

Polymeric Microspheres as Protein Transduction Reagents*[§]

David Nagel‡, Jonathan M. Behrendt§¶, Gwen F. Chimonides§, Elizabeth E. Torr‡, Andrew Devitt‡||, Andrew J. Sutherland§, and Anna V. Hine‡**

Discovering the function of an unknown protein, particularly one with neither structural nor functional correlates, is a daunting task. Interaction analyses determine binding partners, whereas DNA transfection, either transient or stable, leads to intracellular expression, though not necessarily at physiologically relevant levels. In theory, direct intracellular protein delivery (protein transduction) provides a conceptually simpler alternative, but in practice the approach is problematic. Domains such as HIV TAT protein are valuable, but their effectiveness is protein specific. Similarly, the delivery of intact proteins via endocytic pathways (e.g. using liposomes) is problematic for functional analysis because of the potential for protein degradation in the endosomes/lysosomes. Consequently, recent reports that microspheres can deliver bio-cargoes into cells via a non-endocytic, energy-independent pathway offer an exciting and promising alternative for *in vitro* delivery of functional protein. In order for such promise to be fully exploited, microspheres are required that (i) are stably linked to proteins, (ii) can deliver those proteins with good efficiency, (iii) release functional protein once inside the cells, and (iv) permit concomitant tracking. Herein, we report the application of microspheres to successfully address all of these criteria simultaneously, for the first time. After cellular uptake, protein release was autocatalyzed by the reducing cytoplasmic environment. Outside of cells, the covalent microsphere–protein linkage was stable for ≥ 90 h at 37 °C. Using conservative methods of estimation, 74.3% \pm 5.6% of cells were shown to take up these microspheres after 24 h of incubation, with the whole process of delivery and intracellular protein release occurring within 36 h. Intended for *in vitro* functional protein research, this approach will enable study of the consequences of protein delivery at physi-

ologically relevant levels, without recourse to nucleic acids, and offers a useful alternative to commercial protein transfection reagents such as Chariot™. We also provide clear immunostaining evidence to resolve residual controversy surrounding FACS-based assessment of microsphere uptake. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.O113.034900, 1543–1551, 2014.

Many proteomic techniques can be used to build a picture of a protein with unknown function, but eventually the individual protein's activity must be studied. Traditional transfection of encoding DNA permits intracellular expression, but often at uncontrolled, nonphysiological levels. Moreover, DNA transfection can neither deliver protein–inhibitor complexes nor readily deliver multiple proteins in a single experiment and thus exploit knowledge from proteomic protein–protein interaction analyses. In contrast, a truly generic protein transduction reagent could theoretically address all possibilities. We believe that polymeric microspheres could fulfill this role, and we have recently synthesized and characterized dual-functionalized, bio-compatible microspheres that permit intracellular tracking (1). Herein, we now report the development of those microspheres into a protein transduction reagent that can carry protein stably, deliver it efficiently to cells, release the protein in the cytoplasm, and concurrently permit fluorescent imaging of transduced cells.

Phagocytosis of microspheres was first observed over 30 years ago (2). Perhaps more unexpectedly, uptake of polystyrene microspheres has recently been reported in many other, nonphagocytic cell types, some of which are traditionally considered to be resistant to DNA transfection and/or protein transduction. For example, microspheres are taken up readily by primary immune cells (3), embryonic stem cells (4), human neural stem cells (5), differentiating mouse neural stem cells (5), and several nonphagocytic cell lines (3, 6, 7). In all instances, the reported efficiency of cellular uptake is high, with “beadfection” of up to 90% of cells being typical (4, 5, 8). No additional reagents aside from the microspheres themselves are required in order to promote cellular uptake, and critically, no toxicity has been observed in any of the cell types beadfected, including HEK293T and L929 cells 2 days after beadfection (8), E14g2a embryonic stem cells 3 days after beadfection (4), and mouse and human neural stem cells 30

From the ‡School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, UK; §School of Engineering and Applied Science, Aston University, Aston Triangle, Birmingham, B4 7ET, UK; ¶Aston Research Centre for Healthy Aging, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

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days after beadfection (5). In the latter case, the microspheres did not have any deleterious effect on the differentiation of human neural stem cells 30 days after beadfection (5).

The mechanism of microsphere entry is also nontoxic, and compelling evidence has been published recently that polystyrene-based microspheres (from 0.2 μm to as large as 2 μm) enter cells via a non-endocytosis, energy-independent mechanism (8). Although unusual, such a mechanism is consistent with claims for the commercial reagent Chariot™ (9). Interestingly, a non-endocytic, energy-independent mechanism has also been reported for the entry of rhenium cluster/polymer hybrid particles into HeLa cells (10). Failure of the microspheres to be endocytosed, at least via a clathrin-dependent mechanism, is perhaps to be predicted, as their diameter considerably exceeds that of clathrin-coated vesicles (typically 100 nm). Bradley and co-workers (8) propose that the entry mechanism for polystyrene-based microspheres is one of passive diffusion in which the microsphere interacts with the membrane, anchors, and, after membrane reorganization, enters the cell, resulting in direct cytoplasmic localization.

