Bead-Based Cellular Analysis, Sorting and Multiplexing

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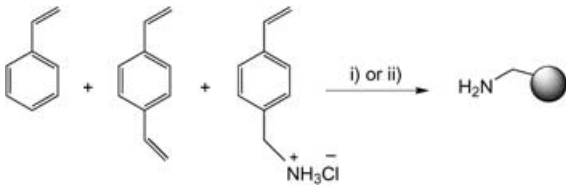
Cellular entry is important for the delivery of potential drugs, small-molecule modulators and sensors. Recently attention has focused on the development of a diverse range of specific delivery systems in order to enhance transport and uptake at the cellular level. Contemporary examples of delivery vectors include peptides, such as those based on fragments derived from the Tat protein, peptidomimetic carriers, such as those based on polymers and dendrimers, and various physical methods, such as micro-injection.[1–7]

Polymer-mediated delivery offers many advantages; for instance, polymeric materials can allow the controlled delivery and release of their cargo at controllable local concentrations, and there are many examples of drugs and sensors being encapsulated within polymer microparticles. However, their introduction into cells is often complex.[7] One type of polymeric material that has been shown to be engulfed and actively transported throughout cells is latex beads; however, surprisingly little work has been carried out on exploiting this phenomenon.[8] One reason for this is that commercially available materials are often unfunctionalized chemically or, if they are functionalized, this is only achievable under mild aqueous conditions, whereas modification for the attachment of a range of more interesting chemical probes requires compatibility with organic solvents.

The aim of this study was the generation of robust biocompatible polymeric particles of defined sizes that could be employed in solid-phase multistep syntheses yet be taken up rapidly by cells and exploited in a variety of applications. Here the application of a range of functionalized, cross-linked, multilabelled polymer microspheres as a mode of traversing the membrane of living cells is described. It allows general cellular labelling, sorting and the potential monitoring of dynamic intracellular processes.

Uniform, monodisperse amino-functionalized microspheres (0.2 and 0.5 μm) with 2% divinylbenzene (DVB) cross-linking were prepared by an emulsifier-free emulsion polymerization, while 2.0 μm microspheres were synthesized by dispersion polymerization (Scheme 1).[9,10] By selecting the relevant polymerization conditions, it was possible to generate functionalized microspheres of any desired size and loading (0.02–0.20 mmol g^{-1}). As expected, the cross-linking component was found to be vital to enhance the robust nature of the microspheres during routine chemical manipulations.



Scheme 1. Preparation of amino-functionalized polystyrene microspheres. i) Emulsifier-free emulsion polymerization:[9] styrene, DVB, *p*-vinylbenzylamine hydrochloride (VBAH), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AIBN·2 HCl; V5O initiator), water, $MgSO_4$, 80 °C, 350 rpm, 2–5 h; ii) Dispersion polymerization:[10] styrene, DVB, VBAH, AIBN, ethanol, poly(vinylpyrrolidone), 65 °C, 250 rpm, 12 h.

The microspheres were labelled with fluorescein, rhodamine, Texas Red or dansyl dyes as necessary (synthesis details are given in the Supporting Information), and a number of cell studies were conducted. Mouse melanoma (B16F10), HeLa, human embryonic kidney (HEK-293T) and neuronal ND7 cells were treated in triplicate with the polymeric microspheres (0.2–2.0 μm) at a range of concentrations (0.2–0.8 mg mL^{-1}) over four different time periods (3, 6, 12 and 24 h). Following incubation, the excess microspheres were removed, and analysis by flow cytometry and fluorescence microscopy showed that cellular uptake of the microspheres was effective in all cases. All sizes of bead could be delivered into the cells, though with varying degrees of success depending on the cell type investigated. Taking B16F10 cells as a representative example, Table 1 shows the rate of uptake of fluorescein-labelled microspheres, while Figure 1 depicts the cellular incorporation of 0.2 μm fluorescein-labelled microspheres after 12 h and 2.0 μm dansyl-, rhodamine- and fluorescein-labelled microspheres after 3 h, as analyzed by fluorescence microscopy.

