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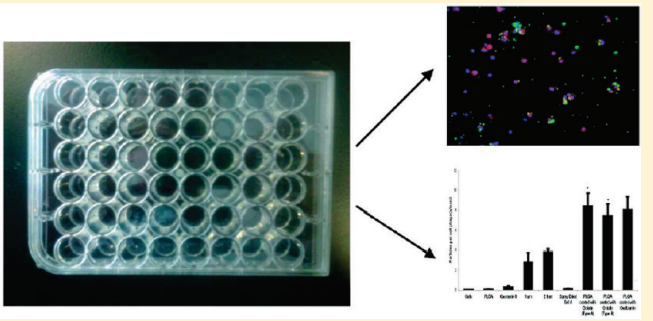
The Application of High-Content Analysis in the Study of Targeted Particulate Delivery Systems for Intracellular Drug Delivery to Alveolar Macrophages

Ciaran Lawlor,[†,‡] Mary P. O'Sullivan,[‡] Neera Sivadas,[†] Seonadh O'Leary,[‡] Paul J. Gallagher,[†] Joseph Keane,[‡] and Sally-Ann Cryan*[,†]

[†]School of Pharmacy, Royal College of Surgeons in Ireland, Dublin 2, Ireland

[‡]Institute of Molecular Medicine, Trinity College Health Science Building, St. James' Hospital, Dublin 8, Ireland

ABSTRACT: With an ever increasing number of particulate drug delivery systems being developed for the intracellular delivery of therapeutics a robust high-throughput method for studying particle−cell interactions is urgently required. Current methods used for analyzing particle−cell interaction include spectrofluorimetry, flow cytometry, and fluorescence/confocal microscopy, but these methods are not high throughput and provide only limited data on the specific number of particles delivered intracellularly to the target cell. The work herein presents an automated high-throughput method to analyze microparticulate drug delivery system (DDS) uptake byalveolar macrophages. Poly(lactic-*co*-glycolic acid) (PLGA) microparticles were prepared in a range of sizes using a solvent evaporation method. A human monocyte cell line (THP-1) was differentiated into macrophage like cells using phorbol 12-myristate 13-acetate (PMA), and cells were treated with microparticles for 1 h and studied using confocal laser scanning microscopy (CLSM), spectrofluorimetry and a high-content analysis (HCA). PLGA microparticles within the size range of 0.8−2.1 μm were found to be optimal for macrophage targeting ($p < 0.05$). Uptake studies carried out at 37 °C and 4 °C indicated that microparticles were internalized in an energy dependent manner. To improve particle uptake, a range of opsonic coatings were assessed. Coating PLGA particles with gelatin and ovalbumin was found to significantly increase particle uptake from 2.75 ± 0.98 particles per cell for particles coated with gelatin. Opsonic coating also significantly increased particle internalization into primary human alveolar macrophages ($p < 0.01$) with a 1.7-fold increase in uptake from 4.19 ± 0.48 for uncoated to 7.53 ± 0.88 particles per cell for coated particles. In comparison to techniques such as spectrofluorimetry and CLSM, HCA provides both qualitative and quantitative data on the influence of carrier design on cell targeting that can be gathered in a high-throughput format and therefore has great potential in the screening of intracellularly targeted DDS.



KEYWORDS: macrophage targeting, microparticle, high-content analysis (HCA), intracellular drug delivery

INTRODUCTION

Intracellular drug delivery is an increasingly important facet of pharmaceutics. In particular the delivery of nucleic acid based therapeutics, e.g. plasmids and siRNA, requires drug delivery systems (DDS) that protect the fragile therapeutic molecule during formulation and delivery and can facilitate a high cellular concentration of the therapeutic.[1,2] With an increasing number of biomaterials and particulate delivery systems being designed for intracellular drug delivery, better tools are required to screen DDS−cell interactions.

Traditionally *in vitro*/*ex vivo* methods used to quantify cellular uptake of DDS have employed techniques such as direct optical microscopy, confocal microscopy and flow cytometry.[3,4] Hasegawa et al. studied the effect of size on polystyrene latex beads and poly(lactic-*co*-glycolic acid) (PLGA) particle uptake into a rat alveolar macrophage cell line (NR8383) by means of direct visual microscopy to count individual particles within cells.[5] This method is labor intensive; where a polydisperse population of particles exists, size exclusion is not possible, and in granular cells the process of quantifying particle uptake visually is further complicated.

Spectrofluorimetry and flow cytometry are also used to quantify cellular uptake of DDS.[6−9] In spectrofluorimetry, cells are treated with fluorescently labeled particles for a set incubation period and lysed and fluorescence levels determined using a spectrofluorimeter in a single cuvette or multiwell format. Cell uptake is determined as increases in cell-associated fluorescence above background, i.e. untreated cells normalized for total protein content. However, this technique does not differentiate

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between particles internalized by the cells versus those bound to the cell surface, and there is no supporting qualitative data to confirm intracellular particle uptake. Flow cytometry or fluorescent-activated cell sorting (FACS) can provide quantitative information about fluorescently labeled particle uptake based on fluorescent intensity.[10,11] The cells must be in suspension for analysis, however, and this has implications when dealing with adherent cell lines particularly with macrophages where the process of removing the cells may damage them and bias the results. Spectrofluorimetry and flow cytometry are useful for generating quantitative data on DDS uptake but the data needs to be supplemented with microscopy to confirm intracellular localization.[8] For example, Coester et al. employed flow cytometry to quantify uptake of cross-linked gelatin nanoparticles in combination with confocal microscopy.[12]

Fluorescent and confocal laser scanning microscopy (CLSM) are widely used techniques for imaging particle−cell interactions using fluorescently loaded particles. CLSM is a particularly useful technique for providing spatial information on particle location within a cell.[13,14] Both techniques are however primarily qualitative. Previous studies using these techniques have employed corroborative quantitative data from flow cytometry or spectrofluorimetry.[12,13]

The use of HCA to better understand particle-cell interactions may provide a better understanding of these interactions through qualitative and quantitative analysis within a given sample with the capability of defining parameters for particle analysis. Herein we describe this new technology for quantifying and imaging cellular uptake of DDS based on high-content subcellular analysis (HCA) imaging analysis, using InCell 1000 (GE Healthcare). This technique is based on high resolution imaging of fluorescently labeled cells, capable of providing analysis at a single cell level, which is important for investigating heterogeneous responses to treatment.[15] Infrared autofocus ensures all samples are within focus and experiments can be carried out in live or fixed formats where images are captured by charged-coupled device (CCD) cameras.[16] This technology combines high image resolution with automated acquisition protocols and can be fully automated by the use of liquid handling systems to provide high-throughput cell analysis.[17] The HCA approach can be employed at low magnification to analyze whole cell populations and by switching to high magnification subcellular analysis can be carried out in the same sample set.[16] HCA therefore quantifies fluorescent signals and images individual cells and can simultaneously analyze multiple parameters in a single sample, including DDS of specific sizes e.g. nanoparticles or microparticles. Special data analysis software converts complex cell parameters into a simple numerical output.