For functional analysis following transduction, the avoidance of endocytosis or phagocytosis is particularly relevant, as endocytosed particles are destined for endosomes and then, normally, for the lysosomes. The lowered pH of the endosome and, more seriously, the acidic and hydrolytic environment of the lysosome risk disruption of the protein structure and/or function. In contrast, for vaccine delivery (where liposomes can be employed), such exposure is advantageous because protein breakdown forms an essential part of antigen presentation. The potential for protein breakdown in endosomes is also irrelevant for the delivery of protein/peptide drugs such as insulin (for which microencapsulation has proven effective for long-term controlled drug release (11, 12)), as these drugs typically function in the extracellular environment, often exerting their effects by binding to membrane-bound receptors. Thus, although vehicles such as liposomes and nanoparticles are employed both extensively and successfully as drug and vaccine delivery vectors *in vivo* (13–16), they are far from ideal for studying the biological effect of a delivered protein *in vitro*. Colloidal particles are also endocytosed (17), and therefore these delivery vehicles may present similar disadvantages.

Traditionally, protein transduction domains such as HIV TAT (18–20) or other cell-penetrating peptides (21–23) are used to deliver proteins to cells. Whereas positively charged peptides such as TAT are thought to enter the cells via macropinocytosis (reviewed in Ref. 24), a recent publication suggests that at least some cell-penetrating peptide/bio-cargo complexes (siRNA) are endocytosed (25). Here, although the cargoes avoid the lysosomes, acidification of the endosome is required for endosomal escape of the delivered cargo, and indeed, acidification appears to be a recurring requirement for endosomal escape of biomolecular cargoes using cell-pene-

trating peptides (reviewed in Ref. 24). Consequently, cell-penetrating peptides are unlikely to become generic tools for functional protein delivery.

In contrast, the recent demonstrations that polystyrene microspheres can carry a variety of molecular cargoes with them into the cytoplasm (4, 5, 7, 26, 27) make them particularly exciting as potential vectors for delivering functional proteins and/or protein complexes. β -Galactosidase retains its activity when delivered via this route (7), confirming the potential of microspheres to act as generic protein-delivery vehicles. However, delivered proteins have to date remained tethered to the microspheres, and thus existing studies are limited to proteins that are active in the cytoplasm and, critically, retain their activity when immobilized on polystyrene. For the broad-based study of protein function, the subsequent release of the delivered protein within the cell is desirable.

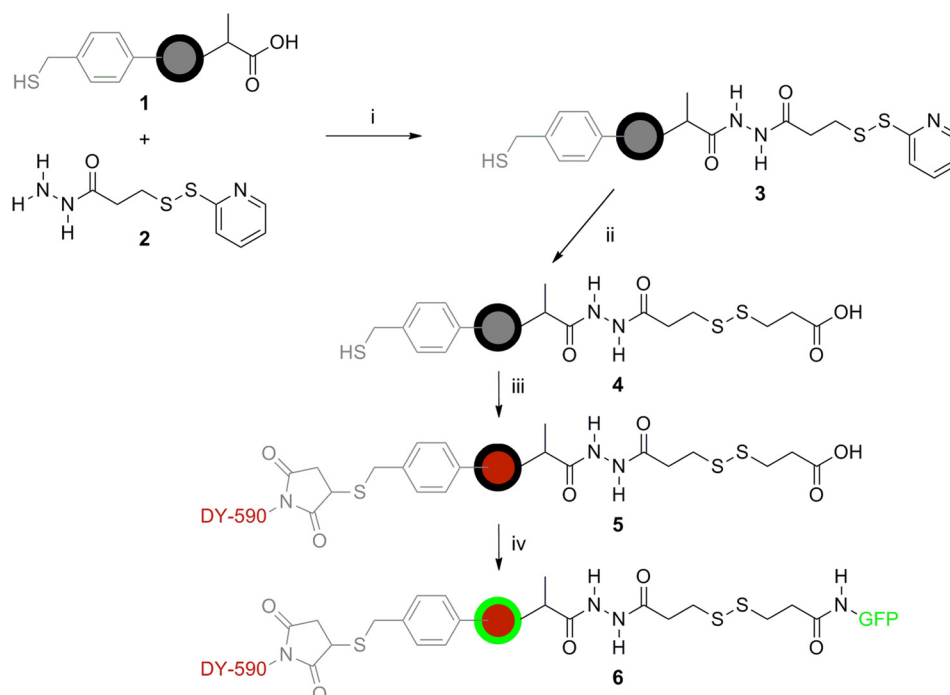
An ideal technology would deliver any protein to any cell type and release that protein in the cell, where it could undertake its normal activity. Here we report the first example of such a microsphere-based approach. Protein is delivered on microspheres and then released in the cell by the reducing cytoplasmic environment. This release is mediated by a linker that attaches the protein stably and covalently to the microspheres *in vitro* but intracellularly is cleaved over a period of hours. It has already been shown that microspheres are taken up with high efficiency by a range of cell types and can carry a variety of cargoes. Because the chemistry of the linker described herein is amenable to linkage with any molecule containing a free amine moiety, the technology provides a new generic platform for *in vitro*, cell-based delivery of individual proteins, protein complexes, protein mixtures, or other amino-functionalized molecules.

EXPERIMENTAL PROCEDURES

All chemical reagents were purchased from Sigma-Aldrich (UK) unless otherwise stated.