Table 1. Cellular uptake of fluorescently labelled microspheres into B16F10 cells over time (0.4 mg mL^{-1}) at 37 °C and under 5% CO_2.

	% Cellular incorporation			
Bead size [μm]	3 h	6 h	12 h	24 h
0.2	24	62	93	95
0.5	27	58	81	85
2.0	6	14	38	51

Cellular uptake was also found to be bead-size, concentration and time dependent (Table 1 and Figure 2). As a general trend, there was a greater percentage uptake of beads in cells incubated with either the 0.2 or 0.5 μm microspheres than in those incubated with the larger 2.0 μm beads (see Table 1).

In terms of cell type, uptake was found to be relatively easy with the HEK-293T and ND7 cell lines; the B16F10 cells were intermediary, whilst uptake with the HeLa cells was found to be the most difficult (see Supporting Information). Investigation of temperature variation on cellular uptake revealed that no

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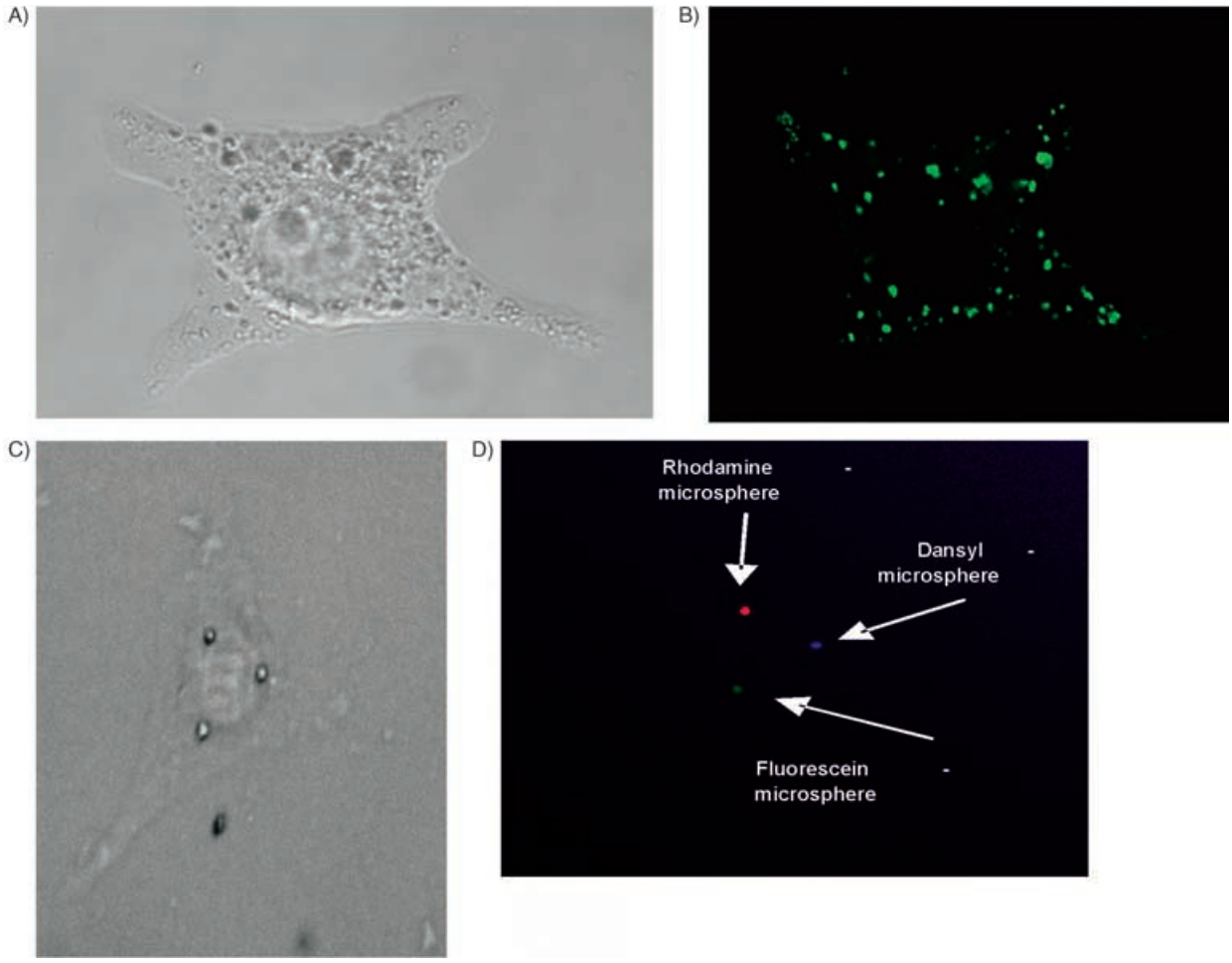


