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Adhesive interactions between cells and biotinylated phospholipid vesicles in alginate: towards new responsive biomaterials

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Abstract Creating tissue-mimetic biomaterials able to deliver bioactive compounds after receipt of a remote and non-invasive trigger has so far proved to be challenging. The possible applications of such "smart" biomaterials are vast, ranging from subcutaneous drug delivery to tissue engineering. Self-assembled phospholipid vesicles (liposomes) have the ability to deliver both hydrophilic and hydrophobic drugs, and controlling interactions between functionalized vesicles and cells within biomaterials is an important step for targeted drug delivery to cells. We report an investigation of the interactions between thermally-sensitive and biotin-coated dipalmitoyl phosphatidylcholine vesicles and 3T3 fibroblast cells. The stability of these vesicles under physiological conditions was assessed and their interaction with the cell membranes of fibroblasts in media and alginate/fibronectin mixtures was studied. Stable vesicle-cell aggregates were formed in fluid matrices, and could be a model system for improving the delivery of remotely released drugs within vesicle-containing biomaterials.

1 Introduction

Phospholipid vesicles are spherical self-assembled capsules that have been widely studied due to their ability to mimic cell membranes and act as delivery vehicles for bioactive compounds like drugs [1]. Their membranes can be engineered in a variety of ways to modulate their interaction with biological targets. Masking vesicle membranes with polyethylene glycol (PEG) lipids creates 'stealth liposomes' and inhibits their recognition by the immune system [2, 3]. On the other hand, groups like antibodies or glycosaminoglycans can be appended to the exterior of vesicles to create drug carriers that will recognize specific cell types [4].

Phospholipid vesicles can act as active stores for both hydrophilic and hydrophobic molecules [5–7], which has generated extensive interest in their use as drug delivery vehicles in vivo and in vitro [1, 8]. Targeting drug-containing vesicles to cells may improve efficacy of smart biomaterials by bringing the carrier and cell into proximity before release of the vesicle contents. However to be used in this manner, selective recognition of the vesicles by cells must be fully understood. A persistent problem in clinical trials is recognition by lymphocytes, leading to clearance of drug-containing vesicles from the body via the reticuloendothelial system (RES) before they reach their target cells [1, 9, 10]. After recognition, vesicles can be engineered to be endocytosed or to fuse with the cell membrane, for example cationic lipids can promote endocytosis while unsaturated phospholipids, e.g. with dioleoyl chains, can promote fusion with cell membranes [10–12]. Vesicles which are endocytosed are usually trafficked to endosomes and lysosomes where they are degraded and their contents destroyed, although some progress is being made developing vesicular carriers that are activated by the low pH in endosomes to release their contents [10, 13].

In recent studies we have shown that assemblies of magnetic nanoparticles and thermally-sensitive vesicles can be used to store hydrophilic compounds, for example

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drug molecules, which can be specifically released by a magnetic or thermal trigger [6, 14, 15]. When the assemblies of magnetic nanoparticles and thermally-sensitive vesicles are incorporated into hydrogel materials, the released compounds can be used as biological actuators in vitro that initiate changes in surrounding cells cultured in the hydrogel. Ascertaining the presence and measuring the strength of interactions between vesicles and cells in this in vitro system is crucial. This may allow targeted cell kill, which has significance in aspects of tissue engineering, particularly for the removal of misdifferentiated stem cells from engineered tissue. It may also allow us to develop the potential of magnetic nanoparticle/thermally-sensitive vesicle conjugates as vesicle-based drug delivery systems in vivo; targeting and remote magnetic triggering of drug delivery to cells is of great interest in the future for clinical drug delivery. Thermally-sensitive vesicles with an adhesive biotin coat will form the next generation of our magnetic nanoparticle/vesicle assemblies [16]. Herein we report investigations into the interaction of these adhesive vesicles with 3T3 fibroblasts, a stable cell line that proliferates rapidly in fibronectin containing calcium alginate gels [16] and can be used as a model for anchorage dependent cells.