HCA technology has traditionally been used in cell biology assays, such as siRNA screens, neuron regeneration assays, stem cell research[18−20] and toxicity[21,22] screening, but remains a relatively untapped resource for drug delivery research. A recent review highlighted the need for improved systems for understanding particle−cell interaction that was capable of more robust size exclusion quantification.[23]

This study aimed to optimize the bioengineering of inhalable particulates designed specifically for targeted and efficient drug delivery to alveolar macrophages in the lungs for application in the treatment of a range of respiratory diseases and infections. Microparticles have been shown to facilitate drug delivery via phagocytosis to macrophages with size (low micrometer range) and surface properties having a significant effect on particle uptake and drug delivery efficiency.[24−26] As these particles were designed to be delivered as an inhalable dry powder, particles in the low micrometer range (<5 μm) are required to achieve an appropriate mass median aerodynamic diameter (MMAD) and avoid the potential toxicological risk associated with nanoparticles.[27,28] Comprehensive comparison of design factor influence on particle uptake has been hampered by the limitations of the more traditional techniques described above. Therefore the aim of this study was to apply HCA technology to study the effects of particle size and surface coatings on particle uptake and compare the data from more traditional techniques. Surface modification of microparticles has been shown to enhance their uptake by macrophages, therefore we designed microparticle delivery systems using opsonic coatings designed to alter zeta-potential and increase particle−cell interaction. The opsonic coatings used on the surface of the PLGA were gelatin (type A), gelatin (type B) and ovalbumin. These coatings have shown potential for increasing particle uptake by macrophages.[24] Gelatin has been suggested to increase particle uptake by macrophages through interactions with the fibronectin receptor on the surface of the macrophage.[29] However, no study has been conducted to quantify the difference in uptake after coating compared to uncoated microparticles or furthermore to identify if different types of gelatin have different uptake profiles. Additionally, a nonspecific glycoprotein (ovalbumin) was included in the study to evaluate if simply altering zeta-potential could enhance cellular uptake. We sought to test the effect of size and coating on particle uptake using traditional and HCA technologies and determine the relative capabilities of the techniques to provide meaningful data on the impact of particle design on biological interactions.

EXPERIMENTAL SECTION

Materials. PLGA 503 was purchased from Boehringer Ingelheim; dichloromethane was obtained from Fluka Chemicals; poly(vinyl alcohol), coumarin-6, BSA-FITC, type A gelatin, type B gelatin, phorbol 12-myristate 13-acetate, phalloidin-TRITC, and Hoechst 33342 were obtained from Sigma Aldrich; Mastersizer 2000, Zeta-Sizer Nano (Malvern); Ultra-Turrax T25 homogenizer (Ultra-Turrax); Micro-BCA Assay (Pierce); THP-1 (LGC Promochem); 12-well plates, 48-well plates (NUNC); confocal laser scanning microscope (Carl-Zeiss); INCell 1000 (GE Systems); Tescan Mira XMU; Buchi 190.

PLGA Microparticle Preparation. Microparticles were prepared using a double emulsion, solvent evaporation method.[29] Briefly, PLGA was dissolved in dichloromethane (2.5% w/v) and the solution was remotely probe sonicated at an output level of 4 for 8 s. A primary emulsion (w_1/o) was created by the addition of 2 mL of 2.5% poly(vinyl alcohol) (PVA). This emulsion was remotely probe sonicated at an output level of 4 for 16 s. A final secondary emulsion ($w_1/o/w_2$) was formed by transferring the primary emulsion into a continuous phase consisting of 1% PVA. The homogenization parameters of the emulsion were adapted (Table 2) to produce PLGA particles of different sizes including 0.8 μm, 2.2 μm, 4 μm, 6.8 μm, 8.4 μm, 14.3 μm and 23.5 μm. A spindle head with a stator diameter of 25 mm and a rotor diameter of 18 mm on the homogenizer was used to produce the microparticles. Modifications to the volume of 1% PVA in the continuous phase, the speed of homogenization and the duration of homogenization allowed for the formulation of microparticles varying in size. Following homogenization, the emulsion was mechanically stirred in the fume hood overnight to allow the

solvent to evaporate and allow microparticle formation. Microparticles were then centrifuged at 7000*g* for 7 min and washed in distilled water three times to remove residual PVA, prior to lyophilization. Where applicable, coumarin-6 dye was added to the organic phase at a ratio of 1:100 (w/w) dye:polymer. Where applicable, bovine serum albumin-fluorescein isothiocyanate (BSA-FITC) was added into the first aqueous phase (10% w/w).

Gelatin Microparticle Preparation. In order to formulate pure gelatin microparticles in the low micrometer range a spray-drying method was used. Gelatin (type A) 1% w/v in distilled water was spray-dried through a 0.5 mm nozzle using a Buchi 190 spray-dryer under the following parameters: inlet temperature 100 °C, outlet temperature 40−41 °C, pump flow 15% and aspirator 75%[30] to produce pure gelatin microparticles.

Particle Characterization. PLGA microparticles were suspended in water and sized using laser diffraction (Mastersizer 2000), and the zeta-potential of the microparticles was determined using a Zetasizer (Malvern). The morphology of the microparticles was determined using scanning electron microscopy (SEM). Briefly, lyophilized microparticles were stud mounted and sputter coated in gold to increase conductivity and visualized using SEM (Tescan MiraXMU).

Microparticle Coating. PLGA microparticles of 2.1 ± 0.3 μm in size that were produced by the solvent evaporation method were coated with gelatin type A, type B or ovalbumin using different stirring methods. Stirring involves dissolving the protein in a solution that will not dissolve the polymer, i.e. dH_2O. The PLGA is gently stirred in the presence of the protein to allow interactions occur at a ratio 3:1 of gelatin:PLGA for 4 h at room temperature with gentle mechanical stirring.[31] Coating was carried out below the isoelectric point of each protein to ensure optimal coating with this method. To evaluate the efficiency of gelatin coating a micro-BCA protein assay was used. This assay is capable of low level detection of protein from approximately 0.5 μg/mL and above. For this assay, a known quantity of PLGA was suspended in dH_2O and placed in a water bath at 37 °C for 4 h to dissolve the gelatin from the PLGA. This solution is centrifuged at 7000 xg for 7 min with 1 mL of the supernatant removed and mixed with 1 mL of the micro-BCA working reagent (WR) and incubated at 37 °C for 1 h in a water bath to allow the reaction to occur. Following incubation the samples were read at 562 nm immediately. All samples were read within 5 min of each other. This is important as the micro-BCA will continue to react even after removal from the water bath.

Cell Culture. A human monocyte-like cell line (THP-1) was cultured in T25, vented flasks. Culture medium was free from antibiotics and consisted of RPMI-1640 with 10% fetal bovine serum. Cells were seeded at a density of 2.5×10^5 cells/mL and allowed to grow to a confluence of 1×10^6 cells/mL before splitting or plating the cells. Cells grew in suspension and were centrifuged at 200*g* for 10 min when changing medium or seeding cells. To differentiate cells into adherent macrophage-like cells 100 nM PMA was used for 72 h at 37 °C/5% CO_2.[32]

Primary alveolar macrophages were obtained by bronchoalveolar lavage (BAL) of 3 nonsmoker donors with the approval of the St. James' hospital ethics committee in accordance with the Helsinki Declaration. The BAL fluid was passed through a 100 μm nylon cell strainer and centrifuged at 200*g* for 15 min. Cells were resuspended in RPMI-1640 with 10% human serum containing 0.1% cefotaxime and 0.2% fungizone and plated at an optimal density of 1×10^6 cells/mL. Cells were incubated overnight at 37 °C/5% CO_2 and washed to remove nonadherent cells prior to carrying out the uptake study. Ethical approval for the study was obtained from the St. James' hospital ethics committee.

Confocal Laser Scanning Microscopy (CLSM). For qualitative microparticle uptake studies, 1 mL of cells were plated on acid-washed cover-slips in 24-well NUNC plates at a density of 1×10^5 cells/mL per well. The cells were differentiated using 100 nM PMA for 72 h in an incubator at 37 °C/5% CO_2. Following differentiation fresh medium was added to the wells followed by treatment with 150 μg of microparticles. The plate was then gently shaken to ensure distribution of the microparticles in the wells. The plate was placed in the incubator for 1 h at 37 °C/5% CO_2. After one hour, the medium was removed and the wells were washed in PBS twice to remove unbound microparticles. Cells were fixed with 200 μL of 2% paraformaldehyde for 20 min at room temperature and washed twice in PBS. Cells were then stained using 200 μL of phalloidin-TRITC to stain F-actin for 45 min protected from light; in the final 5 min 100 μL of Hoechst 33342 was added to stain the nucleus and a drop of DAKO antifade solution.[32] Cover-slides were analyzed using a Carl-Zeiss LSM 510 Meta instrument.

