Chapter 9

Assembly, Characterization, and Delivery of Quantum Dot Labeled Biotinylated Lipid Particles

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Abstract

Lipid nanoparticles composed of mixtures of PEGylated-lipids; cationic and neutral lipids prepared by detergent dialysis can encapsulate biological active molecules and show considerable potential as systemic therapeutic agents. Addition of biotinylated lipids to this formulation allows surface modification of these particles with a suitable ligand or probe conjugated to streptavidin for specific cell targeting. Monitoring long circulating particles and cellular uptake requires stable and bright fluorescent probes. Quantum dots (QDs) constitute a relatively new class of fluorescent probes that overcome the limitations of organic fluorophores in biological imaging applications. Here, a protocol for the encapsulation of QD_{655} (red) in biotinylated lipid particles (BLPs) prepared by a detergent dialysis technique is presented followed by characterization of the loaded liposomal vehicles. Then, a protocol for BLPs surface modification via biotin-streptavidin linkage with preformed complexes of ligand-QD_{525} (green) for specific cell targeting of the nanoparticle is detailed. Conditions for cell binding and uptake of two colors QD labeled BLPs as well as basic microscopic settings for confocal live cell imaging are described.

Key words Biotinylated lipid particles, Quantum dots, Epidermal growth factor, Targeted delivery, Detergent dialysis, Confocal fluorescence microscopy

1 Introduction

Liposomal carriers have become major tools for introducing not only a variety of nucleic acids but also proteins, peptides, and nanoparticles into cells in vitro and in vivo [1]. Lipid composition as well as the method of preparation will determine the average size of the carrier as well as the permeability through biological membranes, and thus the efficiency of uptake by cells [2, 3]. An important issue is the targeting of the carriers to specific cell types for which the delivery approach is intended. The role of receptors as molecular target has opened new opportunities for cellular or intracellular targeting using liposomal systems appended with appropriate ligands [4].

Adriana Fontes and Beate Saegesser Santos (eds.), *Quantum Dots: Applications in Biology*, Methods in Molecular Biology, vol. 1199, DOI 10.1007/978-1-4939-1280-3_9, © Springer Science+Business Media New York 2014

Continuous cell imaging has been substantially improved with the introduction of fluorescent probes such as quantum dots (QDs), colloidal nanocrystals composed of cadmium selenide (CdSe) or cadmium telluride (CdTe) core enclosed within a zinc sulfide (ZnS) passivation shell [5, 6]. The main advantages of QDs over organic fluorophores are the greater photostability and the excitation wavelengths range that extends above 500 nm. The latter feature reduces cell phototoxicity, essential for long-term fluorescence imaging. Whereas absorption spectra are broad, emission spectra are narrow without the extension to the red characteristic of organic dyes. This feature allows for simultaneous detection of multiple color QDs upon illumination with a single light source [7].

We report here the preparation and characterization of BLPs, loaded with QD_{655} and surface-modified with preformed complexes of EGF-QD_{525} (EGF, Epidermal Growth Factor) mediated by biotin-streptavidin linkage. The receptor specificity of biotinylated EGF tagged with streptavidin coated QDs, has been extensively investigated in the laboratory, where this protocol was developed. EGF-QDs preformed complexes are biochemically competent ligands for erbB1, the EGF receptor [8]. BLPs were formulated containing up to 3 % of PEGylated-lipids to stabilize the lipid bilayer, and 2.7 % of fusogenic lipid in order to facilitate endosomal escape of loaded cargo into the cytoplasm. PEG (polyethylene glycol) content above 5 % may interfere for instance with the endocytic mechanism of uptake by either lowering the binding affinity to cell receptors or by preventing the intermembrane contact between liposomal surface and endosomal membranes, required to release the cargo in the cytoplasm [9]. In the present protocol QDs loaded BLPs were prepared using a detergent dialysis technique [10, 11] modified for the encapsulation of 10 nm carboxyl-QD_{655} (red) as an approach for delivering these fluorescent nanoparticles into cells.

