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Microsphere-Mediated Protein Delivery into Cells

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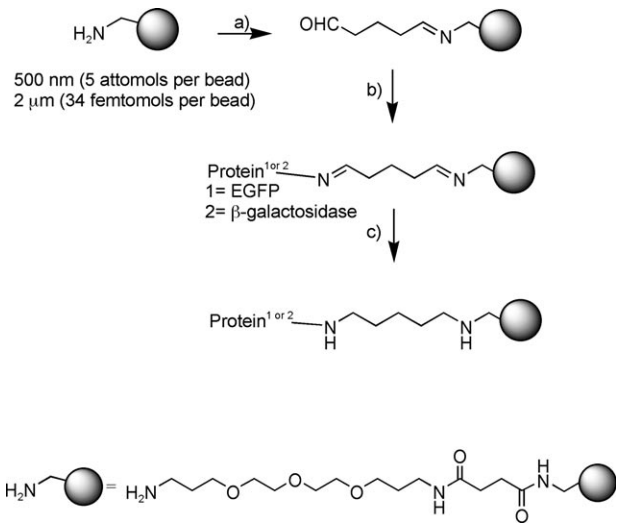
The delivery of functional proteins into cells, although a challenging area of research, offers immense rewards and benefits and has led to the development of a range of delivery systems. Recent examples include conjugation of proteins to cell penetrating peptides (CPP) such as those derived from HIV-1 TAT, "profection" by cationic lipids and the use of several "nanodevices" such as nanotubes or silica nanoparticles.[1–8]

Recently we reported that amino-functionalized, cross-linked polystyrene microspheres of highly defined sizes (200 nm–2 μm) are efficient cellular delivery devices that can enter a broad range of cell types including adherent, suspension and primary cells.[9–10] These beads have been used for cellular "encoding" as well as for intracellular calcium sensing and pH monitoring; in these cases attachment to the bead avoids the dilution and cellular degradation observed with other, more conventional sensors.[11–12] Recently we have also described the use of streptavidin-loaded microspheres for the delivery of various biotinylated molecules, such as DNA, into cells. We have successfully silenced green fluorescent protein (GFP), which is expressed in human ovarian cancer (HeLa) cells, by using siRNA linked to microspheres through cleavable and noncleavable linkers.[13–14] These microspheres are inherently attractive as a carrier/delivery system due to their lack of toxicity and highly controllable cellular loading. Additionally, they offer the opportunity to sort cells at the level of one-bead–one-cell; this allows for defined cellular dosing based on the number of beads per cell. Furthermore, unlike other available delivery methods, these microspheres are released into the cytoplasm without the need for treatment with an endosome-disrupting agent. While the mechanism of uptake is not yet established, we have recently found that these microspheres are unlikely to enter cells through an endocytic pathway. The application of chemical ihibitors of endocytosis and extensive colocalisation studies by microscopy and gene-expression profiling all argue against an endocytic mechanism. Instead, we have proposed an endocytosis-independent uptake mechanism that results in the uncompartmentalised, cytoplasmic localisation of microspheres and their cargo.[15] Microspheres also do not require serum-free conditions and can hence be used for long incubation periods. All these properties make microspheres excellent candidates for the cellular delivery of proteins.

Herein, we demonstrate the ability of protein-loaded microspheres to deliver functional proteins into the cellular environment.

The initial model used to study microsphere-mediated transport of proteins was the extensively studied green fluorescent protein (GFP), which due to its fluorescent properties is an ideal tool to perform an initial quantitative study of cellular uptake of protein-loaded microspheres.[16] Enhanced green fluorescent protein (EGFP) was thus coupled to 500 nm and 2 μm amino-functionalized microspheres derivatised with a polyethylene glycol (PEG) spacer through reaction with glutaraldehyde followed by reduction (Scheme 1).[17–19] The efficiency of the coupling was determined by spectrofluorimetric analysis, while the fluorescence properties of EGFP-loaded microspheres were evaluated by flow cytometry and microscopy analysis; these studies indicated that the microspheres had become highly fluorescent (see the Supporting Information).



Scheme 1. Synthesis of protein-loaded microspheres. a) glutaraldehyde, PBS, 15 h; b) protein (1 = EGFP and 2 = β-galactosidase), PBS, 15 h; c) $NaCNBH_3$, phosphate buffered saline, (pH 7.4):EtOH (3:1), 2 h.