Spectrofluorimetry. THP-1 cells were differentiated using 100 nM PMA and seeded at a density of 1×10^5 cells/mL in a 48-well plate (NUNC). Microparticles loaded with fluorescein isothiocyanate (FITC) were suspended in medium and added to the wells to incubate for 1 h at 37 °C/5% CO_2. Following incubation the cells were washed three times in phosphate buffered saline (PBS) and incubated with 0.1% Triton-X for 30 min.[33] The fluorescence associated with each well was determined on a spectrofluorimeter at excitation/emission wavelengths of (490 nm/520 nm) and expressed as fluorescence per mg of protein determined using a BCA assay.

High-Content Analysis (HCA). To quantify the number of particles per cell a high-content screening system was developed with an INCell 1000 analyzer (GE). Various sized microparticles were evaluated in 48-well plates with 0.5 mL of PMA treated THP-1 cells at a density of 1×10^5 cells/mL per well in triplicate, and primary alveolar macrophages were seeded at a density of 1×10^6 cells/mL. Cells were differentiated with 100 mM PMA for 72 h, followed by replacement with fresh medium, and treated with 75 μg of microparticles. Incubations were carried out for 1 h at 4C and at 37 °C. Cells were then fixed in 4% paraformaldehyde and stained for F-actin with 200 μL of phalloidin-TRITC (5 μg/mL) and for nucleus using 100 μL of Hoechst (10 μg/mL).

Dual object analysis was carried out on the INCell 1000 to quantify the rate of cell uptake. Cells were identified as objects with both a defined radius of cell shape and also a minimum surface area of the nucleus. Positive objects for both these characteristics were then scanned for microparticles of a defined size range, as determined by laser diffraction. Images were analyzed using the dual area object analysis algorithm (GE) to quantify the extent of microparticle uptake (Table 1). The number of particles per cell is quantified by the software first identifying cells; it does so by using two organelle markers, the nucleus and the cytoplasm. In this study the nucleus was fluorescently labeled with Hoechst and the cytoplasm with phalloidin-TRITC, these dyes have specific excitation/emission wavelengths (Table 1). A threshold for area intensity for the respective dyes is employed in the identification of defined cells. Once identified, the software can use a size exclusion algorithm to

Table 1. Dual Area Object Analysis Parameters[a]

param	excitation/emission (nm)	segmentation	min area (μm^2)	exposure time (ms)	HWAF offset (μm)
nuclei	360/460	top-hat	100	200	24
cells	535/620	region growing	130	400	11.1
microparticles	475/535	multiscale top-hat	based on range of size obtained by laser diffraction	250	11.5
nonfluorescent	bright-field	n/a	n/a	10	3.8

[a] The hardware autofocus varied slightly with each experiment and the data included in the table is a representative of the values obtained on a given day.

Table 2. Manufacturing Parameters for Production of PLGA Microparticles of Different Sizes

manufacturing conditions			microparticles	
homogenizer speed (rpm)	vol of 1% PVA (mL)	duration of homogenization (min)	size (μm) ± SD	span
17,500	20	2	0.8 ± 0.1	2.05
17,500	20	1	2.1 ± 0.3	1.23
9,500	20	2	4 ± 0.27	1.72
6,000	20	3	6.75 ± 0.25	1.84
13,500	50	2.5	8.5 ± 0.34	1.76
13,500	50	1.5	14.29 ± 1.61	1.21
6,500	40	0.5	23.95 ± 1.8	0.79

count the number of fluorescently-labeled particles within the cell; the software can be programmed to count only those fluorescent particles within a defined size range. The software will only count particles that are within the defined region, i.e. within the cell, colocalized with the intracellular cytoplasmic phalloidin stain. In addition, to differentiate between cell membrane bound and intracellular particles, treatments were carried out at 4 °C to inhibit active internalization of particles and compared to experiments carried out at 37 °C. The protocol can be modified to utilize different dyes and combinations of dyes, but does require that the dyes used have different excitation/emission wavelengths at 360 nm/460 nm (nucleus), 535 nm/620 nm (cytoplasm), 475 nm/535 nm (fluorescently labeled particles) to avoid artifactual interference.

Statistical Analysis. Statistical analysis was carried out using Student's *t* test. A $p < 0.05$ was taken to be statistically significant.

RESULTS

Particle Characterization. Microparticle size is summarized in Table 2 showing the range of particle sizes produced by varying the solvent evaporation manufacturing method. By adjusting the homogenizer speed, homogenization time and the volume of PVA used, different sized particles could be manufactured. The size of the pure gelatin microparticles produced by spray-drying was 2.48 ± 0.41 μm as determined by laser diffraction. Opsonic coating of the microparticles resulted in a shift in zeta-potential from −33.4 ± 2.62 mV for the uncoated microparticles to +15.9 ± 1.75 mV, +2.12 ± 4.65 mV and +22 ± 3.23 mV for microparticles coated with gelatin (type A), gelatin (type B) and ovalbumin respectively. The coating of PLGA microparticles resulted in a shift from a negative surface charge to a positive charge due to the free amino groups of the proteins coating the surface.[34,35] The size of microparticles changed from 2.1 ± 0.3 μm to 2.5 ± 0.5 μm, and 2.43 ± 0.29 μm and 2.39 ± 0.25 μm for microparticles coated with gelatin (type A), gelatin (type B) and ovalbumin respectively. The effect of coating on surface morphology and the polydispersity of the microparticles associated with this method of particle production can be clearly seen in Figure 1, where the uncoated microparticles, Figure 1A (0.8 μm) and Figure 1B (23 μm), have a smoother surface compared to gelatin (type A) coated (Figure 1C), gelatin (type B) coated (Figure 1D) and ovalbumin coated (Figure 1E), where an opsonin has coated the surface, thereby altering its smooth morphology. Figure 1F shows spray-dried gelatin microparticle with a corrugated surface morphology.

Assessment of the Effect of Microparticle Size on Particle Uptake by Macrophages. *The Effect of Size on Microparticle Uptake Using Spectrofluorimetry and CLSM.* In the first instance, differentiated THP-1 cells were treated with the microparticles of different sizes for 1 h and the effect on uptake was studied using spectrofluorimetry and CLSM. The results of the spectrofluorimetry study shown in Figure 2A suggest that particles in the 0.8−2.1 μm size range are optimal for intracellular delivery to macrophages providing efficient intracellular delivery after only 1 h incubation. However, due to the wide variability of the spectrofluorimetry data it was difficult to determine statistical significance without carrying out a number of time-consuming replicate experiments. Due to the lack of qualitative imaging with spectrofluorimetry, cell membrane bound particles may also be counted. Figure 2C shows a confocal image of THP-1 cells treated for 1 h with fluorescently labeled 2.1 μm microparticles and compared with unlabeled (nonfluorescent) microparticle treated cells (Figure 2B). *Z*-Stack analysis, where images at a series of cell depths are collected, was carried out to verify internalization of the microparticles. Despite the high magnification employed it is difficult to distinguish individual particles, making it more difficult and time-consuming to count particles per cell and ultimately to quantify intracellular drug dosing for therapeutic applications.

The Effect of Size on Microparticle Uptake Using HCA. As described previously, to adapt HCA for drug delivery system screening, the algorithm for detecting particles per cell was based on the size distribution data obtained from the Mastersizer 2000 (Table 2) and a minimum of 8 fields per well was analyzed using a 10× objective lens. This corresponds to roughly 1,000 cells (dependent on cell number in a given field) analyzed between the three wells on a given plate showing HCA's capability as a high-throughput technology. Increasing the number of fields per well would increase the number of cells analyzed. The experimental design was such that cells were treated $n = 3$ at three different passages, starting at passage 6, and controls for passage to passage variation for cell uptake behavior.