2 Materials and methods

2.1 Biotinylated dipalmitoyl phosphatidylcholine vesicle (Bt-DPPC vesicle) preparation

Phospholipid vesicles (800 nm diameter) were formed from dipalmitoyl phosphatidylcholine (DPPC, 14.6 mg, 1.9×10^{-5} mol, Avanti Lipids) and 0.2% mol/mol *N*-(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Bt-DHPE, 200 µl of a 0.2 mM solution in chloroform, Avanti Lipids, USA). The lipids were dissolved in chloroform (1 ml) and the solvent removed under reduced pressure. The lipid film was rehydrated in phosphate buffered saline (PBS, 1 ml, Invitrogen). The solution was vortex mixed and heated to 50°C to fully disperse the lipid film and form a suspension, which was then extruded (19 passes) at 50°C through an 800 nm pore membrane using an Avestin LiposoFast extrusion apparatus.

2.2 Preparation of rhodamine-tagged Bt-DPPC vesicles

Prepared as for standard Bt-DPPC vesicle preparation but the lipids were dissolved in rhodamine-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (rhodamine-DHPE) solution in chloroform (0.02 mM, 1 ml to give 0.1% mol/mol), in the place of chloroform alone. The vesicles produced were sized by fluorescence microscopy and found to be (1.2 ± 0.6) µm in diameter.

2.3 Preparation of Bt-DPPC vesicles encapsulating either 5/6-carboxyfluorescein (5/6-CF) or fluorescein isothiocyanate-tagged dextran (FITC-dextran)

The lipid film was formed as described for standard vesicle preparation, however, the lipid film was rehydrated in a solution of either: 5/6-CF (0.05 M, Sigma UK) prepared in 3-(*N*-morpholino)propanesulfonic acid buffer (MOPS, 1 ml) or FITC-dextran (4 kDa, 1 mg/ml) in PBS (1 ml). The lipid film was vortex mixed and extruded as described previously. These vesicles with encapsulated compounds were purified using a PD-10 Sephadex column which had been pre-equilibrated using PBS (25 ml). The vesicle suspensions (1 ml) were diluted to 2.5 ml with PBS, loaded onto the column and then eluted from the column with a further 3.5 ml of PBS.

2.4 Preparation of Bt-DPPC vesicles in alginate/fibronectin mixtures

Freshly prepared Bt-DPPC vesicles (1 ml) were centrifuged at 500 rpm for 3 min and the supernatant removed. The pellet was re-suspended in sodium alginate solution (2% wt/vol) and fibronectin solution (1 mg/ml) was added at 1% vol/vol. The gel was cured by the infusion of $CaCl_2$ (0.1 M, 1 ml) through an 800 nm polycarbonate membrane into the sodium alginate/vesicle suspension.

2.5 Growth and maintenance of 3T3 fibroblast cultures

3T3 Fibroblast cells (ECACC, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% vol/vol Fetal Bovine Serum (FBS) and antibiotic (1% penicillin, 1% streptomycin). Cells were passaged at 80% confluency using trypsin/EDTA (25%).

2.6 Assaying Bt-DPPC vesicle stability

Suspensions of Bt-DPPC vesicles with encapsulated 5/6-carboxyfluorescein (25 µl, total lipid concentration = 20 mM) were added to PBS (1 ml) in a 24 well culture plate (PAA) and incubated under cell culture conditions (37°C and 5% vol/vol CO_2). Aliquots of the supernatant were removed (200 µl) and the fluorescence was measured using a Fluorostar optima fluorescence plate reader (BMG Labtech) every 60 min for 5 h. After this time Triton X-100 was added (1% vol/vol in PBS, 1 ml) to each well and the plate incubated at room temperature for 20 min. Aliquots of the solutions in each well (200 µl) were then

transferred into a clear 96 well plate and the fluorescence measured.

2.7 Assaying vesicle-cell interactions

Cells were seeded as a monolayer onto 24-well cell culture plate and incubated for 12 h (cell count of 39 cells/μL, total cell count 40,000). Suspensions of vesicles with encapsulated 5/6-carboxyfluorescein (25 μl, total lipid concentration = 14 mM) were then added to: a monolayer of cells; a layer of extracellular matrix (where the cells had been removed using cell dissociation buffer (CDB)); tissue culture polystyrene which had been incubated with media for 12 h. The mixtures were then incubated for 12 h. Aliquots of the media (0.2 ml) were transferred to a 96 well plate and the fluorescence measured using a Fluorostar optima fluorescence plate reader (BMG Labtech). The vesicle samples in the cell culture wells were then washed with PBS (10 × 1 ml), aliquots of the final wash transferred into a 96 well plate and the fluorescence measured. Finally the vesicle samples in the cell culture wells were incubated with Triton X-100 (1% vol/vol in PBS, 1 ml) for 20 min at room temperature. Aliquots of the Triton X-100 solutions (0.2 ml) were transferred into a 96 well plate and the fluorescence measured.

