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Cellular Uptake of Fluorescent Labelled Biotin–Streptavidin Microspheres

M. Bradley · L. Alexander · R. M. Sanchez-Martin

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Abstract Amino functionalized, cross-linked, polystyrene microspheres were covalently loaded with streptavidin to which was coupled fluorescently labeled biotin and biotinylated-tagged DNA. These biotin–streptavidin microsphere conjugates were then successfully delivered into cells. The application of the streptavidin–biotin technology to these microspheres allows the effective delivery of any biotinylated material into intact mammalian cells, without the need for delicate procedures such as micro-injection.

Keywords Multifunctionalised microspheres · Streptavidin–biotin · Flow cytometry · Fluorescent imaging · Intracellular delivery

Introduction

In recent years, a diverse range of delivery systems have been developed in order to enhance transport and uptake at the cellular level. Examples include peptides such as those based on fragments derived from the Tat protein, cell penetrating peptides [1–3], peptidomimetics such as peptoids [4], cationic lipids [5], liposomes [6], dendrimers [7, 8] and several nanodevices such as those based on nanotubes [9].

Polymer mediated delivery offers many advantages over more traditional methods. For instance, polymeric materials can allow the controlled delivery and release of their cargo at locally controllable concentrations and there are many examples where drugs and sensors have been encapsulated within polymer microparticles [10, 11]. However their introduction into cells is often complex. One type of polymeric material that has been shown to be engulfed and actively transported throughout cells are latex beads, although, surprisingly little work has been carried out on exploiting this phenomenon [12]. One reason for this is that commercially available materials are often unfunctionalized chemically, or if they are functionalized, use of this moiety is only achievable under mild aqueous conditions. However, modification for the attachment of a range of more interesting chemical probes requires compatibility with organic solvents, such as dimethylformamide (DMF).

We recently described the synthesis of robust, biocompatible, amino functionalized cross-linked polystyrene microspheres of highly defined sizes (0.5 and 2 μm) that can be employed in solid phase multi-step syntheses and demonstrated their efficient cellular delivery into a broad range of cell including primary cells. We additionally described the efficient fluorescence-activated sorting of cells containing either single or multiple beads with one or more colours, thereby allowing the "encoding" of multiple cell types and subsequent cellular multiplexing [13]. We have also established that amino functionalized polystyrene microspheres can be used successfully for calcium sensing while avoiding the dilution and cellular degradation observed with other, more conventional, sensors [14]. The use of fluorescein-loaded microspheres as intracellular pH sensors in living cells has also been successfully proven using spectrofluorometry, fluorescence microscopy and flow cytometry [15] with the sensors covalently bound to the polymeric particles.

The high affinity of the biotin–streptavidin interaction has allowed many applications to be developed, that make

M. Bradley · L. Alexander · R. M. Sanchez-Martin (✉)
Chemical Biology Section, School of Chemistry,
University of Edinburgh,
Joseph Black Building, West Mains Road,
EH9 3JJ Edinburgh, UK
e-mail: rosario.sanchez@ed.ac.uk

use of this general bridge system, in various branches of biotechnology [16–19]. There are some remarkable properties of this system. Thus the small size of biotin makes it a perfect tag for biologically active macromolecules because biotinylation does not usually alter their biological activity, while the biotin–streptavidin interaction is not disrupted by serum proteins.

The exceptionally tight interaction between biotin and streptavidin (Kd 4×10^{-14} M) [20] suggested that this might be a practical way to add a range of molecules to microspheres and subsequently aid their delivery into cells. Here we show the synthesis and evaluation of biotin–streptavidin based cellular uptake of microspheres using techniques such as flow cytometry and fluorescence microscopy. The application of the biotin–streptavidin technology to our microspheres opens up a huge number of applications such as the binding and delivery of targeting ligands (for example monoclonal antibodies) in an efficient manner.