Figure 1. Microscopy images for B16F10 cells after incubation at 37 °C under 5% CO_2 for 12 h with 0.2 μm fluorescein microspheres: A) normal light; B) fluorescein filter. Controlled bead cellular uptake (3 h) with 2.0 μm dansyl, fluorescein and rhodamine microspheres: C) normal light; D) superimposed image of dansyl, fluorescein and rhodamine filters (concentration of microspheres is 0.4 mg mL^{-1}).

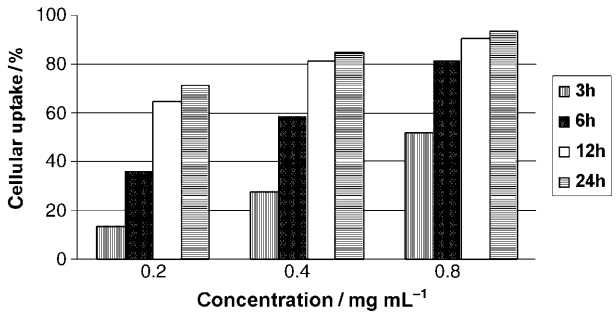
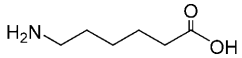
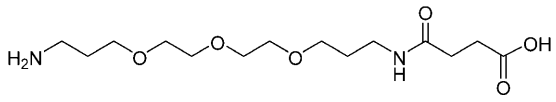


Figure 2. Comparison of cellular uptake (% of labelled cells from total population) of 0.2 μm fluorescently labelled Ahx microspheres on B16F10 cells at different concentrations and incubation times (37 °C and 5% CO_2 atmosphere).

uptake was detected at 4 °C and provides evidence that the microspheres do not simply bind to the cell surface, a fact additionally supported by confocal microscopy studies, as the images explicitly showed internalization of the beads.

Modification of the 6-aminohexanoic acid (Ahx) spacer unit to a more biocompatible hydrophilic poly(ethylene glycol)-based spacer improved cellular uptake (see Table 2), while incubation of cells with microspheres in complete or serum-free medium indicated that uptake was not significantly affected by medium composition.[11] This opens up possibilities for these beads to be used with a variety of cell types and culture conditions. Finally, the cell viability was assayed at any of the concentrations and times tested, as verified by an methylthi-

Table 2. Results of cellular uptake (% of labelled cells from total population) of 0.2 μm Ahx and PEG-based microspheres at varying concentrations on B16F10 cells after 12 h (at 37 °C and 5% CO_2 atmosphere).