2.8 Cell staining and imaging of cells with Bt-DPPC vesicles in media or alginate mixtures using confocal fluorescence microscopy

Harvested cells were re-suspended in fresh medium and were seeded onto glass coverslips at a density of 1×10^5 cm^{-2}. Cells were incubated with rhodamine-labeled Bt-DPPC vesicles suspended in PBS or sodium alginate (total lipid concentration = 20 mM, 200 μl) at 37°C and 5% CO_2 (vol/vol) for 12 h. The culture medium was removed and the samples washed with PBS (10 × 1 ml). Samples were fixed using paraformaldehyde (4% wt/vol in PBS, 1 ml) for 15 min then washed using PBS. The cells were blocked using ICC block (PBS containing Triton X-100 at 1% vol/vol, bovine serum albumin 1% wt/vol) and stained with FITC-tagged phalloidin (0.165 μm phalloidin) in 1% bovine serum albumin for 20 min. Samples were washed with PBS (5 × 1 ml) and then counterstained with DAPI and mounted using Prolong antifade (Invitrogen, UK).

2.9 Flow cytometric analysis of cells with Bt-DPPC vesicles

Cells were grown on tissue culture plastic until 60% confluency was reached. Vesicle suspensions with or without encapsulated FITC-dextran (25 μl, 14 mM) were then added to the samples and the cells cultured for a further 12 h. Cells were harvested using CDB centrifuged at 700 rpm for 3 min and resuspended in FACS buffer (0.5 ml, PBS containing 2 mM EDTA and 0.5% BSA wt/v) and stored on ice. The samples were analyzed by flow cytometry, which was carried out on a Becton–Dickinson FacsCalibar using CellQuest software.

3 Results and discussion

3.1 Stability of Bt-DPPC vesicles under cell culture conditions

The loss of encapsulated material from thermally-sensitive phospholipid vesicles occurs when the bilayers undergo a phase transition upon heating to the membrane melting temperature (T_m), corresponding to the vesicle bilayer entering a liquid disordered phase from the solid ordered phase [17]. The melting temperature is characteristic for each phospholipid bilayer and DPPC vesicles undergo this transition at 41°C [18]. The T_m of DPPC bilayers should ensure stability under physiological conditions (i.e. 37°C), yet the vesicles should still be able to release their contents thermally during culture, a key requirement of our magnetic delivery system [6]. We used Bt-DPPC vesicles with encapsulated 5/6-carboxyfluorescein (5/6-CF, a model for water-soluble bioactive compounds) and assayed membrane disruption of the vesicles under cell culture conditions (Fig. 1). Release of 5/6-CF from compromised vesicles is self-indicating, as a large increase in fluorescence results from the alleviation of self-quenching inside the vesicle. After incubation of Bt-DPPC vesicles for 5 h at 37°C and 5% vol/vol CO_2, no spontaneous release of 5/6-CF was observed; subsequent addition of Triton X-100 at 5 h completely disrupted the membrane and caused full release of encapsulated material. This confirms that as expected, adhesive Bt-DPPC vesicles can successfully store compounds under typical conditions used for cell culture.

3.2 The effect of interactions with cells or surfaces on Bt-DPPC vesicle stability

As well as direct interactions with cells, adhesive Bt-DPPC vesicles could also interact with the extracellular matrix produced by proliferating cells and the surface upon which the cells sit. The interactions of Bt-DPPC vesicles with three relevant surfaces were therefore assessed: tissue culture plastic, cell-free extracellular matrix and a fibroblast monolayer. The surface of tissue culture polystyrene is plasma treated to generate a hydrophilic surface that encourages the culture of anchorage dependent cells, while

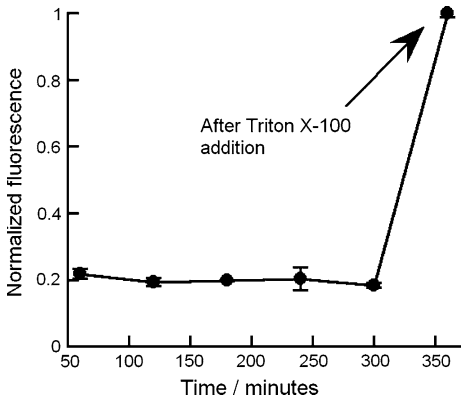

Fig. 1 Stability of Bt-DPPC vesicles with encapsulated 5/6-carboxyfluorescein under cell culture conditions. Triton X-100 was added at 5 h and the fluorescence data was normalised to the maximum fluorescence observed from each sample