Materials and methods

Synthesis and fluorescent labelling of streptavidin-loaded microspheres

The 0.5 and 2.0 μm aminomethyl functionalized microspheres were washed with PBS buffer (pH 7.4), NaOH (200 mM) and finally PBS (pH 7.4). After washing and centrifugation, microspheres were resuspended in a 25% (*w*/*v*) glutaraldehyde solution in PBS buffer and the mixture was shaken for 15 h. After this time the microspheres were washed with PBS (pH 7.4) before addition of streptavidin (5 μg/mL in PBS, pH 7.4) and the mixture was shaken for 15 h. They were then washed with PBS (pH 7.4) and treated with a sodium cyanoborohydride solution (5 mM) in PBS/EtOH (3:1) for 2 h. Finally the streptavidin-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).

Streptavidin-loaded microspheres were centrifuged and re-suspended in a solution of biotin-4-fluorescein in biotin–streptavidin binding buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 M NaCl) and the mixture was shaken for 1 h, after which time the microspheres were washed with the same buffer and resuspended in PBS (pH 7.4).

Streptavidin-loaded microspheres were centrifuged and re-suspended in a solution of biotin-dsDNA-Cy3 in biotin–streptavidin binding buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 M NaCl) and the mixture was shaken for 1 h after which time the microspheres were washed with the same buffer and resuspended in a 20 mM HEPES buffer.

Efficiency of streptavidin coupling

A series of biotin-4-fluorescein (MW: 644.7) dilution solutions in PBS buffer were prepared and the absorbance was measured at 494 nm to build a calibration curve (see Fig. 1).

After coupling of biotin-4-fluorescein to microspheres the supernatants (biotin released) were combined and the absorbance at 494 nm (A_{494}) was recorded.

Cell culture conditions and general protocol for cellular uptake experiment

HeLa cells were cultured in RPMI containing high glucose (4.5 mg/mL) and supplemented with 4 mM glutamine, 100 units/mL of penicillin/streptomycin and 10% FBS. The cells were maintained in a humid chamber at 37 °C in an atmosphere of 5% CO_2.

HeLa cells were grown in RPMI medium supplemented with 4 mM glutamine, 10% FCS and 100 units/mL of penicillin/streptomycin in T75 cell culture flasks until 70% confluency. Cells were suspended using trypsin/EDTA and plated in a 24 well-plate at a density of 4×10^4 cells per well. After overnight incubation, cells were incubated with 0.5 and 2 μm biotin-4-fluorescein-labelled microspheres or biotin-dsDNA-Cy5-labelled microspheres at different concentrations, for 24 h at 37 °C with 5% CO_2. After the incubation time, cells were washed twice with PBS, harvested with trypsin/EDTA, washed again with PBS and resuspended in 2% FCS in PBS buffer. The internalization of labeled-microspheres was analyzed by flow cytometry analysis using

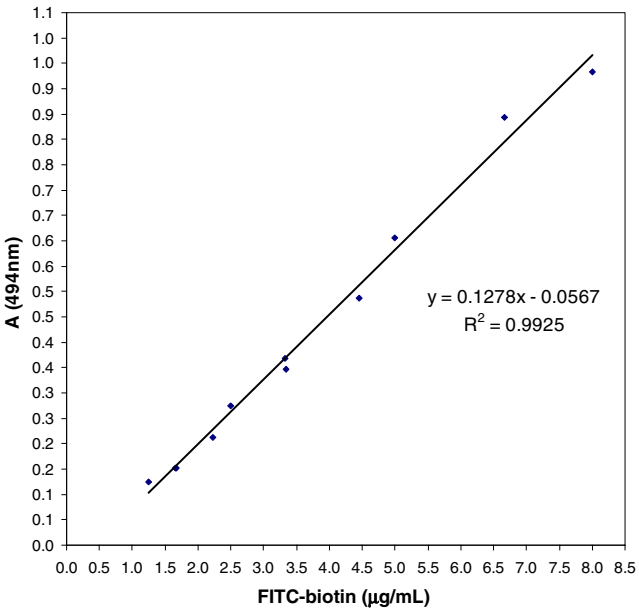
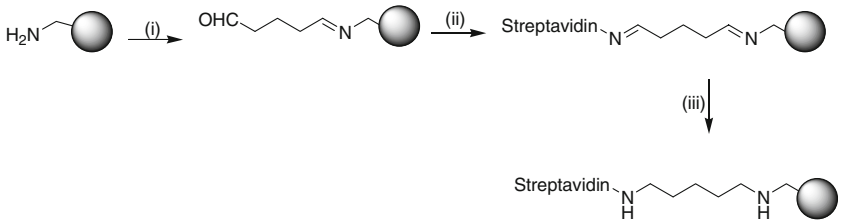