Spacer	% Cellular incorporation 0.1 mg mL^{-1}	0.2 mg mL^{-1}	0.3 mg mL^{-1}
 Ahx	49	64	90
 PEG-based	95	97	98

azolyldiphenyl tetrazolium bromide toxicity and a trypan blue assay.[12, 13]

To assess the potential use of these microspheres as biomarkers, a time-course study followed by confocal microscopy revealed that, 72 h after uptake, all cell types retained the fluorescently labelled microspheres (of all sizes) without loss of the microspheres. This makes these excellent candidates as cellular markers and opens up possibilities in the area of cellular multiplexing. To demonstrate the potential of these labelled microspheres in a cellular multiplexing assay, HeLa, B16F10 and HEK-293T cell lines were individually encoded with unique fluorescent identifiers (single rhodamine-, Texas Red- or dansyl-conjugated beads per cell), and then the cells were combined. After incubation of the mixed population of cells, flow cytometric analysis distinctly revealed all three labelled mixed cell populations, which could be sorted at will. These mixed cells were then used in a multiplexing transfection study with a plasmid (pEFGP-N1) that encodes green fluorescent protein (GFP). Transfection efficiency was evaluated by subjecting the three mixed cellular populations (HeLa, B16F10 and HEK-293T) in a single well to a standard transfection protocol (Superfect as the transfecting agent complexed with pEGFP-N1). After overnight incubation, fluorescence-activated cell sorting (FACS) analysis showed the intensity of GFP expression along with the specific bead-based tag that was the unique identifier for the specific cell line. It was observed that, after 24 h, transfection of HEK-293T cells was much greater than for B16F10 and HeLa cells (Figure 3). Importantly, the results obtained on the mixed "tagged" population were identical to the transfection efficiencies obtained in the absence of the beads. These result open up the possibility of simultaneous biological analyses of a number of cell lines specifically encoded with either single or combinations of fluorescent microspheres.

To explore the possibility of using these microspheres as an internal cellular-delivery system, switchable within a cellular environment, a simple nonfluorescent-to-fluorescent model

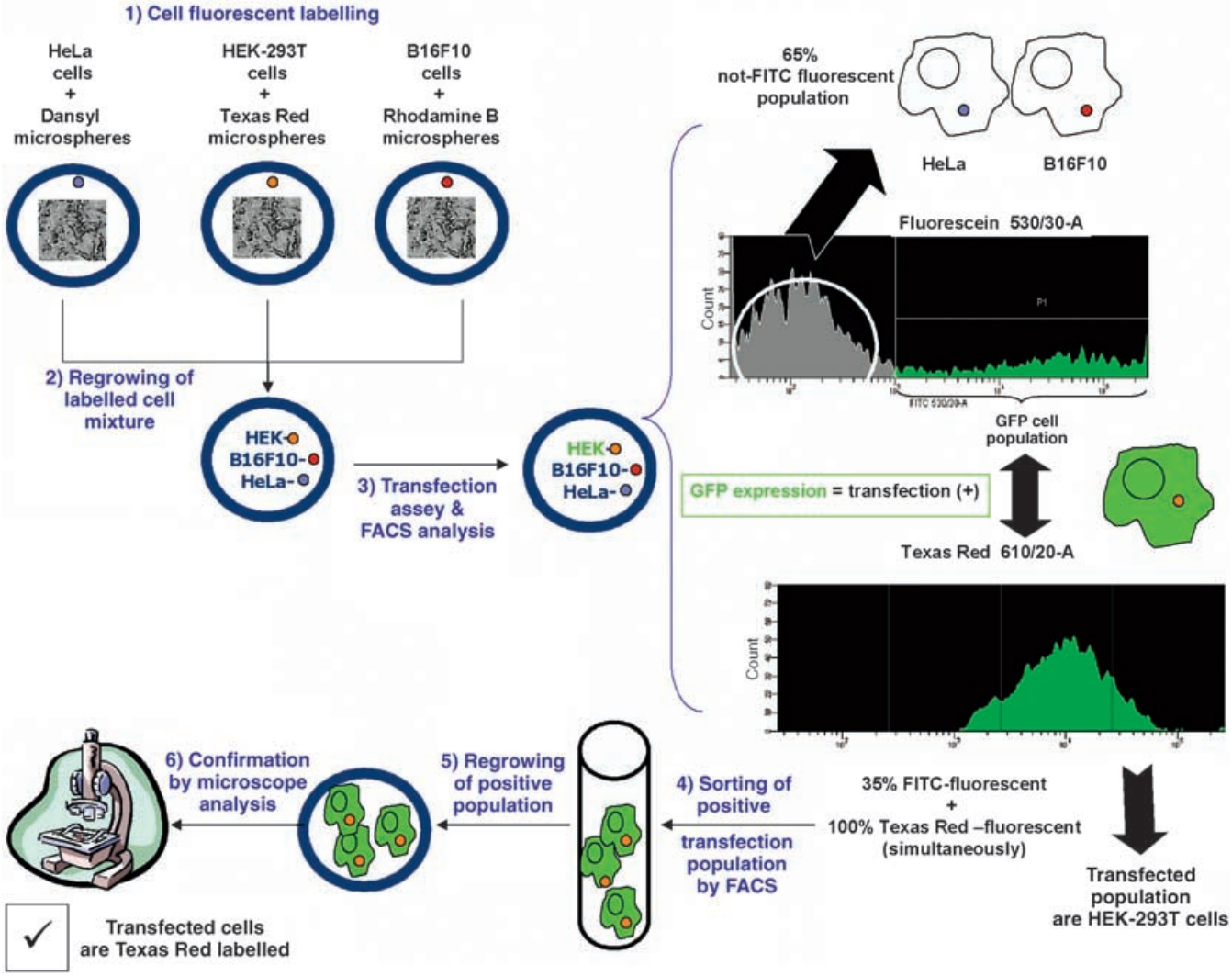
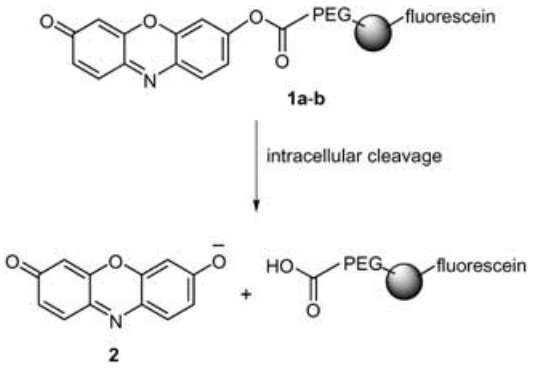