Derivatization of Microspheres with a Cell-cleavable Linker—Carboxyl shelled microspheres **1** (Scheme 1, 30 mg) were synthesized as described previously (1) and then isolated via centrifugation (2 min, 12,000g), resuspended in 1 ml of DMF¹ via ultrasonication (2 min), and added to a reaction mix containing 3-(2-pyridyl)dithio)propionyl hydrazide **2** (Pierce, Loughborough, UK) (9.2 mg, 40.1 μmol) and *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoro-borate (TBTU) (12.9 mg, 40.1 μmol) in 1 ml of DMF, to which *N,N*-diisopropylethylamine (7.2 μl , 40.1 μmol) was then added. After washing (twice with 1 ml of DMF each time) the microspheres were resuspended in the reaction mixture and incubated for 1 h at room temperature with shaking. Microspheres were then washed with DMF (three times with 1 ml each time) and collected via centrifugation. The resulting pellet of thiol-reactive microspheres **3** was resuspended in 1 ml of DMF, and 3-mercaptopropionic acid (3.5 μl , 40.1 μmol) was added.

¹ The abbreviations used are: DMF, *N,N*-dimethylformamide; EDAC/EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GFP, green fluorescent protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoro-borate.



SCHEME 1. Derivatization of core-shell microspheres 1 with a cell-cleavable linker, subsequent internal labeling, and protein attachment. Reaction scheme showing the derivatization of core-shell microspheres 1. Reaction conditions: (i) TBTU, *N,N*-diisopropylethylamine, DMF; (ii) 3-mercaptopropionic acid, DMF; (iii) DY-590-maleimide, DMF; (iv) GFP, EDAC, MES, pH 6.0, NaOH.

The reaction was incubated at room temperature for 1 h with shaking. Microspheres 4 were isolated via centrifugation and subsequently washed with DMF (five times with 1 ml each time) using centrifugation and ultrasonication.

Coupling of DY-590 Maleimide-derivatized Dyes to Microspheres—Microspheres 4 (Scheme 1, 5.0 mg) were washed with DMF (twice with 1 ml each time), with centrifugation (5 min, 6000 rpm) and decantation of the supernatant after each wash, and then resuspended in DMF (1 ml). A solution of fluorescent dye (DY-590 maleimide, Dyomics, Jena, Germany) in DMF (10 μ l, 1.32 mM) was added to the suspension of microspheres, which was then shaken at room temperature for 2 h. The resultant internally labeled microspheres 5 were isolated via centrifugation (2 min, 6000 rpm) and decantation of the supernatant, purified with several wash/centrifugation cycles (DMF (five washes with 1 ml each) and water (two washes with 1 ml each)), and finally resuspended in water (1 ml).

Conjugation of GFP to Microspheres—GFPuv bearing an *N*-terminal His₆ tag was expressed in *Escherichia coli* Tuner DE3™ cells (Novagen, Nottingham, UK) and purified using nickel-nitrilotriacetic acid agarose (Novagen) before dialysis into 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (50 mM, pH 6.0). Fluorescent microspheres 5 (1 mg) were pelleted via centrifugation (2 min, 12,000g) and resuspended in 100 μ l of MES buffer (50 mM, pH 6.0). 200 μ l of MES buffer (50 mM, pH 6.0) containing GFPuv (500 μ g, 18.5 nmol) was added to the microspheres, and the reaction mixture incubated at room temperature with rolling for 15 min. EDAC (0.2 mg, 1.1×10^{-3} mmol) dissolved in 100 μ l of MES (50 mM, pH 6.0) was added to the reaction and the pH was adjusted to 6.5 by the addition of aqueous NaOH (0.5 M). After incubation for 2 h at room temperature with rolling, microspheres were recovered via centrifugation and washed with sodium phosphate buffer (100 mM, pH 7.2; five washes with 500 μ l each) prior to resuspension in sodium phosphate buffer (100 mM, pH 7.2) at a concentration of 5 mg/ml.

Beadfection—HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 1 \times nonessential amino acid supplement, and 4 mM L-glutamine. Cells were seeded as required and incubated for 24 h at 37 °C with 5% CO₂. The medium was then replaced with fresh medium containing conjugated microspheres (50 μ g/ml) as indicated, and the cells were incubated as required.

Epifluorescent Microscopy Imaging of Beadfected Cells—HeLa cells were seeded (6×10^4 cells/well) in four-well chamber slides (Nunc, Loughborough, UK), beadfected, and incubated as specified. Cells were imaged directly in medium using a Zeiss Axiovert 200 m fluorescence microscope ($\times 63$ objective) fitted with a Hamamatsu Orca charge-coupled device camera driven by Volocity 4.2.1 software (Improvision, Coventry, UK) and equipped with an ASI Z stage (Zeiss, Cambridge, UK). GFP fluorescence was detected using Zeiss filter set 10 (excitation 450–490 nm, emission 515–565 nm), and DY-590 fluorescence was detected using Zeiss filter set 15 (excitation 546/12 nm, emission LP 590 nm). For z-stack series, images were taken in ~ 0.7 - μ m z-steps by using the motorized Z stage, from the bottom to the top focal plane of the cells (10 to 12 slices).