Fig. 1 Calibration curve of biotin-4-fluorescein at different concentrations



Scheme 1 Preparation of streptavidin-loaded microspheres. (*i*) glutaraldehyde, PBS, 15 h; (*ii*) streptavidin, PBS, 15 h; (*iii*) $NaCNBH_3$, PBS, ethanol, 2 h (phosphate buffer saline (PBS))

a FACSAria flow cytometer (Becton Dickinson). A total of 10,000 events per sample were analyzed. Cell samples were excited with a 488-nm (Coherent® Sapphire™ solid state) laser and 530/30 nm (Fluorescein) and 575/26 nm (Cy3) band pass filters were used for fluorescence analysis of the cellular uptake of biotin-4-fluorescein derivatives and biotin-dsDNA-Cy3 respectively.

The fluorescence images of cellular biotin-labelled streptavidin-loaded microspheres were acquired on a Zeiss pseudoconfocal microscope (Axiovert 200M Inverted microscope) excited by a mercury arc lamp. The luminescence images were recorded with a set of fluorescence filters with excitation and emission band-pass filters: 470/40 nm and 525/50 nm for fluorescein, 535/50 nm–610/75 nm for AlexaFluor® 568 and 365/10 nm and 460/50 nm for DAPI. The corresponding differential interference contrast (DIC) image was detected simultaneously to show the cell morphology. The nucleus was stained with blue-fluorescent bis-benzimide derivative Hoechst 33342. AlexaFluor® 568-phalloidin was use to stain the cytoskeleton (actin filaments) in vitro.

For the analysis of oligonucleotide intracellular delivery, the fluorescence images of cellular Cy3-DNA-biotin-labelled streptavidin-loaded microspheres were acquired on a Leica microscope (DM IRB). The nucleus was stained with blue-fluorescent bis-benzimide derivative Hoechst 33342 and Cell Tracker™ Green (5-chloromethylfluorescein diacetate) was used for cell labelling.

Results and discussion

The first stage was the preparation of streptavidin-loaded microspheres. For this purpose, the streptavidin was coupled to 0.5 and 2 μm aminomethyl microspheres by overnight reaction in the presence of glutaraldehyde [21]. Subsequently, a solution of streptavidin in PBS buffer (pH 7.4) was added and allowed to react overnight. Finally, the reversible Schiff base was reduced with sodium cyanoborohydride to give the covalently loaded streptavidin-microspheres (Scheme 1).

In order to be able to evaluate the cellular uptake, the microspheres were labelled with biotin-4-fluorescein, which has been reported to be a superior fluorescent biotin derivative for accurate measurement of streptavidin in crude biofluids [22]. This fluorescent labelling allowed us to follow the microspheres inside the cells and to study their cellular uptake. Additionally, the fact that we were able to label the microspheres with this biotinylated compound also demonstrated the integrity of the streptavidin when bound to the microspheres. It also allowed determination of the loading of streptavidin on the microspheres and as a consequence the coupling efficiency. The streptavidin coupling to microspheres was approximately 90% (see Table 1).