Figure 3. FACS analysis of the level of GFP expression in a mixed cell population. B16F10, HeLa and HEK-293T cells were labelled with rhodamine, dansyl and Texas Red microspheres, respectively. Decoding was achieved by analysis of the individual histograms by using the relevant filters: 530/30 nm filter for GFP expression and 450/50 nm, 576/26 nm and 610/20 nm filters for HeLa cells, B16F10 and HEK-293T cells were encoded with dansyl, rhodamine and Texas Red microspheres, respectively.

was chosen.[14] Microspheres (0.5 and 2 μm, **1a** and **1b**, respectively) were dual labelled with fluorescein and resorufin through an amide and an ester linkage, respectively, as shown in Scheme 2 (details in Supporting Information). Free resorufin



Scheme 2. Intracellular cleavage of ester-bound resorufin leading to the release of fluorogenic resorufin (**2**) from fluorescein-labelled PEG microspheres (0.5 μm **1a** and 2.0 μm **1b**).

is highly fluorescent ($\lambda_{ex=}$585 nm) but cell impermeable. On the other hand, the ester form of resorufin, such as in **1a**, **b**, is not fluorescent. Thus, on cellular entry, intracellular fluorescence would be due to the liberation of free resorufin (**2**) by endogenous esterases and/or background hydrolysis. After 6 h incubation, the resorufin fluorescence increased 11-fold compared with the control parent resorufin (Figure 4). This establishes these materials as effective delivery devices of exogenous cellular cargo. In addition, the ability to doubly label the microspheres allows trafficking of the loaded beads and release to be continuously monitored within the cells.