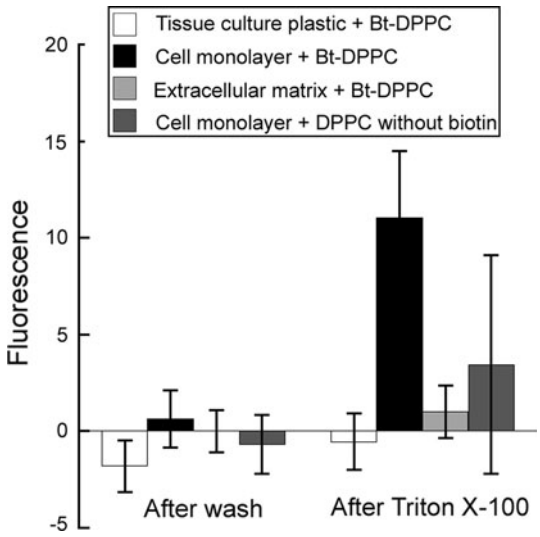

Fig. 2 Release of 5/6-carboxyfluorescein (5/6-CF) from Bt-DPPC vesicles (Bt-DPPC) or blank vesicles (DPPC without biotin) that have been exposed to different surfaces, as monitored by fluorescence (background fluorescence has been subtracted). *Left* after washing with PBS buffer. *Right* after Triton X-100 was added to the samples

the extracellular matrix of 3T3 fibroblasts is a complex mixture of polysaccharides (such as glycosaminoglycans) and proteins that are secreted from cells. Fibroblast cells grown as a monolayer also display a range of membrane-anchored receptors which control cellular interactions, for example detecting and binding to the surroundings.

Biotinylated dipalmitoyl phosphatidylcholine vesicles encapsulating 5/6-CF were used to monitor the adhesive interaction between the vesicles and the surfaces. The presence of intact vesicles can be revealed by the addition of Triton X-100, which lyses the vesicles to release the 5/6-CF and give a fluorescent signal. Addition of Bt-DPPC vesicles to each of the three surfaces did not release 5/6-CF, indicating that any adhesive interactions with the surfaces did not disrupt the vesicles. Each sample was then washed thoroughly with PBS to remove unbound Bt-DPPC vesicles, and Triton X-100 added to each. A significant fluorescence signal from the lysis of bound vesicles was only obtained from the sample with both Bt-DPPC vesicles and a fibroblast monolayer (Fig. 2); the fraction of Bt-DPPC vesicles that had adhered to the cells could be calculated as 3–4% of the number added. The addition of non-biotinylated DPPC vesicles to a fibroblast monolayer gave a smaller fluorescence signal after washing and Triton X-100 addition, indicating that the adhesive interaction was largely occurring via the biotin coating. Given there was no recognition of the extracellular matrix or tissue culture plastic, this suggests the biotin coating on the Bt-DPPC vesicles is recognized by membrane-bound biotin receptors on the fibroblasts.

The stability of these fibroblast-vesicle assemblies over a 72 h period was then assessed. The fluorescence due 5/6-CF released from cell-bound vesicles was monitored both before and after the addition of Triton X-100. Over this period the population of cells will rapidly increase, as 3T3 fibroblasts can double their population over 120 h even when cultured in alginate/fibronectin gels [16]. Every 24 h the fluorescence of each of the samples was measured, followed by Triton X-100 addition to lyse the bound vesicles and release 5/6-CF. Despite ongoing cell division, Bt-DPPC vesicles attached to cells showed a high level of membrane integrity, with no release of 5/6-CF over a 72 h period. Subsequent vesicle lysis with Triton X-100 produced a similar level of fluorescence over the 72 h period; fluorescence measurements after Triton X-100 addition showed $2.9 \pm 0.4\%$ of the originally added Bt-DPPC vesicles had bound to the cells after 1 h, while after 3 days this value had only slightly declined ($2.6 \pm 0.4\%$). This showed that although the number of cells had increased, the number of intact Bt-DPPC vesicles had remained largely constant within the cell culture wells.