Fluorescence-based analysis of the biotin-4-fluorescein-streptavidin-loaded microspheres was carried out to evaluate how immobilization of the streptavidin onto the polymer

Table 1 Efficiency of streptavidin coupling to microspheres

Samples	A_{biotin} (494 nm)[a]	Concentration (μg/mL)	Biotin released (nmol)	Streptavidin coupled[b] (nmol)	Coupling efficiency (%)
Starting solution (1)	0.263	2.5	1.9	–	–
Starting solution (2)	0.742	6.25	1.9	–	–
0.5 μm (1)	0.170	1.8	1.4	0.125	89[c]
0.5 μm (2)	0.506	4.4	1.4		
2 μm (1)	0.140	1.54	1.2	0.175	88[d]
2 μm (2)	0.456	4.01	1.2		

[a] Mean value from 3 measurements for each sample
[b] Each molecule of streptavidin binds 4 biotins (MW 644.7)
[c] SC 2%, 100 μL/sample, 0.07 mmol/g of amino groups
[d] SC 2%, 100 μL/sample, 0.10 mmol/g of amino groups

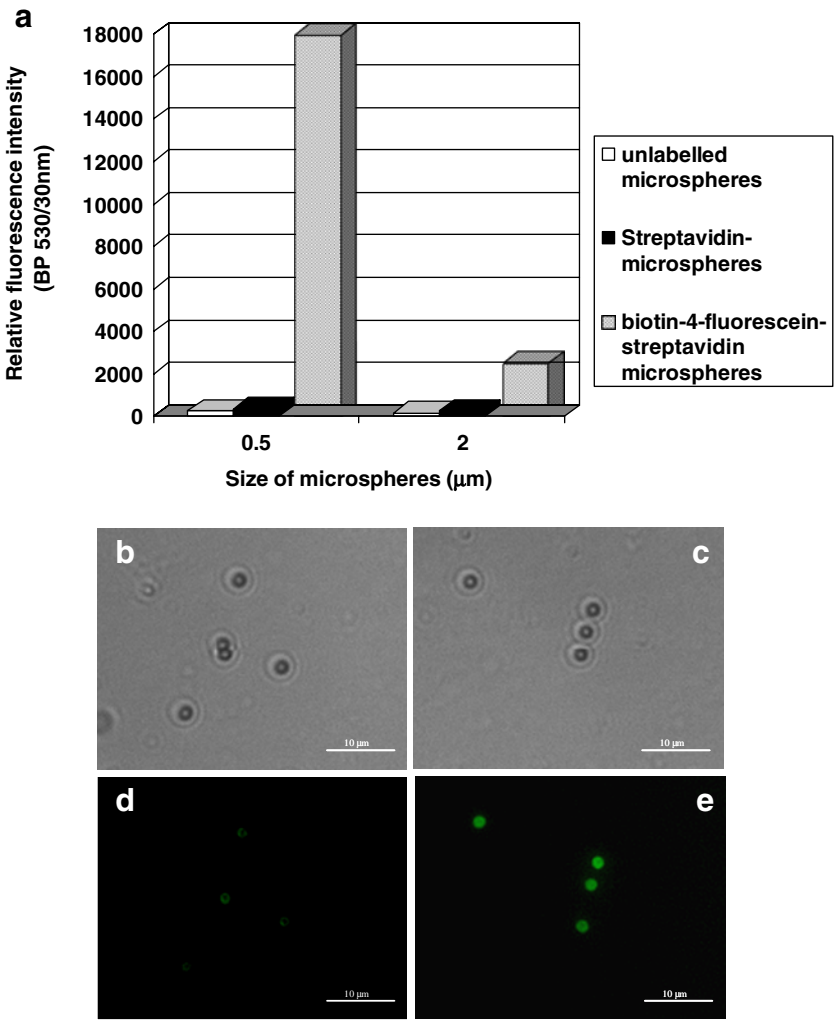


Fig. 2 **a** Flow cytometry analysis of streptavidin-loaded microspheres labelled with biotin-4-fluorescein. Microscopy pictures of streptavidin-loaded microspheres (**b** and **d**) and biotin-labelled streptavidin-loaded microspheres (**c** and **e**)

particles affected its binding properties. Flow cytometry results show that the fluorescent intensity signal increased dramatically after treatment of the streptavidin-loaded microspheres with the fluorescently labelled biotin (Fig. 2a) suggesting that attachment had not altered its binding properties. Microscopy-based analysis showed identical behaviour (see Fig. 2b and c).