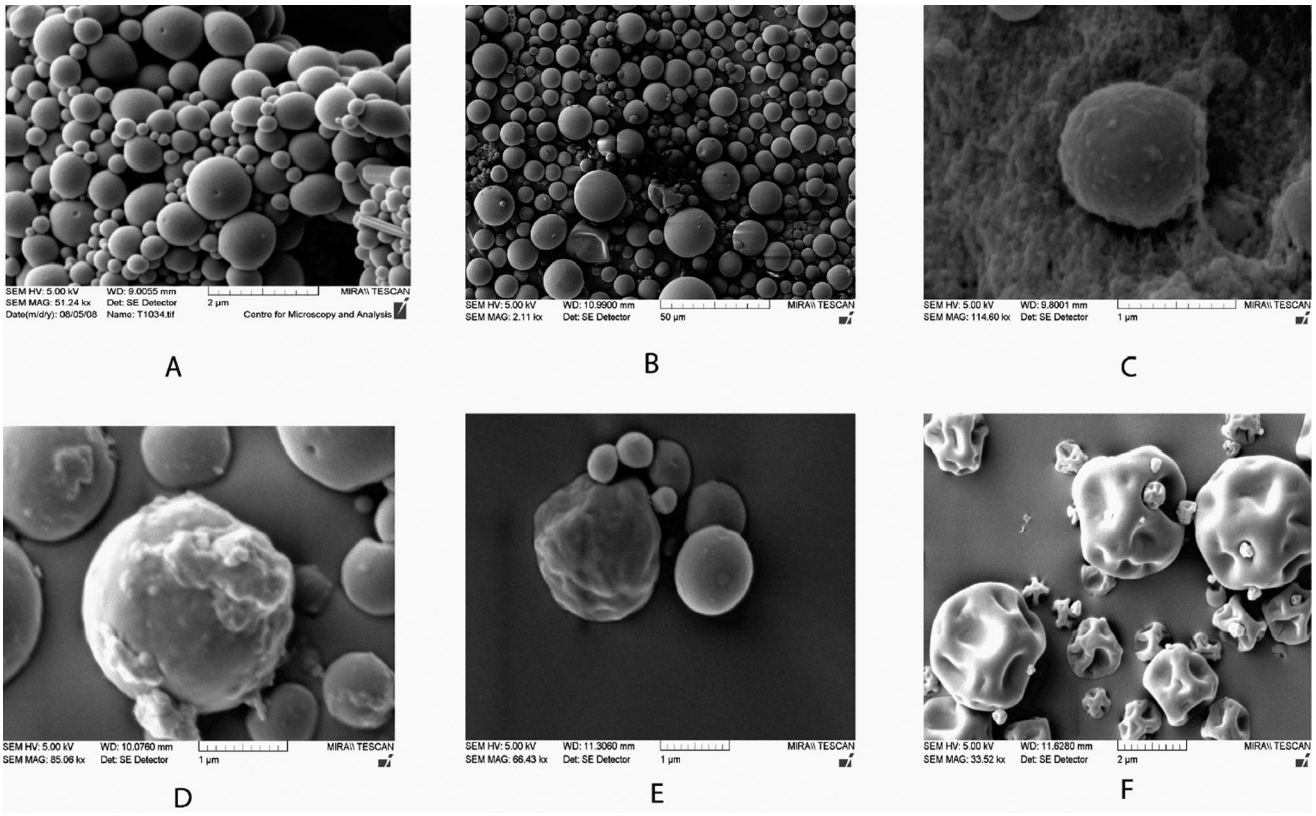


Figure 1. Scanning electron micrographs (SEM) for PLGA microparticles. Different magnifications were used for each size: (A) 0.8 μm sized particles at 51.24k× magnification, (B) 23 μm particles at 2.11k×, (C) 1 μm sized PLGA microparticles coated in gelatin (type A) at 85.06k× magnification, (D) 1 μm sized PLGA microparticles coated in gelatin (type A) at 114.6k× magnification, (E) 1 μm sized PLGA microparticles coated in gelatin (ovalbumin) at 66.43k× magnification, (F) 2 μm sized spray-dried gelatin (type A) microparticles at 33.52k× magnification.

Figure 3B shows the segmentation of cells based on the two parameters of cell cytoplasm (red) and nucleus (blue); only when these two parameters were identified were particles quantified, i.e. only when a cell was identified were internalised particles counted, thereby eliminating the risk of quantification of extracellular particles. Incubation time for this assay was 1 h, and negative controls of untreated cells showed no coumarin-6 fluorescence at the wavelengths (475/535 nm) for particle detection, as expected. Nonencapsulated coumarin-6 showed barely detectable levels of cell uptake. The results shown in Figure 3A show an optimal size range of 0.8−2.1 μm for uptake of microparticles under normal body temperature of 37 °C in the differentiated THP-1 cell model. The 0.8 μm sized PLGA particles facilitated a significant increase in coumarin-6 uptake ($p < 0.05$) (Figure 3D). These particles showed a 5.7-fold increase compared to the 4 μm particles and at least an 11-fold increase compared to any other sized particles except for the 2.1 μm sized particles. The 2.1 μm sized particles were also shown to be taken up significantly more than any other particle, except for the 0.8 μm sized particles ($p < 0.05$). These particles showed a 6.2-fold increase compared to the 4 μm particles and at least a 12-fold increase compared to any other sized particles (except for the 0.8 μm sized particles).

Using the HCA software it is possible to derive the average number of particles internalized per cell. The 0.8 μm sized particles were taken up at an average rate of 3.97 ± 0.5 particles per cell, while the 2.1 μm particles were taken up at an average rate of 4.32 ± 0.54 particles per cell ($n = 3$). This high-throughput method enabled screening of a greater size range of microparticles than would normally be feasible within a single experiment and provided both qualitative and quantitative data from the same experiment, thereby decreasing interexperimental variability.

In addition, to differentiate between cell membrane bound and intracellular particles, treatments were carried out at 4 °C to inhibit active internalization of particles and compared to experiments carried out at 37 °C. The comparative uptake is shown in Figure 3A and indicates there is no significant internalization of any of the particles in the size range studied at 4 °C. It is worth noting that this is not a test to show that active transport is responsible for cell uptake, as at 4 °C, the cell membrane fluidity is altered to inhibit uptake.[36] In order to understand the mechanism of cellular uptake, pharmacological inhibitors of different pathways would be required. This data does however support the ability of HCA to exclude membrane bound particles during quantification.

Assessment of the Effect of Microparticle Coatings on Particle Uptake by Macrophages. *The Effect of Coatings on Microparticle Uptake Using Spectrofluorimetry and CLSM.* Based on the data from the effect of size on uptake following 1 h incubation, 2.1 μm sized microparticles were coated with gelatin (type A), gelatin (type B) and ovalbumin to assess if these opsonic coatings could increase macrophage uptake as previously suggested for microparticles coated in proteins.[24] In the first instance, differentiated THP-1 cells were treated with the coated microparticles for 1 h and the effect on uptake was studied using spectrofluorimetry and CLSM. The spectrofluorimetry data