Bead cellular uptake was evaluated by the "beadfection" of several cell lines (B16F10, HeLa, HEK293T and ND7; in triplicate) with both sizes of microspheres (500 nm and 2.0 μm) at a range of concentrations (included untreated cells, cells incu-

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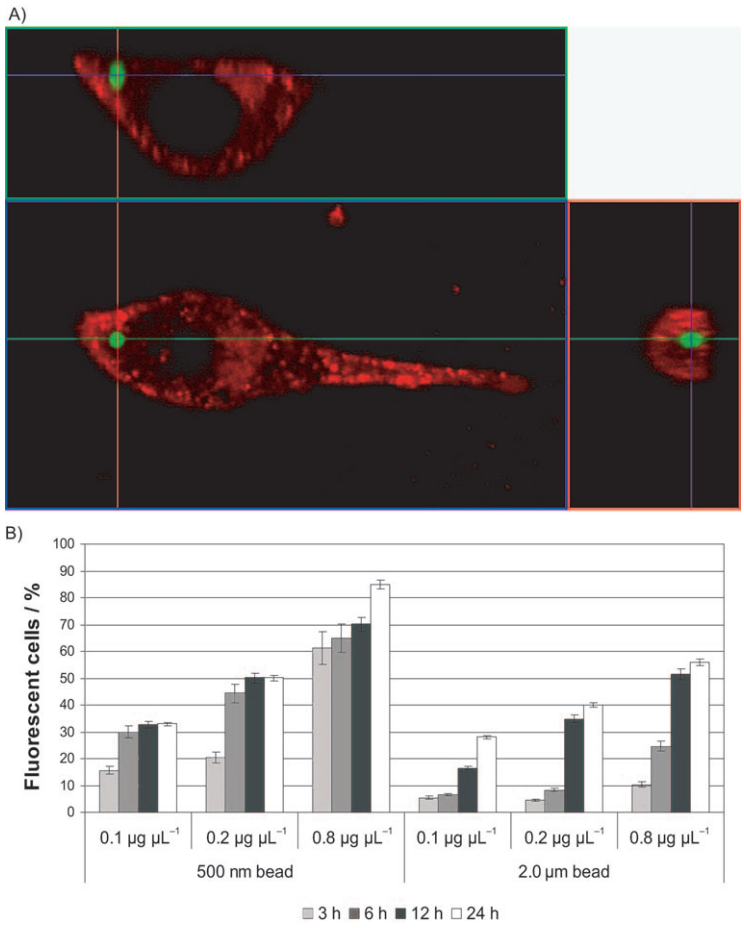

Figure 1. A) Confocal microscopy image of a single mouse melanoma cell (B16F10) loaded with a 2.0 μm EGFP-loaded microsphere after 24 h of incubation. The cell membrane was stained with a red fluorescent dye (PKH26). B) Flow cytometry analysis of B16F10 cellular uptake of 500 nm and 2 μm EGFP-loaded microspheres at different concentrations (μg μL^{-1}) after 3, 6, 12 and 24 h.

bated with unlabelled microspheres and free GFP in solution). Analysis by flow cytometry and fluorescence microscopy showed that cellular uptake of the microspheres was highly effective. Figure 1A shows a confocal image of B16F10 cells after incubation with 2.0 μm GFP-loaded microspheres (after fluorescence-assisted cell sorting (FACS) and overnight regrowth). As a general trend, it was found that 500 nm microspheres loaded with the protein could be delivered into cells with a higher efficiency than 2 μm beads. Uptake was improved by increasing the concentration and the time of incubation (see Figure 1B). As expected, cellular uptake was lower compared to microspheres loaded with smaller cargos such as a sensor (Indo-1), while the degree of cellular uptake was influenced by cell type (see the Supporting Information for flow cytometry analysis).[11–12]

To further validate beadification as an effective tool for protein delivery, we tested the ability of microspheres to deliver a functional protein (β-galactosidase) into cells. This is a robust model system due to the number of well-known and defined protocols and techniques to analyse its activity in mammalian cells.[4,6,20] Following the coupling of β-galactosidase to PEG-microspheres (see Scheme 1), B16F10 and HeLa cells were treated (in triplicate) with 500 nm and 2.0 μm enzyme-loaded microspheres at a range of concentrations. Following incubation, intracellular enzymatic activity was analysed by flow cytometry following treatment with fluorescein di-β-D-galactopyranoside (FDG), a specific β-galactosidase fluorogenic substrate that releases fluorescein upon hydrolysis and allows an accurate determination of enzymatic activity. The fluorescence emission from this substrate is directly proportional to the amount of enzyme present.[21] Based on both fluorescence microscopy and flow cytometry, we found that cellular uptake of these β-galactosidase-loaded microspheres was effective and that the enzyme within the cells was active (Figure 2, over). None of the controls showed any significant fluorescence intensity (see Figure 2B). Figure 2C shows an image of cells loaded with β-galactosidase-microspheres and treat-