The next stage of this research was an evaluation of the cellular uptake of the biotin–streptavidin-loaded microspheres. A number of studies were conducted using human ovarian cancer (HeLa) cells. These cells were treated in triplicate with microspheres (0.5 and 2.0 μm) at a range of concentrations. Following incubation, the excess microspheres were removed and analysis by flow cytometry and fluorescence microscopy showed that cellular uptake of the microspheres was effective. Both sizes of bead could be delivered into the cells, though with varying degrees of success depending on the bead size and concentration (Figs. 3 and 4). Cells incubated with the 0.5 μm microspheres resulted in a greater uptake of beads compared to the larger

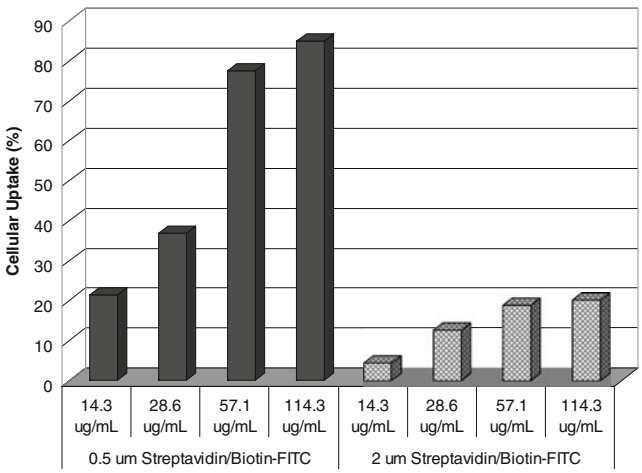


Fig. 3 Comparison of cellular uptake (percent of labelled cells from total population) of 0.5 and 2.0 μm fluorescently biotin-labeled streptavidin-loaded microspheres with HeLa cells, at different concentrations after 24 h of incubation (at 37 °C and 5% CO_2 atmosphere). Results obtained by flow cytometry analysis (530/50 nm BP filter)

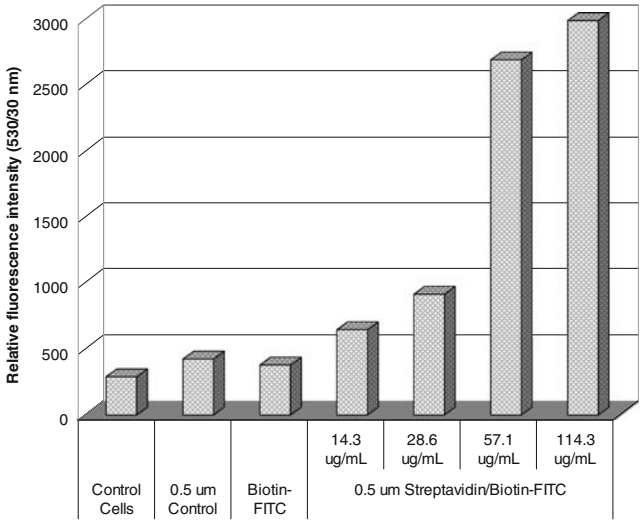


Fig. 4 Comparison of cellular uptake (relative fluorescence intensity) of 0.5 μm fluorescently biotin-labeled streptavidin-loaded microspheres at different concentrations. *Controls* Untreated cells and cells incubated with streptavidin-loaded microspheres compared to cells incubated with biotin-4-fluorescein, after 24 h of incubation (at 37 °C and 5% CO_2 atmosphere). Results obtained by flow cytometry analysis (530/50 nm BP filter)