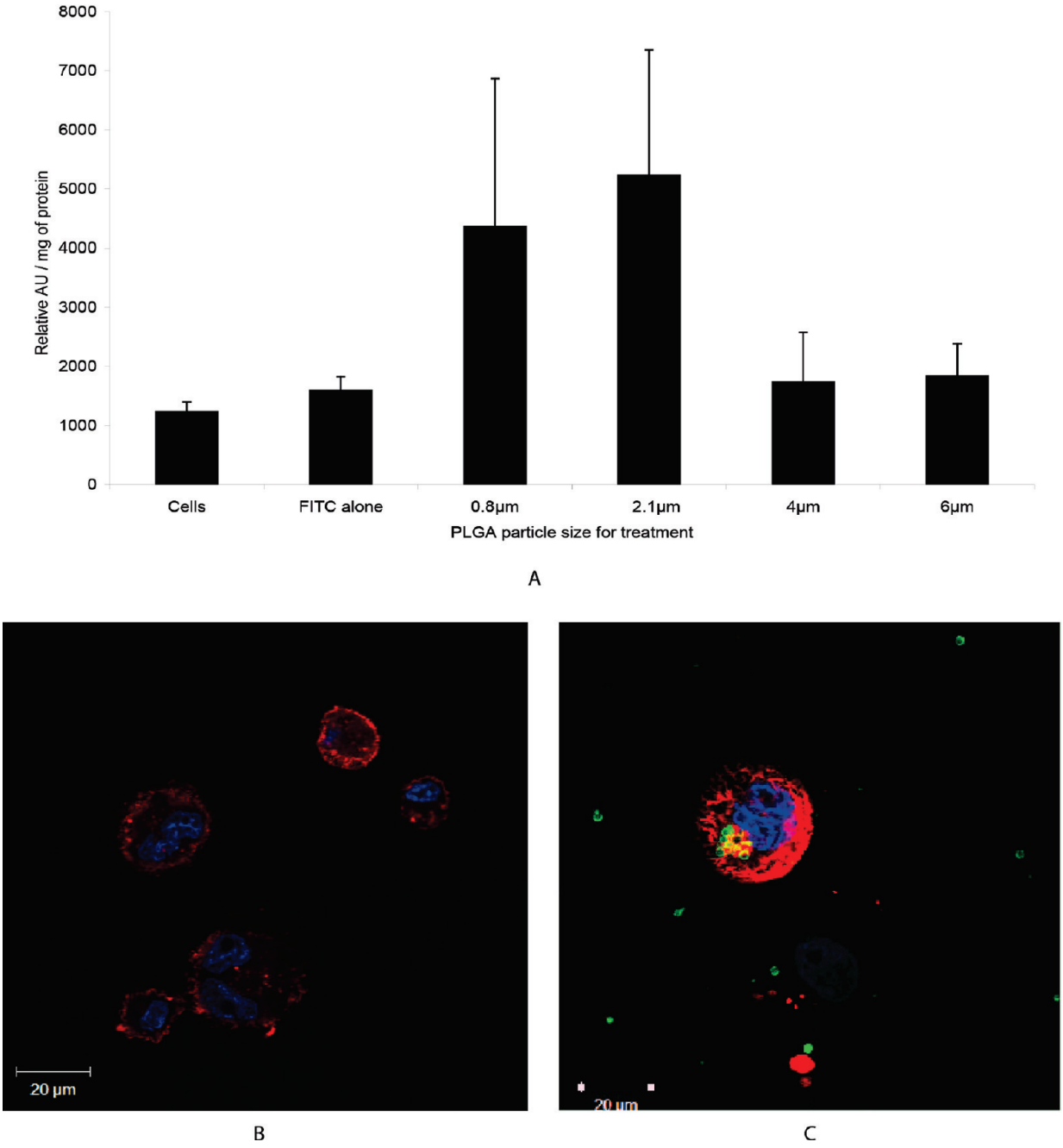


Figure 2. Spectrofluorimetry and CLSM analysis of microparticle uptake by THP-1 cells. (A) Spectrofluorimetric analysis of FITC-labeled microparticles ranging in size, 0.8 μm, 2.1 μm, 4 μm and 6 μm, were incubated with PMA-treated THP-1 cells for 1 h. The cells were rinsed and lysed, and the relative absorbance units/mg of protein was determined by spectrofluorimetry ($n = 3 \pm$ SD). (B) Confocal image of nonfluorescent microparticle treated THP-1 cells counterstained for F-actin (red), the nucleus (blue) and (C) confocal image of fluorescent 2 μm microparticle (green) treated THP-1 cells counterstained for F-actin (red), the nucleus (blue).

in Figure 4A suggests that coating microparticles with gelatin (type A), gelatin (type B) and ovalbumin does not result in a significant increase in particles per cell after 1 h compared to uncoated microparticles. CLSM images in Figure 4B show uptake of microparticles coated with gelatin (type A), and Figure 4C is a cropped zoom confocal image of the cells in order to see the microparticles within the cell. Figure 4D shows the uptake of gelatin (type B) coated microparticles, and there appear to be fewer internalized particles in this cell. The images from the CLSM would indicate a high number of noninternalized particles in the sample (Figure 4B) after 1 h.

The Effect of Coating on Microparticle Uptake Using High-Content Analysis. The same coated microparticles were used to treat differentiated THP-1 cells for 1 h, and the effect on uptake was studied using high-content analysis (HCA). Once again cells were incubated $n = 3$ at three different passages. The results of these experiments showed a highly significant increase in cellular uptake of all coated particles compared to uncoated particles ($p < 0.01$)

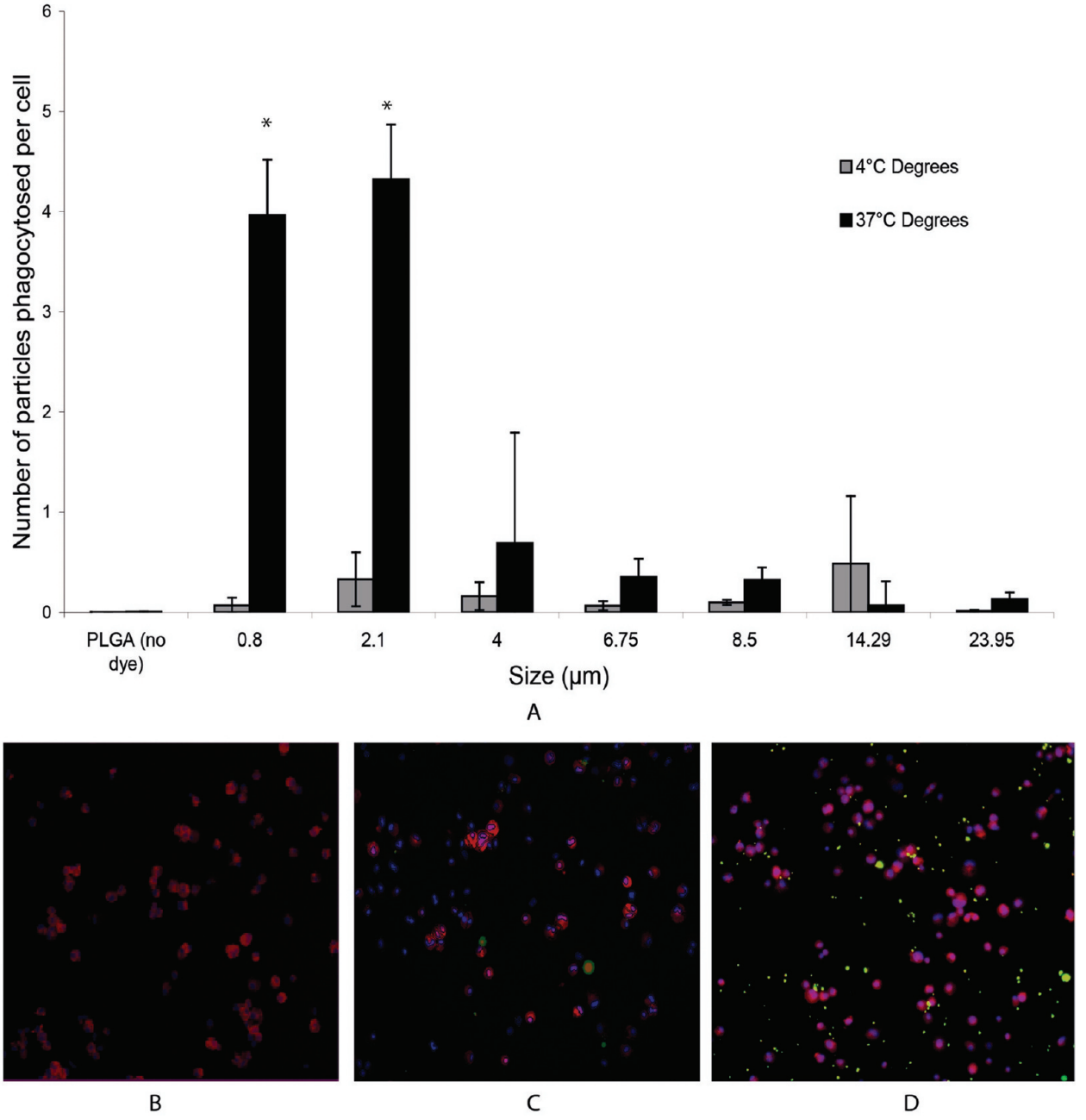
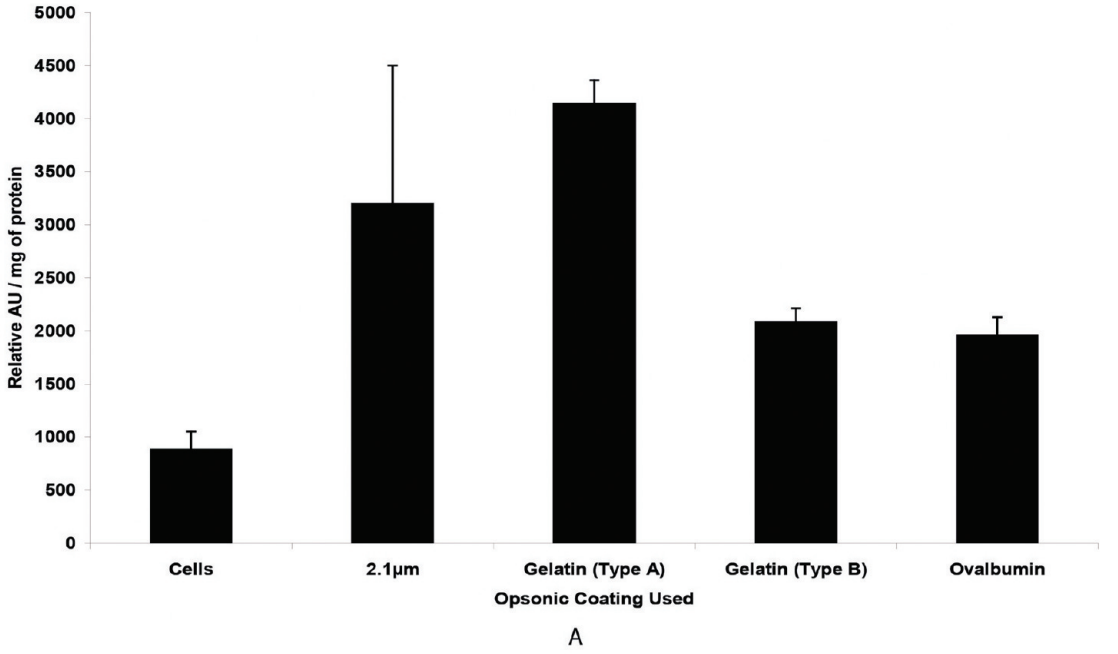


