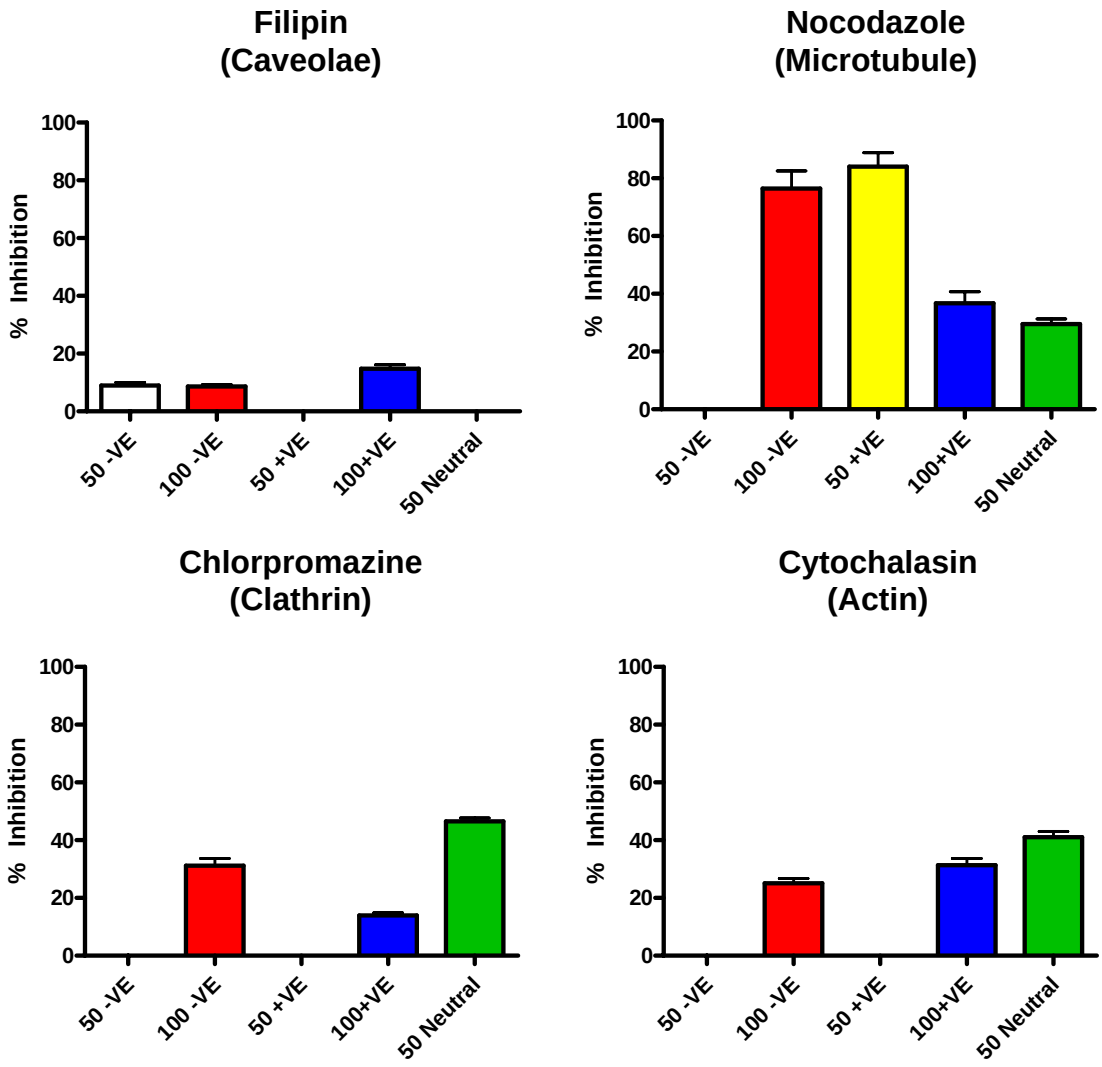

Figure 12. Effect of inhibitors of endocytosis on particle uptake. Cells were pre-treated with either Filipin, Nocodazole, Chlorpromazine or Cytochalasin for 30 minutes prior to addition of nanoparticles. Following pre-treatment cells were exposed to nanoparticles of various size and charge for 24 hours and the degree of uptake measured. n=3

Objective 3. To determine whether nanoparticles influence the functional integrity of the alveolar epithelial barrier. Months 4-6.

Methods

In order to investigate the effect of particle exposure on barrier function, AT1 cells were grown to confluency on porous transwell inserts and exposed to nanoparticles of varying size and charge as described above. To assess the degree of permeability fluorescently labelled dextran or albumin was placed in the upper chamber with the nanoparticles and the cells incubated for four hours. After four hours the inserts were removed and the fluorescence in the lower chamber measured.

Results

These studies yielded inconclusive data which may be due to a number of reasons discussed below.

Objective 4: To determine the influence of the lung lining liquid (lung surfactant) on these processes. Months 2-6.