2.0 μm beads. Various controls were run including untreated cells, cells incubated with streptavidin-loaded microspheres and cells incubated with 4-biotin fluorescein in solution. None of the controls showed any significant fluorescence intensity when compared with cells treated with the fluorescently labelled microspheres. As mentioned, cellular uptake was found to be concentration and size dependent, to this effect Fig. 5 shows the cellular incorporation of 0.5 and 2.0 μm biotin-4-fluorescein-labeled streptavidin microspheres after 24 h, as analyzed by fluorescence microscopy.

A further study was carried out applying this biotin–streptavidin approach, in which the cellular uptake of oligonucleotides loaded onto the microspheres were analysed. A biotinylated fluorescently-labeled dsDNA was used as a model system (biotin-dsDNA-Cy3). Following the binding of the dsDNA to the streptavidin-loaded microspheres, HeLa cells were treated in triplicate with 2.0 μm microspheres at a range of concentrations. Following incubation, the excess microspheres were removed and analysis by flow cytometry and fluorescence microscopy showed that cellular uptake of these DNA-loaded microspheres was effective (Fig. 6). The oligonucleotide-loaded microspheres were delivered into the cells, though with varying degrees of success depending on the concentration. Various controls were run including untreated cells, cells incubated with streptavidin-loaded microspheres and cells incubated with biotinylated ds-DNA in solution. Again, none of the controls showed any significant fluorescence intensity when compared with cells treated with the fluorescently labelled microspheres (see Fig. 6a). Figure 6b shows the incorporation of 2.0 μm dsDNA-loaded microspheres, as analyzed by fluorescence microscopy.