Figure 3. High-content analysis of microparticle uptake. Cells were treated with microparticles loaded with coumarin-6 (green) at 4 °C or 37 °C for 1 h, counterstained for F-actin with phalloidin-TRITC (red) and the nucleus with Hoechst 33342 (blue), and the uptake of microparticles was imaged and quantified using the INCell 1000 with a 10× microscope objective. Data represents (A) uptake quantified using the INCell 1000 ($n = 3 \pm$ SD) and corresponding images of (B) dual object analysis segmentation for 14 μm sized microparticles (particles not internalized are not segmented), (C) nonfluorescent microparticles (control), and (D) fluorescent 0.8 μm PLGA microparticles loaded with coumarin-6 (green). *Significant difference compared to other microparticles ($p < 0.05$).

as shown in Figure 5. Again it is worth noting that based on the algorithm employed only internalized particles were counted.

Treatment of THP-1 cells with uncoated particles of 2.1 μm microparticles led to 3.7 ± 0.5 particles internalized per cell. However, gelatin (type A) coating increased this uptake to 8.3 ± 1.32 particles per cell, gelatin (type B) coating to 7.4 ± 1.18 particles per cell and ovalbumin coating to 8 ± 1.32 particles per cell ($p < 0.01$). As the coating of PLGA microparticles enhanced the rate and extent of uptake significantly, particles composed entirely of the gelatin were prepared by spray-drying to see if pure gelatin particles were taken up to the same extent by macrophages as gelatin-coated PLGA microparticles. The spray-dried gelatin (type A) particles were shown to have a low level of cell uptake (<0.08 particle per cell). This may be explained by the fact that the gelatin particles solubilize instantly in the preheated medium and therefore lacked the 3-D structure/size required for phagocytic uptake. Conversely, the PLGA coated particles retained their coating and their three-dimensional structure during incubation to maximize phagocytosis and ultimately uptake.



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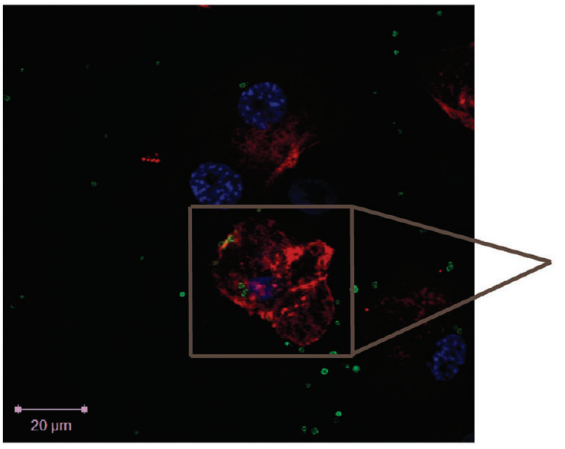

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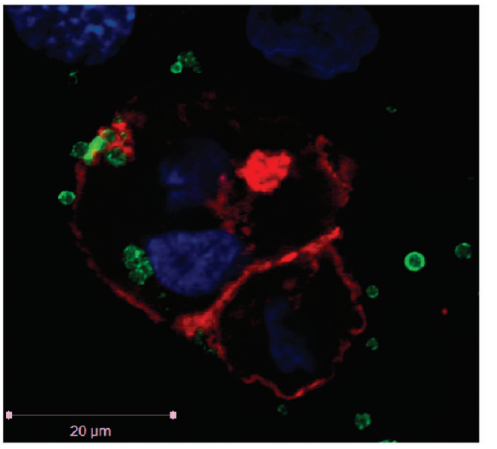
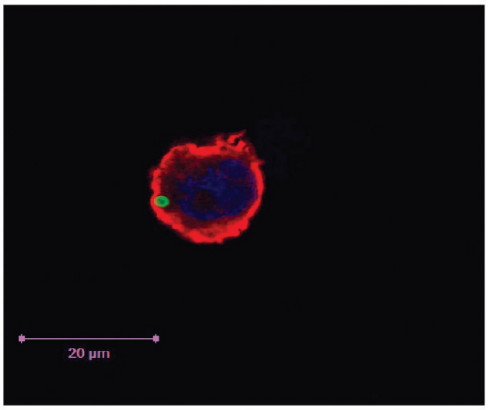


C



D

Figure 4. (A) Fluorimetric analysis of uptake of coated microparticles was carried out as on cell lysates and normalized for relative absorbance units/mg of protein ($n = 3 \pm$ SD). Confocal images of treated THP-1 cells counterstained for F-actin (red) and the nucleus (blue): (B) gelatin (type A) coated microparticles, (C) a cropped image focusing on a cell from panel B and (D) gelatin (type B) coated microparticles using a 63× oil objective.