Methods

Prior to addition to the cells, stock solutions of nanoparticles were mixed with bronchoalveolar lavage (BAL) fluid and incubated for 30 minutes. Following this, nanoparticles were diluted to a working concentration and added to the cells for four hours. Particle uptake was measured as before

Results

These studies showed that the addition of BAL fluid to neutral charge particles significantly increased uptake of particles below 200nm ($P<0.026$) but prevented uptake of particles above 500nm compared to control ($P<0.02$). When the study was repeated with negatively charged particles, the addition of BAL fluid increased uptake of all particles below 500nm compared to control ($P<0.0001$) and had no effect on 1µm negatively charged nanoparticles (Figure 13).

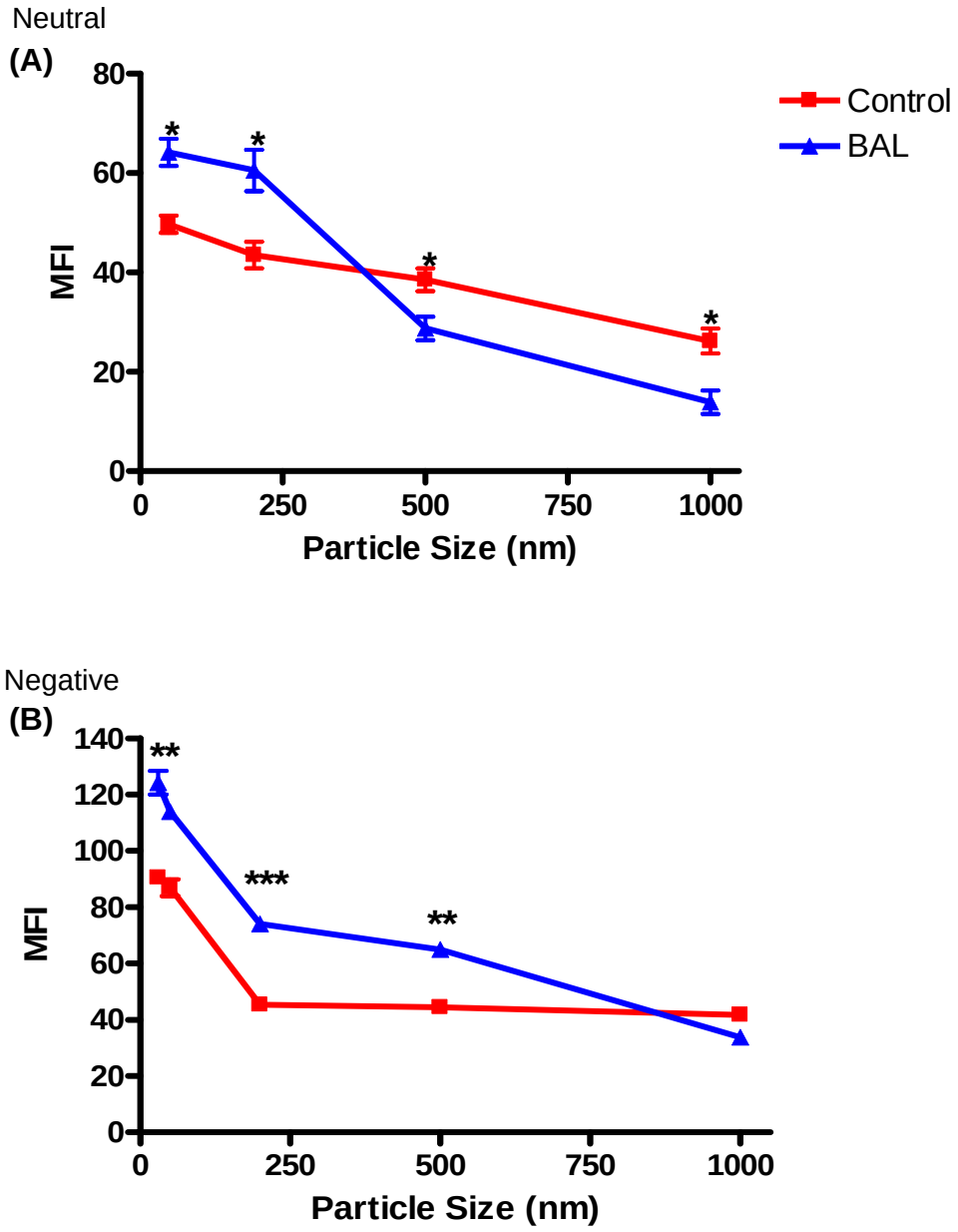

Figure 13. Effect of BAL fluid on AT1 cell nanoparticle uptake. Neutral charge (A) and negative charge (B) nanoparticles of increasing size were pre-incubated with BAL fluid for 30 minutes prior to addition to AT1 cells. Cells were incubated for 4 hours and the degree of particle uptake measured. n=3 ***P<0.001, **P<0.002

Preliminary Summary of Findings

Objective 1:

- Neutral charge and negatively charged 50nm beads were internalised more avidly by ATI cells than larger beads of the same charge. This was not the case for positively charged beads where 100nm beads were internalised to a greater extent than 50nm beads. However, our studies suggest that 50nm positively charged beads are cytotoxic (this is why some later studies do not include positively charged particles).
- Positively charged 100nm beads were taken up more avidly than 100nm negative and neutral charge beads. Interestingly, these positively charged beads were not toxic
- Negatively charged beads were internalised to a greater extent by ATI cells than neutral charge beads of the same size
- For negatively charged and charge neutral beads, as the size of the particle increases the degree of particle uptake decreases.
- ATII cells do not internalise nanoparticles to such a degree as that it may be measured. Only a very small number of internalised particles could be observed in less than 5% of cells

We hypothesise that 50nm particles, in particular those that are negatively charged, are internalised to a greater extent than larger particles as their size allows them to be internalised by a number of active and passive mechanisms that do not allow larger sized particles to enter. Our studies of 100nm beads, however, show that positively charged beads are internalised more readily than negative and neutral charge beads of the same size. These results along with results from studies using 50nm beads clearly show that both size and charge determine the degree of particle uptake.

Objective 2:

- All beads co-localised with clathrin.
- All beads except negatively charged 50nm particles co-localised with cytosolic actin but were not found to co-localise with the intense region of actin staining at the cell surface.
- All beads except negatively charged 50nm particles co-localised with Golgi.
- A significant proportion of internalised 50nm particles enter ATI cells by passive diffusion.
- Significantly more negatively charged beads enter by passive diffusion than charge neutral beads.
- Inhibitors of endocytosis had little effect on uptake of negatively charged and, to a lesser extent, positively charged beads.
- Uptake of larger sized beads and charge neutral beads of all sizes was significantly affected by inhibition of endocytosis.
- Microtubule disruption of caused the greatest inhibition of particle uptake.

	Filipin (Caveolae)	Chlorpromazine (Clathrin)	Nocodazole (Microtubule)	Cytochalasin (Actin)
50nm neutral	**-**	**++**	**+**	**++**
50nm –ve	**+**	**-**	**-**	**-**
100nm –ve	**+**	**++**	**+++**	**+**
50nm +ve	**-**	**-**	**+++**	**-**
100nm +ve	**+**	**+**	**++**	**+**

Table 1. Summary of the effect of inhibitors of various cell vesicle trafficking proteins on particle uptake.

Results of confocal microscopy to detect co-localisation of internalised particles with proteins associated with vesicular trafficking demonstrated that all particles co-localised with clathrin, indicating that clathrin-mediated endocytosis is an important mechanism in particle uptake. All nanoparticles except negatively charged 50nm beads co-localised with actin and golgin indicating that these two intracellular trafficking proteins are also important in directing particles through the cell cytosol for the cell membrane. The observation that negatively charged 50nm beads did not co-localise with actin and golgin may relate to our findings that a large proportion (70%) of these particles enter passively and not via active mechanisms that may direct the particles towards these proteins involved in vesicular trafficking. Furthermore, our data suggest that approximately half the 50nm neutral particles are taken up by active processes, the remainder by passive mechanisms.

Thus, these studies support the concept that particles below 50nm can easily enter the cell via non-metabolic processes such as passive diffusion due to their small size. Furthermore we believe that the addition of a negative charge allows these particles to more readily interact with the cell membrane, or cell secretions, or other extracellular components, to facilitate their uptake. Larger beads cannot pass through the membrane by passive mechanisms and thus are internalised by active processes such as endocytosis. Pharmacological inhibition of these pathways results in a significant decrease in particle uptake. Disruption of microtubules (not studied by confocal) and inhibition of clathrin-mediated (as seen with confocal, although not for the 50nm negatively charged beads) endocytosis appeared to have the greatest effect. These two mechanisms of endocytosis are closely linked; following clathrin-mediated endocytosis microtubules at the cell surface carry endosomes towards the nuclear region of the cells. These results suggest this pathway may account for a significant proportion of the active uptake of particles. The actin data coincide with the confocal findings, suggesting that it is important for 50nm neutral and 100nm particles, but not for negatively charged 50nm particles.

Objective 3:

- These studies were unsuccessful suggesting that a tight barrier was absent in AT1 cells.

A recent collaboration with Ben Forbes group has shown that these cells do not form a tight barrier. Discussion with Leland Dobbs, who works on primary mouse AT1 cells, suggests that these cells might be trying to form pores of Kohn in vitro, thus reducing tight barrier function, which is what he has also discovered. Furthermore the fluorescent label on the dextran and albumin deteriorated during the four hours at 37°C so that it could not be measured in the lower chamber.

Objective 4:

- Addition of lung lining fluid increased uptake of neutral charge particles below 200nm in size but inhibited uptake of particles greater than 500nm in diameter.
- Addition of lung lining fluid to negative charge particles increased uptake of all sizes of particles below 500nm and had no effect on larger particles.

We believe that the addition of lung lining fluid may coat/opsonise the particles with molecules that the cells recognise and therefore “mark” the particles for endocytosis. The addition of a negative charge to the particles may increase the ability of the particles to interact with, and bind to, factors such as proteins and phospholipids and therefore make the particles more susceptible to internalisation.

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