Beadfection with Subsequent Fixation/Permeabilization and Confocal Microscopy Imaging—HeLa cells were seeded (5×10^3 cells/well) into six-well cell culture dishes containing sterile coverslips, beadfected, and incubated for 48 h at 37 °C with 5% CO₂. The coverslips were then washed with PBS (three 2-ml washes; Invitrogen, Paisley, UK) and fixed with 1% paraformaldehyde (20 min, 4 °C). Coverslips were again washed (three washes, each with 2 ml of PBS), permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature (permeabilized samples only), and incubated with anti-GFP monoclonal antibody (Chemicon, Watford, UK) at 1:1000 dilution in PBS containing 4% w/v BSA with gentle rocking for 2 h at room temperature. Coverslips were washed as before and incubated with

phycoerythrin-conjugated secondary antibody (Sigma, Poole, UK) (1:500 dilution in PBS containing 4% BSA) for 1 h with gentle rocking. Coverslips were washed and dried before being mounted in mounting medium (Vector Laboratories, Peterborough, UK). Slides were imaged with a Zeiss LSM 510 Meta confocal microscope using a Plan Apo-chromat $\times 63/1.40$ objective mounted on an Axioplan 2 motorized upright stand, with images collected by LSM software (Zeiss). GFP fluorescence was detected using a Zeiss bandpass (505–550 nm) filter after excitation at 488 nm. Phycoerythrin fluorescence was detected using a long-pass 560-nm filter after excitation at 543 nm.

Assessment of Cell Viability by CellTiter-Blue® Assay—HeLa cells were seeded in quadruplet into 96-well plates (Corning, Birmingham, UK) at 5×10^3 cells per well and incubated/beadfected as described. After incubation at 37 °C/5% CO₂ for 50 h, cells were washed (three 100- μ l washes) with phenol red-free DMEM (Invitrogen) supplemented with FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 1 \times nonessential amino acid supplement, and 4 mM L-glutamine. The media was then replaced with fresh phenol red-free medium containing CellTiter-Blue® reagent (100 μ l of medium + 20 μ l of reagent) per well. Cells were incubated for a further 3 h at 37 °C/5% CO₂, and then the absorbance at 620 nm was measured. After background subtraction, absorbance values were used to calculate the percentage viability, expressed as a percentage of untreated controls (which were set as 100% viable). The viability of cells beadfected with microspheres was compared with that of controls by means of one-way analysis of variance with Dunnett's multiple comparison test, with a *q* value less than 0.05 considered significant.

Assessment of Cell Membrane Integrity via Propidium Iodide Staining—Sterile coverslips (12-mm diameter) were placed into 12-well plates, and 5×10^4 cells were seeded into each well in 1.5 ml of DMEM (Invitrogen) supplemented with FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, 1 \times nonessential amino acid supplement, and 4 mM L-glutamine. After 24 h of incubation, cells were beadfected with microspheres **5** and **6**, and control cells were left untreated. After a further 48-, 72-, or 96-h incubation, media was removed from the wells and the coverslips were washed three times with fresh phenol red-free medium. Propidium iodide at a final concentration of 1 μ g/ml dissolved in phenol red-free medium (1.5 ml) was then placed into each well, and the plates were returned to the incubator for 30 min at 37 °C. The medium–propidium iodide solution was removed, and the coverslips were washed three times with fresh phenol red-free medium and then placed inverted on microscope slides. Propidium iodide fluorescence was imaged on a Leica TCS SP5 II confocal system with a DMI 6000B microscope and an HC Plan Apo-chromat $\times 20/0.7$ dry objective using excitation at 543 nm with emission collected between 625 and 780 nm. Three fields of view were randomly obtained for each sample and imaged at the focal depth of detectable propidium iodide fluorescence. The numbers of cells in each image that were stained by propidium iodide and unstained were then counted using ImageJ software (28), and these numbers were used to calculate the percentage of viable cells.

RESULTS

Confirmation of Cellular Uptake—We have recently described the synthesis and characterization of 1- μ m, core-shell, polystyrene-based microspheres possessing a fluorescently labeled, hydrophobic core and a carboxylic-acid-functionalized, hydrophilic shell with a surface carboxyl loading of ~ 60 μ mol/g (1). The microspheres have a smooth appearance (under scanning electron microscopy), are reasonably monodisperse, are readily taken up by HeLa cells, and are nontoxic to those cells (1). Previous, preliminary experiments had shown

that mouse neuronal stem cells also take up these naked microspheres and do so with 98.8% efficiency, confirming that the core-shell microspheres behave similarly in terms of cellular uptake to more conventional polystyrene-based microspheres (5). However, current evidence that polystyrene-based microspheres enter cells relies mainly on FACS-based data (1, 4, 5, 7, 8). Although these data have been confirmed by various means, including heparin or heparin-sulfate stripping (to remove microspheres adhering to the outside of the cell membrane) (5), trypan blue quenching of external microspheres (3–5, 8), and live cell imaging of beadfected cells with Dil-stained membranes (5), some still consider the concept of microsphere uptake controversial. We therefore sought alternative means to confirm both that our microspheres are taken up by cells and that they can import a biological cargo.