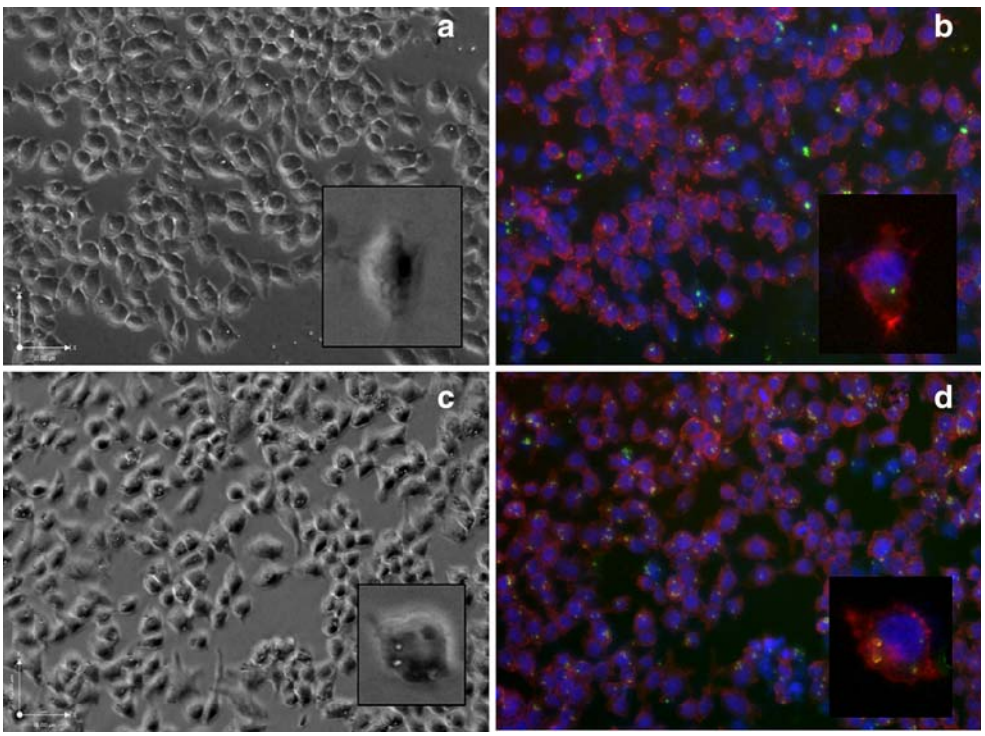


Fig. 5 Pseudoconfocal microscopy analysis of cellular uptake of 0.5 μm (**a** and **b**) and 2.0 μm microspheres (**c** and **d**) fluorescently biotin-labeled streptavidin-loaded microspheres (*green dots*), in HeLa cells, at 57.1 μg/mL after 24 h of incubation (at 37 °C and 5% CO_2 atmosphere). DIC images are **a** and **c**. The cytoskeleton was stained with a red fluorescent dye (AlexaFluor® 568-phalloidin) and the nucleus was labelled with Hoechst 33342 (**b** and **d**)

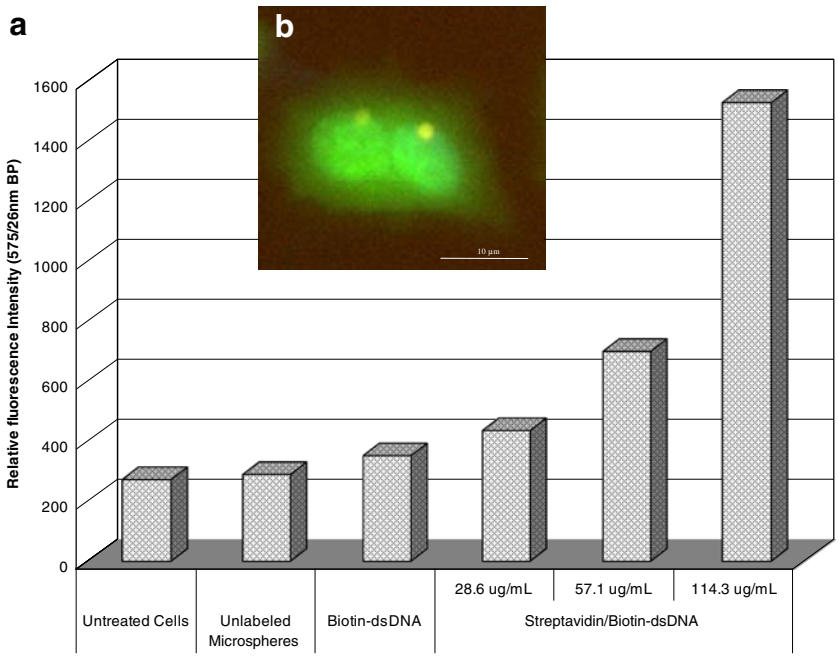


Fig. 6 **a** Comparison of cellular uptake (relative fluorescence intensity) of 2.0 μm Cy3-dsDNA biotin-labeled streptavidin-loaded microspheres at different concentrations in HeLa cells, after 24 h of incubation (at 37 °C and 5% CO_2 atmosphere). *Controls* Untreated cells and cells incubated with streptavidin-loaded microspheres and biotin-dsDNA in solution. Results obtained by flow cytometry analysis (575/26 nm BP filter). **b** Fluorescence image of 2.0 μm Cy3-dsDNA biotin-labeled streptavidin-loaded microspheres in HeLa cells (superimposed imaged of DAPI, green and red filters)

Conclusions

The use of streptavidin-loaded microspheres as a carrier system able to enter living cells has been successfully proven using a biotinylated fluorophore by fluorescence microscopy and flow cytometry. Moreover, the cellular uptake of a biotinylated oligonucleotide has been successfully accomplished. The fact that there are several biotinylation strategies to bind different molecules such as proteins, peptides, antibodies, nucleic acids, etc. to the streptavidin-loaded microspheres opens a huge range of applications for this approach such as intracellular detection of a diverse number of targets.

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