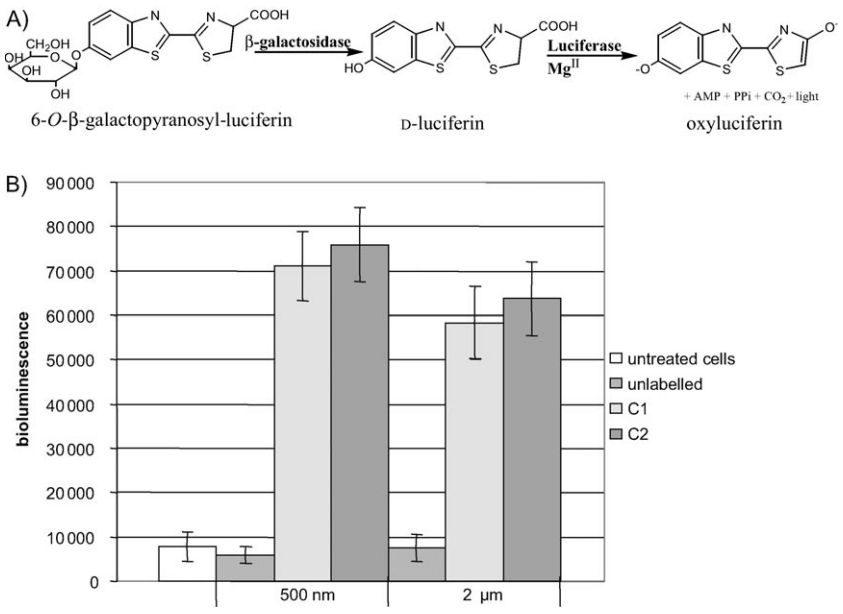

Figure 3. A) Principle of bioluminiscence detection of β-galactosidase activity with a coupled assay consisting of the luciferin-galactoside substrate (6-*O*-β-galactopyranosyl-luciferin), and luciferase (Beta-Glo, Promega). B) β-galactosidase activity by using bioluminescence analysis of HeLa cells incubated with β-galactosidase-loaded microspheres after treatment with 6-*O*-β-galactopyranosyl-luciferin (C1 and C2 are 0.1 and 0.2 μg μL^{-1}, respectively).