Our published core-shell microspheres (in which the core is labeled with DY-630 and the shell carries covalently bound GFP (1; microspheres **10a**)) were used as a starting point in the current study. These microspheres were beadfected into HeLa cells, which were then imaged as a Z stack. As successive focal depths were traversed and the center of the cells was approached, microspheres came into focus. That focus was lost as the focal plane moved further up the cells, indicating successful beadfection (supplemental Fig. S1). This evidence was indicative of internalization, but it was not absolute proof, because imaging had been performed on an epifluorescent microscope. Thus, to confirm internalization, additional HeLa cells were beadfected with DY-630/GFP derivatized microspheres and then either fixed or fixed *and* permeabilized. The cells were then examined via indirect immunofluorescence with mouse anti-GFP antibody and a secondary anti-mouse/phycoerythrin conjugate antibody. In fixed cells, this should result in extracellular microspheres being stained red and internalized microspheres remaining unstained (the cell membrane forms a barrier to antibody access). In contrast, cells that are fixed *and* permeabilized prior to immunostaining should permit antibody access to the cytoplasm and thus to the internalized microspheres. In such a case all microspheres, whether internal or external, should stain red. As shown in Fig. 1, the majority of microspheres in the fixed cells fluoresced green (GFP) but were not stained red (anti-GFP) (see Figs. 1ai and 1a_{ii}), demonstrating both that the microspheres had been internalized and, from the GFP signal, that the internalized GFP remained intact (lack of a GFP signal would indicate either denaturation or degradation of the GFP on the microspheres). Microspheres that were clearly outside these nonpermeabilized cells fluoresced both green and red (white arrow, Fig. 1a_{iv} with Figs. 1ai–1a_{iii}). The presence of internalized GFP was confirmed by the dual green and red staining within permeabilized cells, where the antibody had access to the intracellular environment (Figs. 1bi–1b_{iv}). Thus these immunostaining data both support the validity of the epifluorescent data taken at a focal plane within the cells and, more important, demonstrate unambiguously that our core-

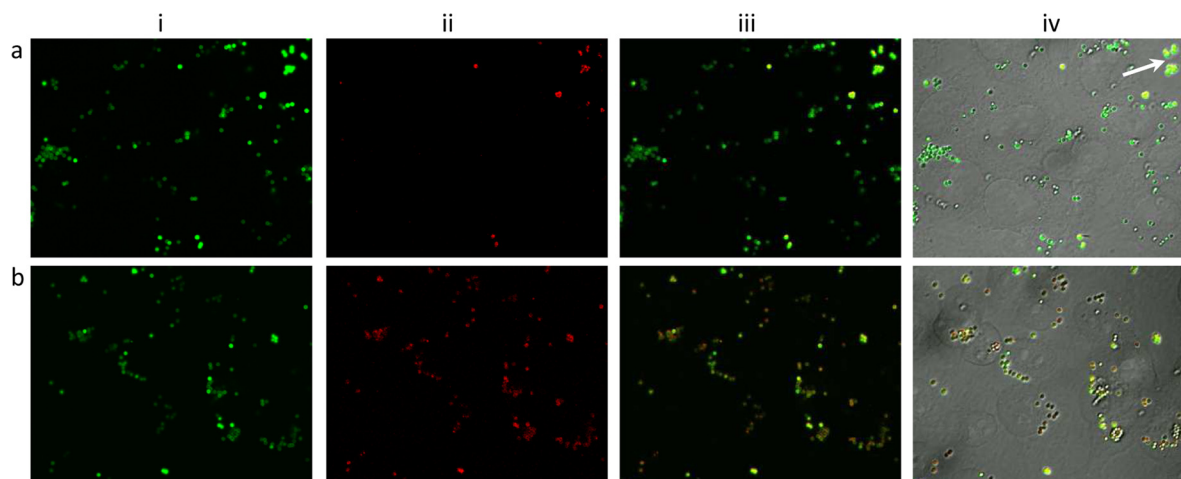


FIG. 1. Indirect immunofluorescence analysis of beadfected, fixed HeLa cells. HeLa cells were beadfected with DY-630/GFP derivatized core-shell microspheres. Samples of the cells were either fixed or fixed *and* permeabilized. They were then examined via indirect immunofluorescence using mouse anti-GFP antibody and an anti-mouse/phycoerythrin secondary antibody conjugate. Confocal images of GFP and/or phycoerythrin fluorescence illustrate (a) non-permeabilized cells, (b) permeabilized cells, (i) GFP fluorescence, (ii) phycoerythrin fluorescence, (iii) co-localization of GFP/phycoerythrin fluorescence, and (iv) combined fluorescent/bright field image to show cellular location. The white arrow indicates non-internalized microspheres. (Note: microspheres shown in this figure were internally labeled with DY-630, which was selected solely to imbue the microspheres with chemical characteristics similar to those used in subsequent fluorescence microscopy studies. It was first confirmed that DY-630 was undetectable by the filter set/wavelengths employed within these experiments.)

shell microspheres were able to enter cells and transduce an intact protein cargo.

Development of Generic Bioconjugation and Delivery Chemistry—Facile bioconjugation between the carrier (here, the microsphere) and the protein is required for a generic protein-delivery protocol. The many techniques available with which to immobilize protein covalently to support surfaces are the subject of a recent review (29), but two of the most common involve either EDC/EDAC or glutaraldehyde/cyano-borohydride-mediated couplings with any amine available on the protein surface to immobilize protein via amide or amine bonds, respectively. As previously demonstrated (1, 5, 7), such methods enable effective protein delivery via beadfection, but subsequently the protein remains permanently tethered to the microspheres.