3.3 Influence of matrix fluidity on the evolution of Bt-DPPC vesicle-cell interactions

These preliminary studies suggested that cell-Bt-DPPC vesicle links form quickly in media, but increases in the viscosity of the environment surrounding the cultured cells would be expected to slow the rate at which these cell-vesicle interactions develop. In particular, the period over which the Bt-DPPC vesicles (or assemblies of magnetic nanoparticles and Bt-DPPC vesicles [16]) are incubated with the cell/sodium alginate mixture before gelation, which will immobilize the vesicles, should determine the number of vesicle-cell assemblies formed. Longer incubation times before curing with Ca(II) may improve the efficacy of these smart biomaterials by

bringing the drug carrier and cell into proximity before magnetic release.

The Bt-DPPC vesicle membranes were doped using rhodamine-DHPE, which allowed visualization of the vesicles and contrast with cells stained with FITC-phalloidin and DAPI. Confocal fluorescence microscopy revealed that rhodamine-labeled Bt-DPPC vesicles in alginate gels that had been cured as soon as the fibroblast solutions were added exhibited very few interactions between the Bt-DPPC vesicles and cell membranes. However cells seeded into sodium alginate solution and incubated with Bt-DPPC vesicles for 4 h prior to gelation showed a marked increase in the number of vesicle-cell interactions. This 4 h period allowed movement of the components through the alginate matrix whilst it was still fluid enough to allow diffusion (Fig. 3a). The effect of the viscosity of the solution was also clear, as cells seeded onto glass without sodium alginate present exhibited more vesicle-cell interactions after 4 h than the sample with sodium alginate (Fig. 3b), showing the anionic polysaccharide chains of alginate hinder the diffusion of vesicles and cells through the samples.

3.4 The cellular location of Bt-DPPC vesicles bound to fibroblasts

Vesicles bound to cells are known to be subsequently endocytosed or fuse with membranes [10, 11], so confocal microscopy was used to establish if either of these processes had occurred in the Bt-DPPC vesicle-cell assemblies. As previously, the vesicle membranes were doped using rhodamine-DHPE to contrast with cells stained with FITC-phalloidin and DAPI. Confocal microscopy was used to image through the cell structure with slices every 0.5 μm, which should allow the exact location of the vesicles to be ascertained.

As anticipated from the studies with 5/6-CF loaded Bt-DPPC vesicles and fibroblasts, the vesicles were not observed to have merged with the membrane of the cells and instead appeared as discrete entities (Fig. 4). The vesicles were observed in the same plane as the cells, but on the upper face of the cell membrane. The vesicles covered the top of the cytoskeleton and the nuclei yet the vesicles were not discernable inside any cell structure, which supports the hypothesis that bound Bt-DPPC vesicles neither fused nor were internalized by the cells.

3.5 Flow cytometric studies of Bt-DPPC vesicle-cell assemblies

Flow cytometry was used to ascertain if the adhesion of Bt-DPPC vesicles to 3T3 fibroblasts gave a measurable increase in size in the resulting aggregates. The Bt-DPPC vesicle/cell mixtures were monitored for changes in size and fluorescence against two control samples; cell-free vesicles and vesicle-free cells (Fig. 5). As well as mixing with blank Bt-DPPC vesicles, Bt-DPPC vesicles containing FITC-tagged dextran (4 kDa) were also mixed with 3T3

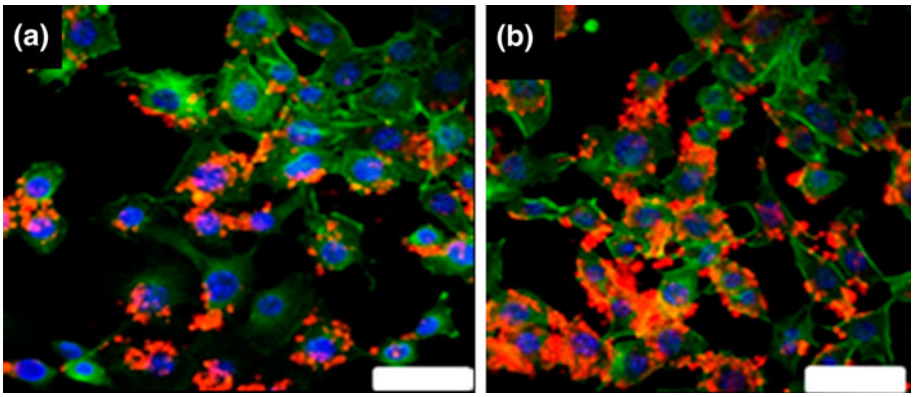

Fig. 3 Formation of Bt-DPPC vesicle/fibroblast assemblies in different matrices. **a** Sodium alginate solution containing Bt-DPPC vesicles (labeled with *red* fluorescent rhodamine-DHPE, *bright spots*, *red* in online version) which was seeded with cells and cultured for 4 h prior to gelation with Ca(II). **b** Bt-DPPC vesicles and cells seeded onto glass and incubated in media for 4 h. *Scale bar* 20 μm