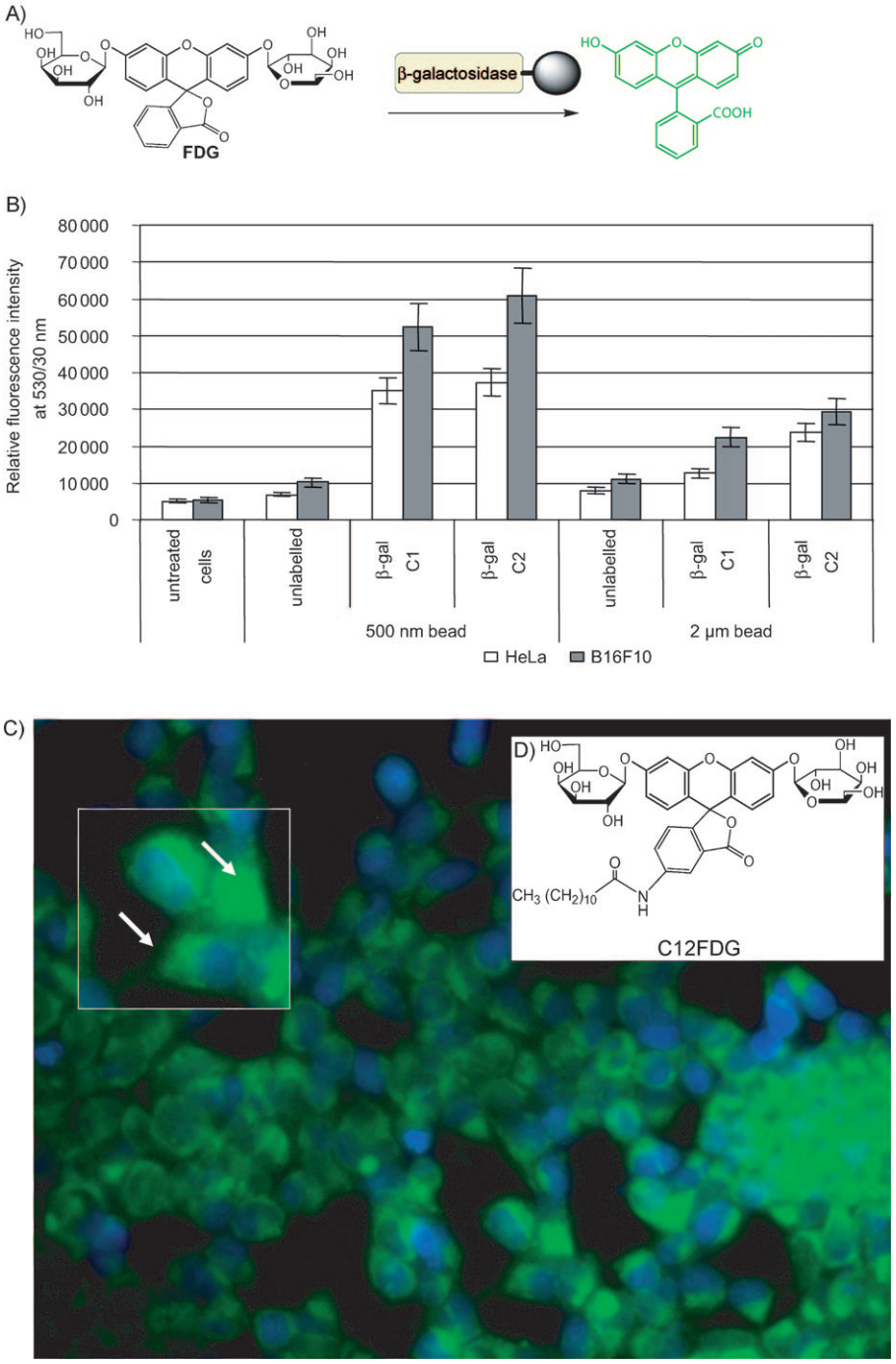

Figure 2. A) Principle of fluorescent detection of β-galactosidase activity using FDG as a substrate. B) Flow cytometry analysis of cellular uptake of 500 nm and 2 μm β-galactosidase-loaded microspheres (C1 and C2 are 0.1 and 0.2 μg μL^{-1}, respectively) by HeLa and B16F10 cells after treatment with the fluorogenic substrate FDG. C) Fluorescence image of HeLa cells incubated with 500 nm β-galactosidase-loaded microspheres after treatment with the fluorogenic substrate C12FDG (white arrows indicate the green fluorescent related to cytoplasmatic release of fluorescein due to β-galactosidase activity). The nucleus was labelled with Hoechst 33342 (blue). D) Structure of the fluorogenic substrate C12FDG.

ed with a membrane-permeable derivative of FDG (C12FDG) that, after enzymatic hydrolysis, is retained within the cell.[22]

The enzymatic activity of β-galactosidase-loaded microspheres was also accessed using a coupled assay consisting of the luciferin-galactoside substrate (6-*O*-β-galactopyranosyl-luciferin), and luciferase (Beta-Glo, Promega).[23] The substrate was selectively cleaved by β-galactosidase to release luciferin, which subsequently serves as a substrate for luciferase (Figure 3 A). The luminescence results reinforced those obtained using the fluorescent FDG substrate (see Figure 3 B). Finally, an evaluation of cell viability for B16F10 and HeLa cells treated with 500 nm and 2.0 μm β-galactosidase-loaded microspheres was carried out by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.[24] These polymeric particles were found to be nontoxic at all concentrations tested in this study (see the Supporting Information).

In this paper we have shown that beadfection is an effective method for the delivery of functional proteins to a variety of cells types. Proteins were coupled to microspheres following a simple and reproducible procedure without altering the activity of the protein. The microspheres were efficiently taken up by cells, and even after only a few hours incubation, cellular uptake was already established and cells could be sorted by FACS, regrown and analysed by confocal microscopy. β-Galactosidase bound to the microspheres and delivered into cells maintained its activity as analysed by flow cytometry, fluorescence microscopy and bioluminescence. These results indicate that microsphere-mediated protein delivery can be performed in an efficient and reproducible manner and reinforces the use of these constructs as protein delivery system in vitro and in vivo. Future work will be focused on the development of strategies for the targeted delivery of proteins, exploiting the multifunctionality of these microspheres. Additionally, synthetic strategies to release the protein from the polymer particles for specific applications, such as nuclear localization, are under investigation.

Experimental Section

General protocol for protein loading of microspheres: Aminomethyl functionalized microspheres (500 nm and 2.0 μm) were washed with PBS buffer (pH 7.4), NaOH (500 mM) and finally PBS (pH 7.4). After washing and centrifugation, microspheres were resuspended in a glutaraldehyde solution (25% (*w/v*)) in PBS buffer, and the mixture was shaken for 15 h at room temperature. After this time the microspheres were washed with PBS (pH 7.4), or a specific protein buffer, before addition of protein (5 $\mu g\,mL^{-1}$ in PBS, pH 7.4). The mixture was shaken for 15 h at room temperature prior to washing with PBS (pH 7.4) and treatment with a sodium cyanoborohydride solution (20 mM) in PBS/EtOH (3:1) for 2 h. Finally the protein-loaded microspheres were washed with PBS and treated with a quenching solution (40 mM ethanolamine with 1% (*w/v*) BSA in PBS). The streptavidin-loaded microspheres were washed and stored in PBS (pH 7.4) or a specific protein buffer.

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Keywords: carrier system · fluorescent probes · intracellular protein delivery · microspheres · nanoparticles

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