Disulfide linkers have been used effectively in the delivery of nucleic acids to cells (27, 30) and in releasing protein from nanocapsules (31). Such linkers permit intracellular release from a carrier because the cytoplasm of mammalian cells is maintained as a reducing environment, containing high concentrations of reduced glutathione (tripeptide γ -glutamyl-cysteinyglycine), which cleaves disulfide bonds to generate free thiols. Thus, an obvious approach would be to conjugate protein to microspheres via disulfide-based cleavable linkers such as *N*-succinimidyl 3-(2-pyridyldithiopropionate). Although linkers of this type are readily available, their advantage is negated by the requirement that a free thiol be accessible for coupling on the protein's surface. Consequently, this often necessitates mutagenesis/engineering of the protein to introduce the thiol moiety in the form of a free cysteine residue. Nevertheless, we elected to assess this conventional,

disulfide-based methodology. A suitable free thiol near the amino terminus of GFP was engineered, and the resulting Cys-GFP protein was coupled to microspheres via an *N*-succinimidyl 3-(2-pyridyldithiopropionate) linker. Although the presence of the *N*-terminal cysteine did not affect protein fluorescence, only weakly fluorescent microspheres resulted, suggesting that protein loading of the microspheres was low. Moreover, long incubation times (4 to 6 h) at room temperature were necessary in order for the protein to attach to the microspheres with this method. Such lengthy incubations are clearly undesirable in terms of the stability of many purified proteins.

Accordingly, we designed a linker that contains a cell-cleavable disulfide linkage but can couple to any primary amine on the protein, rather than requiring a free cysteine residue for immobilization. This approach negates the need to modify the target protein and combines the speed and generic application of EDC/EDAC methodology (all proteins have amino groups available for coupling) with the reversibility engendered by a disulfide-based linker. The linker was synthesized *in situ* on the shells of the microspheres **1** (Scheme 1). Specifically, a TBTU-mediated procedure was employed to couple 3-(2-pyridyldithio)propionyl hydrazide **2** to the carboxylic acid groups within the outer shells of these orthogonally functionalized microspheres. The resultant thiol-reactive pyridyl derivative **3** was then subjected to disulfide exchange to generate the corresponding 3-mercaptopropionic acid-derived linker **4**. The hydrophobic maleimide-modified dye DY-590 (Dyomics) was then coupled to the thiol groups within the polystyrene cores of the microspheres to generate fluor-labeled beads **5**. Finally, GFP was coupled to the cleavable

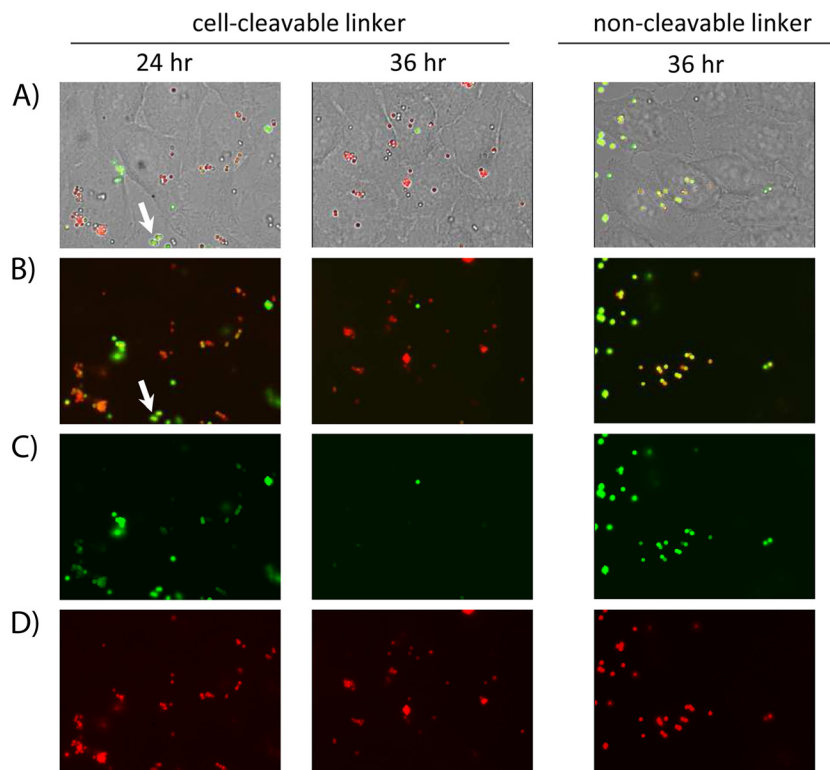


FIG. 2. **Epifluorescent microscopy of beadfected HeLa cells conjugated to GFP via cell-cleavable and non-cleavable linkers.** Samples of HeLa cells were beadfected with DY-590-labeled microspheres conjugated to GFP via a cell-cleavable linker (microspheres **6**) or via a non-cleavable linker (**1**). All images represent a z-slice taken at a focal plane approximating the center of the cells. *A*, combined fluorescent/bright field image. *B*, DY-590 fluorescence (internal bead label) and GFP fluorescence. *C*, GFP fluorescence only. *D*, DY-590 fluorescence only.

linker in aqueous medium using conventional carbodiimide chemistry to generate GFP-loaded microspheres **6**.

Delivery and Release of Protein within Cells—To test the delivery and release of protein in cells using the cell-cleavable linker system, core-shell microspheres were labeled with DY-590 and freshly loaded with GFP **6** (Scheme 1). As a control, core-shell microspheres **1** were labeled with DY-590 and subsequently loaded with GFP using conventional EDAC chemistry to yield a non-cleavable linkage using methodology described previously (1). Previous studies (7, 32) indicate that cellular uptake occurs within about 24 h. Given this time frame, the onset of GFP cleavage from the microspheres should be apparent within 24 h if the cell-cleavable linker is capable both of delivering a protein cargo into cells and subsequently releasing that cargo within the cytoplasm. We therefore examined samples of beadfected cells after 24 and 36 h using fluorescence microscopy (Fig. 2).