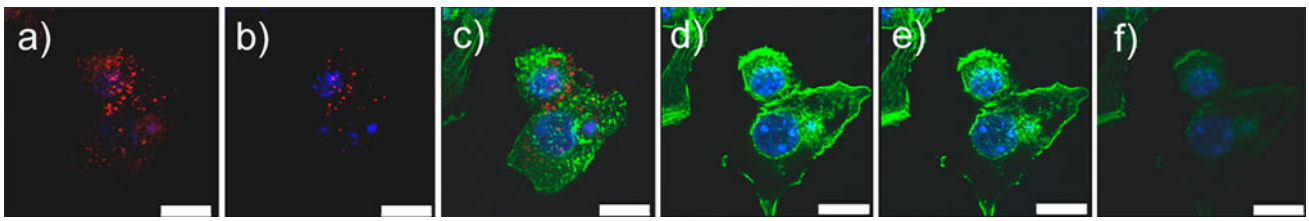

Fig. 4 Z-stack of confocal microscopy images showing rhodamine-DHPE tagged vesicles (*bright spots*, *red* in online version) linked to 3T3 fibroblasts stained with FITC-phalloidin and DAPI. Slice depth (**a**) 0 μm; (**b**) −0.5 μm; (**c**) −1.0 μm; (**d**) −1.5 μm; (**e**) −2.0 μm. *Scale bar* 25 μm