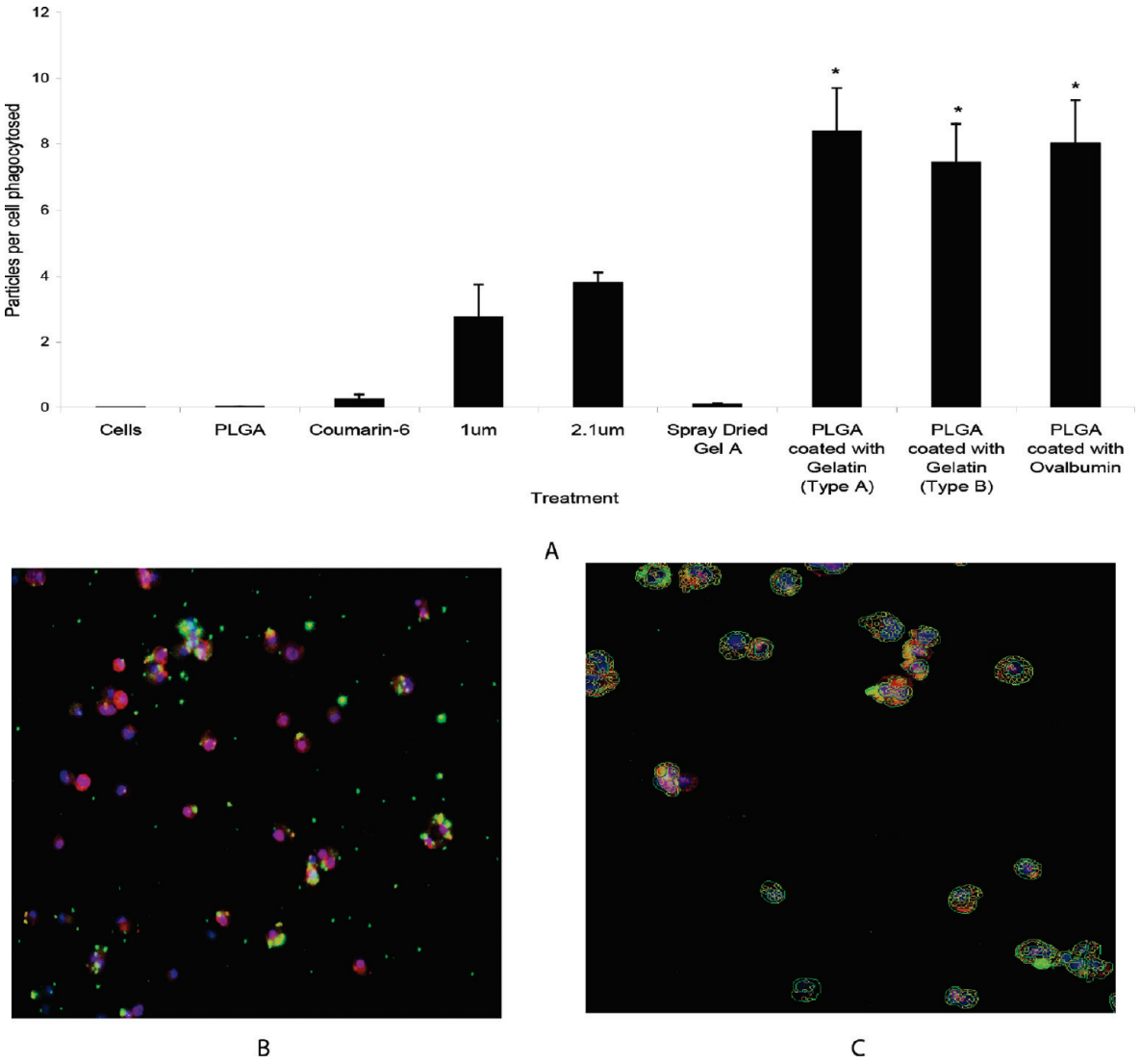


Figure 5. High-content imaging of coated microparticle uptake by THP-1 cells using a 10× objective. (A) Cells were treated with microparticles at 4 °C or 37 °C for 1 h, and uptake was quantified using an INCell 1000 ($n = 3 \pm$ SD). Images represent (B) microparticles coated with gelatin (type B) and (C) dual object analysis showing segmentation and quantification of internalized particles only (gelatin type A). *Significant difference compared to uncoated microparticles ($p < 0.05$)

Figure 5B shows the uptake of gelatin type B coated microparticles, and Figure 5C shows the uptake of gelatin type A coated microparticles using a 10× objective. Dual area object analysis software's capability to segment and count coated microparticles within the cell based on their fluorescent and size properties is shown again in Figure 5C. A green collar is drawn around the cell perimeter and a blue collar around the cell nucleus. These are calculated by the software on the basis of minimum area and fluorescent intensity thresholds to define the cells, and once the cell parameters have been established, a final yellow collar circles particles in the cell that are within this region. The effect of microparticle dose on uptake was also determined, and a significant increase in uptake for coated microparticles was seen even at much lower treatment doses than for uncoated microparticles. For example, after 1 h incubation of cells with 75 μg of gelatin (type B) coated microparticles, the level of uptake was twice that seen for a 300 μg dose of uncoated microparticles of the same size ($p < 0.001$) (data not shown). This decrease in dose could have important translational implications with a lower dose to deliver because of improved targeting and therefore a decreased likelihood of polymer accumulation and toxicity.

Assessment of Microparticle Uptake by Primary Alveolar Macrophages from Bronchoalveolar Lavage (BAL) Using HCA. Human primary alveolar macrophages are difficult to obtain and culture. As a result most studies have employed cell lines to model the behavior of drug delivery systems in macrophages. To test our THP-1 cell line as a model of alveolar macrophages and to screen our delivery systems in a more physiologically relevant model, primary alveolar macrophages were obtained from bronchoalveolar lavage. The ability of primary alveolar macrophages to phagocytose uncoated versus coated microparticles following a 1 h incubation was assessed using HCA. The images (Figure 6B−D) show the high number of cells obtained for quantification, and, with the unique dual object analysis algorithm employed, the extracellular microparticles can be disqualified from quantification. Quantitative data from