Fig. 2A demonstrates that the majority of microspheres were internalized within the cells in under 24 h (note the white arrow, which indicates external microspheres). Moreover, the difference between the 24-h and 36-h samples is striking with regard to microspheres bearing the cleavable linker. Examination of Figs. 2A and 2B shows that at 24 h, the internalized microspheres were mostly red, suggesting that the GFP protein cargo had already been released. However, when green

fluorescence is studied in isolation (Fig. 2C, 24 h cleavable), it is evident that the internalized beads were all faintly green, suggesting partial protein release. After 36 h, green fluorescence from the internalized microspheres disappeared, indicating that all of the protein had been delivered (Fig. 2C, 36 h cleavable). (Note that it is unlikely that GFP fluorescence would be detected after release from the microspheres, because the protein would be diffuse within the cells, and thus its cytoplasmic concentration would be too low; in optimized, extracellular experiments, the lowest concentration of free GFP that we have been able to detect is 0.4 nM (33).) In contrast, when GFP was attached to the microspheres via the non-cleavable linker, green fluorescence remained unchanged after 36 h (Fig. 2C, 36 h non-cleavable), demonstrating that the protein was neither degraded nor released from the microspheres.

To compare the uptake efficiency of these newly derivatized beads with that of those examined previously, we performed multiple beadfection experiments and analyzed the resultant fluorescent images. Beads that were in focus within the central sections of z-stacked fluorescent images of the cells were counted (Table I). As also demonstrated in Fig. 2 and previously (6, 32), these data again confirmed that cellular uptake occurred within 24 h. Moreover, we were interested to note not only that uptake of our GFP-derivatized beads was similar

TABLE I

Efficiency of beadfection of HeLa cells with DY-590 labeled microspheres conjugated to GFP via a cell-cleavable linker (microspheres 6) or a non-cleavable linker (1)

Linker	24 h (%)	36 h (%)
Cell-cleavable	74.3 ± 5.6	68.1 ± 12.9
Non-cleavable	64.7 ± 4.9	71.2 ± 15.4

Numbers are the percentage of cells that successfully took up bead(s) (± S.D.) and were calculated from four experiments performed independently, using cells from three different passage numbers. These percentages were calculated by counting beads in focus within the central section of a z-stack of the cells (e.g. the internal region of the cells). A total of 600 to 1100 cells were counted for each determination.

regardless of linker chemistry (supplemental Table S1), but also that the degree of uptake was remarkably similar, whether calculated by counting fluorescent images or measured by FACS (1). We therefore conclude that on the order of 65% to 75% of cells will take up our core-shell microspheres when loaded with GFP protein.

Beadfection Does Not Compromise Cell Viability or Membrane Integrity—Previous work has shown that microspheres are nontoxic to a variety of host cells, as described in the introduction. To confirm that these earlier findings also apply to our core-shell microspheres, we tested the effect of beadfection in HeLa cells, by means of both CellTiter-Blue® cell viability assay (Promega, Southampton, UK) and propidium iodide staining, to examine membrane integrity. HeLa cells were beadfected with either microspheres 5 (derivatized with the cell-cleavable linker) or microspheres 6 (derivatized with the cell-cleavable linker coupled to GFP) and compared with non-beadfected controls. To examine viability, cells were cultured for 50 h after beadfection and then analyzed via the CellTiter-Blue® assay. No difference in viability was observed between control cells and those beadfected with either microspheres 5 or microspheres 6 (Fig. 3A). Similarly, propidium iodide exclusion demonstrated that beadfection with microspheres 5 and 6 had no effect on HeLa cell membrane integrity at 48, 72, or even 96 h after beadfection (Table II).

The Cell-cleavable Linkage Is Stable When Outside of the Cell—The final requirement of a generic delivery system is stable extracellular bioconjugation of the protein cargo. We therefore incubated GFP-loaded microspheres 6 in used cell culture medium for 90 h at 37 °C. The microspheres were then examined via fluorescence microscopy. Fig. 4 demonstrates both that the GFP remained attached to the microspheres and that the internal labeling of the microspheres remained effective even after such prolonged incubation. We conclude that we have generated microspheres that satisfy all of our requirements for generic protein delivery via bioconjugation between solid particles and soluble protein.

DISCUSSION

The common approach to studying cellular protein activity *in vitro* is transfection with nucleic acid, but this can lead to

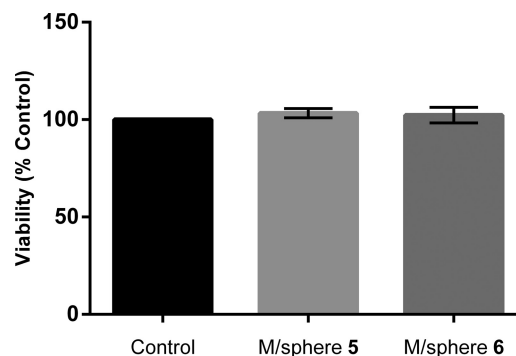


FIG. 3. **CellTiter-Blue® analysis of cell viability after beadfection.** HeLa cells from five different passage numbers were beadfected in quadruplet with microspheres 5 (derivatized with the cell-cleavable linker only) and 6 (loaded with GFP, linked via the cell-cleavable linker), cultured for 50 h, and compared with non-beadfected controls by means of CellTiter-Blue® analysis, as described under “Experimental Procedures.” Data represent means of resazurin reduction, expressed as a percentage of nontreated controls ± S.D.