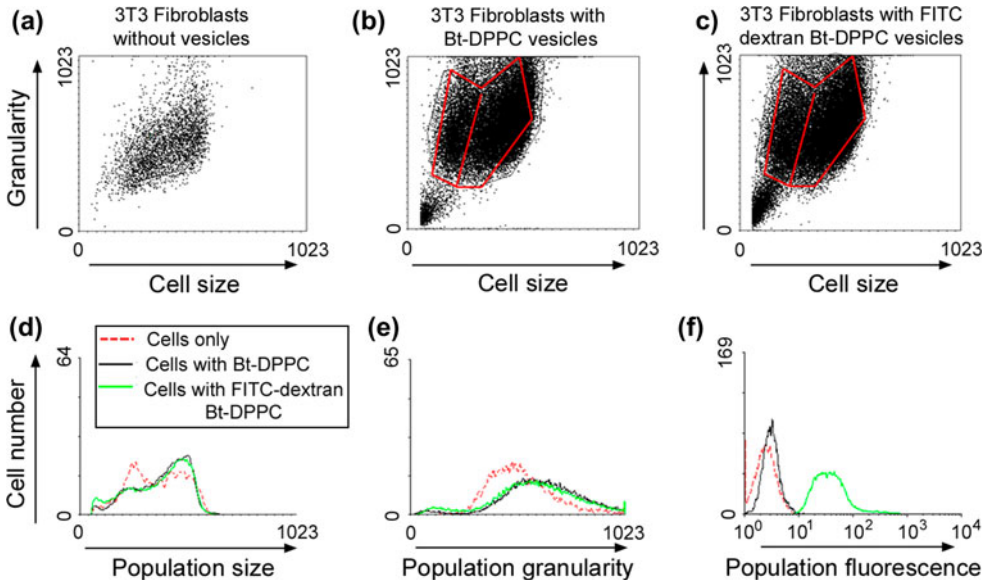

Fig. 5 Analysis of Bt-DPPC vesicle/fibroblast assemblies by flow cytometry. 3T3 Fibroblasts were cultured for 24 h with or without empty Bt-DPPC vesicles (control) or Bt-DPPC vesicles encapsulating FITC-dextran, and processed for flow cytometry as described in the Methods section. **a** A density plot (particle size (*x*-axis) versus granularity (*y*-axis)) of 3T3 fibroblasts, which gate in a single population on the plot. **b** Density analysis of 3T3 cells cultured with empty Bt-DPPC vesicles and **c** FITC-Dextran encapsulating Bt-DPPC vesicles. In both cases the 3T3 fibroblasts now comprise two size-distinct populations (marked in *gray*, *red* online). Unbound Bt-DPPC vesicles are smaller and less dense than cells, thus appearing in the *bottom corner* of the plots. **d**, **e**, **f** Comparison of distribution profiles in each of the three conditions: *dashed trace* (*red* online) = 3T3 fibroblasts alone; *black trace* = 3T3 fibroblasts cultured with empty Bt-DPPC vesicles; *gray trace* (*green* online) = 3T3 fibroblasts cultured with FITC-dextran encapsulating Bt-DPPC vesicles. **d** Comparison of the size distributions. **e** Comparison of the granularity distributions. **f** Comparison of the fluorescence distributions

fibroblasts, enabling the vesicle-cell assemblies to be tracked fluorescently. For each of the samples, particle size and granularity was obtained from the flow cytometry data. Biotinylated dipalmitoyl phosphatidylcholine vesicles that were not interacting with cells were smaller and less granular than cells, appearing at the low size/low granularity region in the bottom left of the plots, while 3T3 cells were observed in a higher size/higher granularity region. Density analysis of 3T3 fibroblasts cultured with empty Bt-DPPC vesicles and FITC-dextran encapsulating Bt-DPPC vesicles appeared to show three different populations compared to cells with no vesicles present, which had a tight population (Fig. 5a–c). In addition to cell-free Bt-DPPC vesicles, density analysis showed the 3T3 fibroblasts now comprised two size-distinct cell-containing populations (outlined in Fig. 5b, c). The increases in the size and granularity profile of 3T3 fibroblasts support the suggestion that Bt-DPPC vesicles had bound at the cell surface (Fig. 5d, e). The lower granularity population was slightly smaller in size and less fluorescent, suggesting the differences between the two cell-containing populations could be ascribed to the number of Bt-DPPC vesicles attached to the cells, with larger vesicle-cell aggregates appearing at higher size and granularity. Furthermore only 3T3 fibroblasts cultured with FITC-dextran containing Bt-DPPC vesicles showed green fluorescence (Fig. 5f), confirming the adhesive link between intact vesicles and the cells. After flow cytometric analysis had been carried out, the samples were collected and imaged to ensure the vesicle structures remained intact [19]. Microscopy on the samples after flow cytometric analysis showed large variation in the number of FITC-dextran containing Bt-DPPC vesicles attached to cells, with some cells bound to large numbers of vesicles and others attached to very few vesicles.

4 Conclusions

We have demonstrated that Bt-DPPC vesicles can retain their contents in vitro under cell culture conditions and that cell surface interactions with the biotin coating can target these vesicles to cells. These interactions only occurred with the cell membranes, and were not observed between the Bt-DPPC vesicles and extracellular matrix or tissue culture plastic. These interactions between Bt-DPPC vesicles and the fibroblasts required the presence of the biotin coating around the vesicle membrane, suggesting biotin lipids can mediate vesicle attachment to cell surfaces. A number of biotin-recognizing receptors are known to be present in cell membranes, including fibroblasts, and several are overexpressed in cancerous cells [20]. Pleasingly the Bt-DPPC vesicles adhere to the fibroblasts intact, without fusion or

endocytosis, which has important implications for remotely triggered drug delivery. However for this interaction to be maximized within a hydrogel cell culture scaffold, the cells and vesicles must be co-incubated for several hours before the mixture is gelled into a robust biomaterial. This stands in contrast to previous studies of *N*-(biotinyl)-dioleoyl phosphatidylethanolamine vesicles with mesenchymal stem cells that had indicated membrane fusion was the primary outcome, but this may have been due to high vesicle fluidity and the fusogenic nature of unsaturated dioleoyl phosphatidylethanolamine lipids used in the vesicles [11, 12]. The cell-vesicle interactions demonstrated here maybe useful for further applications of vesicles in vitro and in vivo for targeted drug release and directed tissue culture. In particular we hope to use the results of these studies to improve the efficiency of remotely triggered drug delivery in our magnetically responsive alginate biomaterials [6]. Through using biotinylated magnetic nanoparticle/vesicle assemblies and incubating these assemblies with cells prior to curing of the alginate gel, we should be able to bring the cells and vesicles into proximity, increasing the local concentration of magnetically released drug around to the cultured cells. Further studies in this area are ongoing.

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