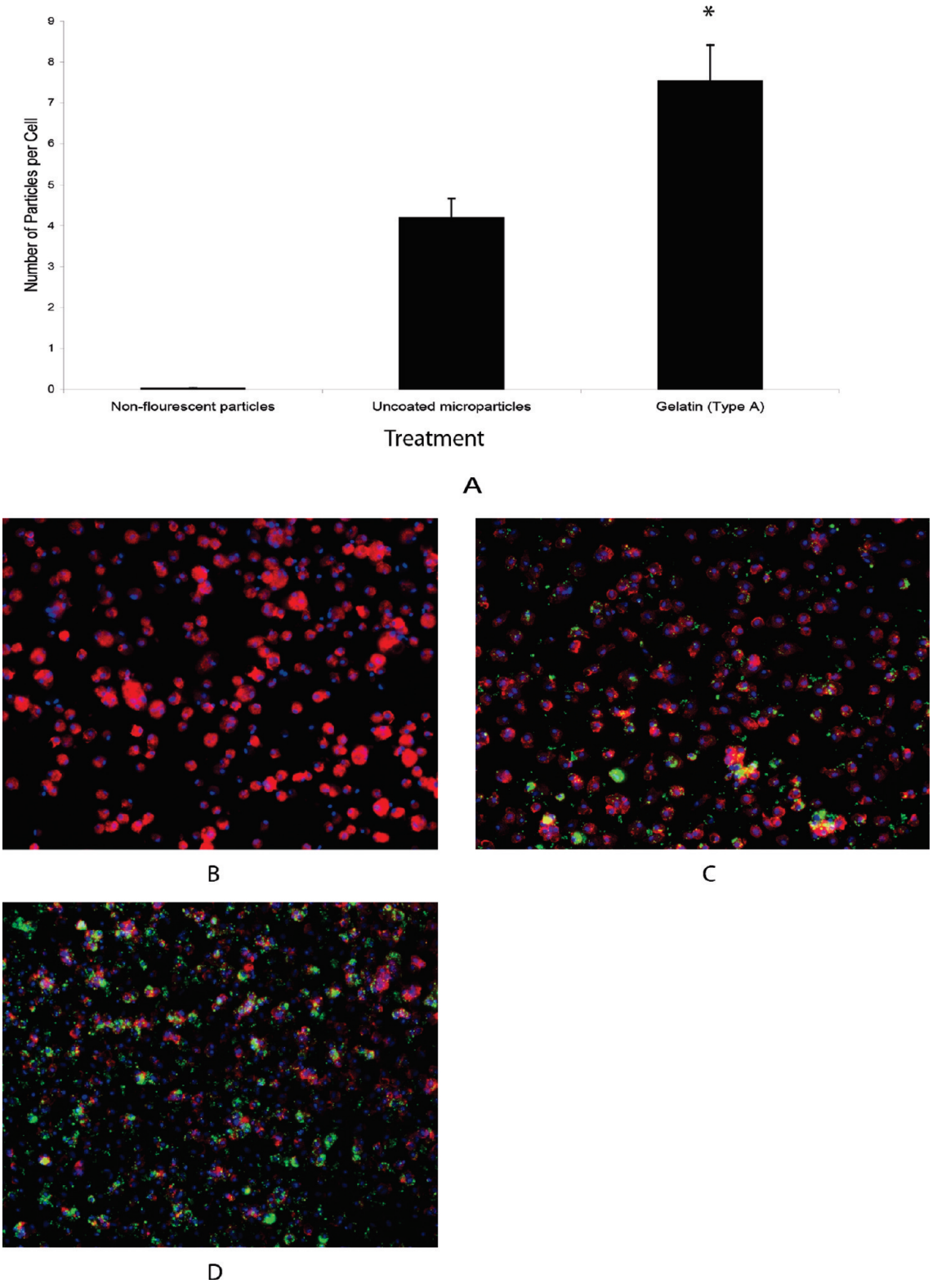


Figure 6. Microparticle uptake by primary alveolar macrophages obtained from bronchoalveolar lavage. (A) Coated microparticle uptake was found to be significantly increased compared to the uncoated particle of the same size ($n = 3 \pm$ SD). Alveolar macrophages were treated with microparticles for 1 h, and the number of particles per cell was quantified by dual object analysis on an INcell 1000 system. Images show (B) untreated cells, (C) cells treated with 2 μm uncoated microparticles and (D) microparticles coated with gelatin (type A). *Significant difference compared to uncoated microparticles ($p < 0.05$)

the HCA (Figure 6A) indicated that the microparticles were internalized efficiently by primary alveolar macrophages with a similar number of particles internalized per cell as was seen in the THP-1 cell model. Primary alveolar macrophages internalized

4.1 ± 0.47 particles per cell after treatment with uncoated 2.1 μm sized PLGA microparticles and 7.53 ± 0.88 particles per cell after treatment with microparticles coated with gelatin (type A), an almost 2-fold increase in the number of particles per cell when a gelatin coating was used ($p < 0.001$).

DISCUSSION

Although there has been a rapid growth in the development of particulate drug delivery systems (DDS), an understanding of how these systems interact with and are internalized by cells has lagged behind. Understanding particle−cell interactions is important for early *in vitro* biopharmaceutical characterization and screening of DDS. Phagocytic cells, such as macrophages, represent the initial defense of the innate immune system. An understanding of the particle characteristics that impact on particle uptake by macrophages is critical for avoiding macrophages, which is an important consideration when attempting to enhance systemic bioavailability[29] but also for targeting drugs to macrophages when therapeutically advantageous, e.g. in tuberculosis *M. tuberculosis* bacilli reside within alveolar macrophages in the lung. In the case of tuberculosis treatment, targeting macrophages can lead to increased intracellular concentrations of therapeutics that can potentially enhance efficacy and reduce systemic toxicity.[37] A comprehensive understanding of the impact of physicochemical properties of DDS, e.g. size and surface coating, on macrophage uptake processes would provide key information for the smart design of DDS.

The work presented here indicates that above a certain size cutoff of 4 μm sized microparticles macrophage uptake of microparticles is significantly diminished. These results corroborate the development of large porous particles (LPPs) for pulmonary delivery to avoid alveolar macrophage clearance. By manufacturing particles with a geometric size of greater than 10 μm these LPPs are less susceptible to macrophage clearance but their low density produces particles with an aerodynamic size suitable for respiratory drug delivery to the alveolar region of the lungs, thereby improving systemic bioavailability.[27,38] The data presented shows a much lower size range for optimal macrophage uptake compared to previous work, which used direct optical microscopy to physically count the number of particles per cell in 100 cells.[3,5] These studies carried out their analysis in a rat alveolar macrophage cell line (NR8383) and determined the optimal size to be between 3 and 6 μm, but the authors did cite they had difficulty in assessing if microparticles were internalized using this quantification method. In comparison, the method employed in our study is high throughput, capable of quantifying internalized microparticles using algorithm protocols. A large variety of different treatments could be assessed in parallel in the same experiment, and in our HCA study cell uptake was examined in $n = 3$ wells at 3 different passages, providing sufficient sample size for meaningful statistical analysis. Variability and sampling bias associated with methods such as spectrofluorimetry and fluorescent microscopy can also be eliminated by using this automated system.[40]

Furthermore, the results presented herein provide quantitative data to support the optimization of physicochemical properties of microparticles for alveolar macrophage targeting. Coating particles with opsonic proteins led to a minimum 2.5-fold increase in cell uptake compared to uncoated microparticles ($p < 0.05$), and additionally lower doses of coated particles were required to achieve highly significant cell uptake. The increase in uptake of coated particles supports the hypothesis that coating particles in opsonins reduces the particle−cell repulsion, thereby facilitating enhanced uptake of microparticle delivery systems by macrophages.[24,25,36]

We compared different techniques for assessing microparticle−cell interactions in terms of their ability to provide both qualitative and quantitative data. HCA data identified an optimal size range of 0.8−2.1 μm for targeting macrophages while spectrofluorimetry could not give a statistically significant answer for optimal size range when used to assess even a narrow size range of particles. This can be attributed to a number of limitations in the spectrofluorimtery technique including the inability to measure individual particles of a specific size per cell and to exclude extracellular particles.

The results of the CLSM studies (Figure 2 and Figure 4) show the limitations of this method for uptake analysis insofar as particles coated in gelatin (type B) appear less efficiently internalized than uncoated particles or those coated with gelatin (type A). The results of the HCA screen (Figure 5 and Figure 6) show this not to be the case, highlighting the issue that confocal imaging has limited utility for quantitative analysis of microparticle uptake due to the subjectivity involved in cell selection and counting and the limited number of cells that can be counted. Optical methods may be difficult to employ for analysis of nano- and microparticulate uptake due to their size, especially due to the difficulty in visualization of particles in granular cells such as macrophages, and in addition these methods are labor intensive. When using confocal microscopy, fluorescently labeled nano-/microparticulates located in proximity may appear as a single fluorescence, making it difficult to distinguish individual particles. Automated systems allow for faster, more accurate results. Flow cytometry may be used for the rapid, automated detection of internalized fluorescently labeled microparticles, but this technique is limited by its inability to carry out size exclusion. HCA incorporates a size exclusion algorithm, which allows for the quantification of only the desired size range and which has also been adapted by us for the study of a range of DDS including nanoparticles.[39] This algorithm then gives the average number of particles per cell in all fields examined.

Our analysis of microparticle uptake into human primary alveolar macrophages confirms the increase in particle uptake for gelatin (Type A) coated microparticles compared to uncoated, with the extent of uptake significantly increased compared to uncoated particles. The effects of particle design appear to translate from the cell line THP-1 into primary macrophages with no significant difference in the number of particles per cell seen in primary alveolar macrophages (4.1 ± 0.47 particles per cell) and THP-1 cells (4.32 ± 0.54 particles per cell). The effect of particle coating in primary cells with a 2-fold increase in particle uptake after coating with gelatin A was the same as that seen in the THP-1 cells. This shows that PMA treated THP-1 cells are a good model of human alveolar macrophages as shown in other studies[41] and that HCA can in the first instance offer meaningful data in cell lines for extrapolation to primary cells.

Overall, we have designed an efficient platform for targeting alveolar macrophages using HCA analysis to assess the effect of particle properties on cell uptake. Macrophages are a notoriously difficult cell type to which to deliver cargoes intracellularly, and this platform therefore has huge potential for targeted drug delivery in respiratory disease and more broadly in immunology. In comparison to previously used techniques, HCA provides both qualitative and quantitative data on the influence of carrier

design on cell targeting in a high-throughput format and therefore has great potential in the rapid and accurate screening of a range of targeted drug delivery platforms.

AUTHOR INFORMATION

Corresponding Author

*Royal College of Surgeons in Ireland, School of Pharmacy, York House, York Street, Dublin 2, Ireland. E-mail: scryan@rcsi.ie. Tel: 00-353-1-4022741. Fax: 00353-1-4022765.

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