TABLE II

Cell membrane integrity after beadfection with microspheres 5 (cell-cleavable linker) and microspheres 6 (cell-cleavable linker conjugated to GFP), as determined via propidium iodide staining

Time (h)	Sample	Unstained cells (%)
48	Control	97.7 ± 2.7
	Microspheres 5	97.1 ± 2.5
	Microspheres 6	98.3 ± 1.7
72	Control	99.3 ± 0.7
	Microspheres 5	99.7 ± 0.2
	Microspheres 6	99.8 ± 0.3
96	Control	98.1 ± 0.9
	Microspheres 5	98.1 ± 1.4
	Microspheres 6	98.9 ± 0.7

Cells were grown on coverslips, beadfected, incubated for time periods as indicated, and stained with propidium iodide as described in “Experimental Procedures.” Numbers represent the mean percentage ± S.D. of unstained cells calculated from three experiments performed independently in triplicate, using cells from three different passage numbers. Totals of 74 to 529 cells (48 h), 305 to 1140 cells (72 h), and 414 to 1193 cells (96 h) cells were counted for each field of view.

problems of dosage, transiency, and/or unwanted genetic manipulation. Moreover, the number of genes that can be delivered to a single cell is strictly limited. Thus, protein transduction remains an attractive alternative, but again, the number of proteins that can be delivered is limited, and as described above, avoidance of endocytosis is desirable when the ultimate aim is to study protein function after delivery.

Microsphere-based protein transduction is attractive because of the facile and high-efficiency mechanism of entry. Microspheres are taken up with high efficiency by a wide variety of cells, apparently without any toxic side effects, and therefore they show great promise for the delivery of intact, functional protein into cells. The microspheres described herein fulfill all of our specified criteria for a protein transduction reagent. They allow for facile covalent linkage of protein

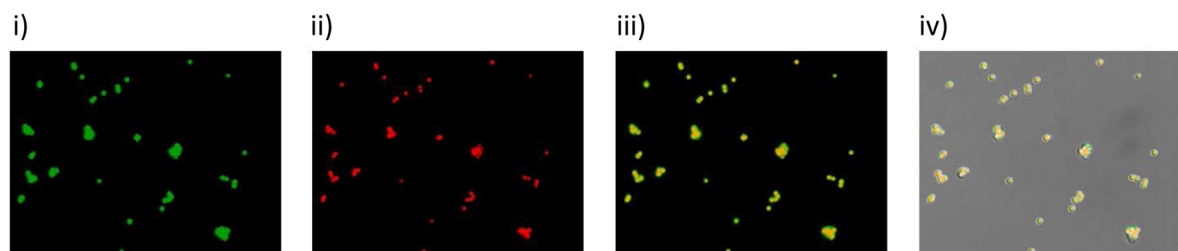


FIG. 4. Epifluorescent microscopy to examine the extracellular stability of the cell-cleavable linker. GFP-loaded microspheres 6 (cell-cleavable linker) were examined via epifluorescent microscopy after incubation at 37 °C for 96 h in used DMEM medium. (i) GFP fluorescence only. (ii) DY-590 fluorescence (internal bead label) only. (iii) Combined GFP and DY-590 fluorescence. (iv) Combined fluorescent/bright field image.

(via a generic, aqueous, carbodiimide-based coupling procedure), which we have demonstrated to be nontoxic to the cells and stable, extracellularly. We have also shown that the delivery efficiency of these microspheres is good, with typically 65% to 75% of the cell population being beadaffected successfully. Protein may be attached to the microspheres via a disulfide linkage, which enables spontaneous release within the cytoplasm, mediated by the reducing intracellular environment. Alternatively, protein may be linked irreversibly to the microspheres if required. During and after delivery, the location of the microspheres can be tracked simply via the independent fluorescent signature of their cores.

Future applications of our microspheres can exploit their versatility. Although not demonstrated herein, because protein is joined simply via a free amino group, our microspheres could in theory carry a mixture of proteins (perhaps those identified via protein–protein interaction analyses) or indeed protein(s) complexed with irreversible inhibitors. Alternatively, the orthogonal nature of their core and shell chemistries means that any molecule bearing a free amine group can be linked to the outer shell, and the core can be labeled with any hydrophobic, thiol-reactive dye/fluor. Thus, our microspheres should also permit facile delivery/release of nonproteinaceous molecules such as RNAi, as demonstrated recently (27, 34), but without the need to attach additional hydrophilic linkers to confer aqueous compatibility. As discussed above, the different chemical reactivities of the core and shell of our microspheres also offer potential for multiplexing using multiple cargo–fluor combinations.

Finally, although polystyrene microspheres have been employed in *in vivo* studies (3, 35) and other, nonbiodegradable silica-based nanoparticles have been used for drug delivery (36), the microspheres described herein are not biodegradable and thus are not intended for *in vivo* use. Rather, they have been developed as a research tool to facilitate *in vitro* functional protein analysis to manipulate cell activity/phenotype via the introduction of exogenously derived proteins.

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** To whom correspondence should be addressed: Tel.: 44-121-204-3961, Fax: 44-121-204-4187; E-mail: a.v.hine@aston.ac.uk.

¶ Current address: School of Materials, University of Manchester, Oxford Road, Manchester, M13 9PL, UK.

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