In order for this strategy to be successful as a means of cell delivery, the microparticles must not interfere with the normal functioning of the cell, such as metabolism and DNA translation. The influence of the microspheres on cellular metabolism following cellular uptake was examined by immunostaining to study the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme important in the essential metabolism process of glycolysis. This enzyme is also involved in a great number of intracellular processes.[15] Thus, various sizes of fluorescently labelled beads were incubated with HeLa, HEK-293T and B16F10 cell lines for 24 h, following which the cells were fixed, and GAPDH was analysed by immunostaining (for details see the Supporting Information). Control experiments with unlabelled cells demonstrated that the intensity of fluorescence was the same in the presence or absence of microspheres; this suggests that, in this regard at least, the metabolic activity of the cells was not affected by internalization of the microspheres. Additionally, the above transfection studies showed that the uptake of the microspheres did not inhibit GFP expression; this implies that the DNA machinery of the cells was not affected.

Commercially available microsphere particles lack the physical properties necessary for multistep functionalization with small organic molecules. We have described the multifunction-

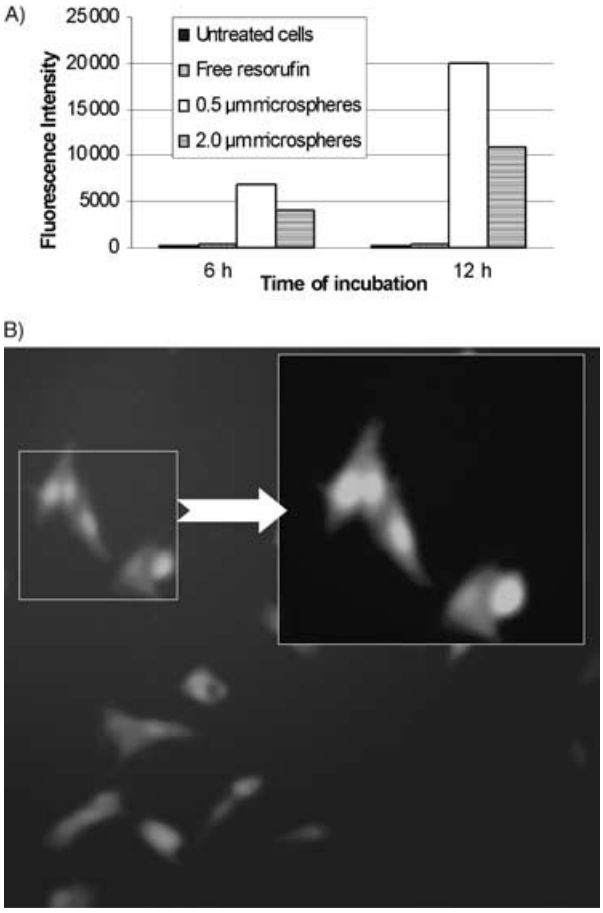


Figure 4. A) FACS results of B16F10 cells following 6 h or 12 h incubation (37 °C and 5% CO_2 atmosphere) with 0.5 μm (**1a**) and 2.0 μm (**1b**) PEG microspheres loaded with resorufin (0.4 mg mL^{-1}), untreated cells and cells incubated with free resorufin. B) Fluorescence images of B16F10 cells incubated with 0.5 μm resorufin-loaded PEG microspheres at 37 °C and under 5% CO_2 for 6 h.

alization of cross-linked polymeric microsphere particles and their analysis as potential cell delivery vehicles. Varying sizes of amino-functionalized cross-linked polystyrene microspheres were generated, fluorescently labelled and used as cellular tags and probes. They allowed the effective delivery of foreign materials into cells, without the need for delicate micro-injection procedures, and were found not to disrupt normal cell physiology. Importantly cellular uptake was shown to be reproducible, nontoxic and general, the microbeads being able to successfully penetrate different cell types independent of the media used, a factor of utmost importance when compared to many traditional cell-penetrating agents. Their ability to internalize was dependant on bead size, concentration and temperature, as well as time of incubation and cell type. Thus, by selecting the appropriate beads/conditions, it becomes possible to fine-tune the cellular uptake of these beads and analyse the beads within the cells—a step towards bead-based internal cellular sensors and allowing the controlled release of bead-based compounds for direct cellular delivery at the single-bead level.

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