QD_{655} loaded BLPs were purified by ultracentrifugation in discontinuous sucrose gradient and then characterized according to size, QDs encapsulation efficiency, and biotin incorporation.

Surface labeling and targeting of BLP-QD_{655} were achieved by coupling preformed complexes of biotin-EGF with a second color of Streptavidin coated QD_{525} (green) (Fig. 1) providing a monitoring tool for the specificity of BLPs uptake and cargo release in live cells by confocal laser scan microscopy (CLSM). The two-color particles, hereafter referred to as EGF-QD_{525}-BLP-QD_{655} were targeted to A431 cells, a human epidermoid carcinoma cell line that overexpresses the EGF receptor. The rationale behind this two-color labeling strategy is that only QD_{525} and QD_{655} that are colocalized would indicate the intracellular distribution of the targeted BLPs whereas time dependent loss of colocalization would suggest endosomal escape of QD_{655}.

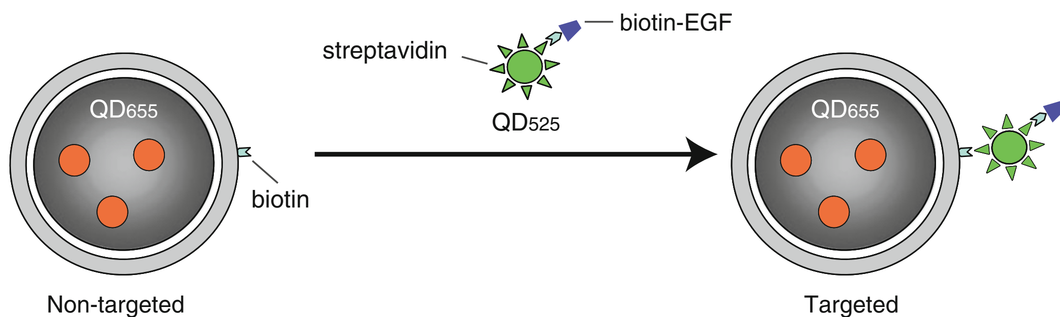

Fig. 1 Scheme depicting the targeting and dual color labeling strategy of BLPs with encapsulated QD_{655}. Preformed complexes of EGF-QD_{525} are conjugated to biotin molecules on the BLP surface. Specific uptake is evidenced as colocalized dots by confocal fluorescence microscopy. *Red fluorescence* indicates unspecific binding of non-targeted BLP-QD_{655}. Reproduced from Sigot et al. (2010) [15], with permission from American Chemical Society

2 Materials

Prepare all solutions using sterile ultrapure water and analytical grade reagents. Manipulate lipids dissolved in chloroform under a fume hood until they are desiccated and transfer volumes employing appropriate Hamilton syringes, avoiding plastic lab-ware. Protect lipid solutions; BLPs and fluorophores should be protected from direct light and maintain refrigerated.

2.1 Buffers

1. Tyrode's buffer without glucose: 135 mM NaCl, 10 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM of (2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.2. Autoclave before use and store at room temperature (20–25 °C).
2. Tyrode's plus: add sterile solutions of 20 mM glucose and 0.2 % of bovine serum albumin (BSA) just before use.
3. HEPES buffered saline (HBS): 10 mM HEPES, 150 mM NaCl, pH 7.4. Sterilized by filtration through a 0.22 μm-pore-size membrane.

2.2 Preparation of QDs Loaded and Targeted BLPs

Synthetic Lipids (Avanti Polar Lipids, Alabaster, AL, USA) were used as lyophilized powders or dissolved in chloroform and stored under argon in a desiccator at −20 °C.

1. DOPE: 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (neutral lipid).
2. DOTAP: 1,2-Dioleoyloxy-3-trimethylammoniumpropane chloride (cationic lipid).
3. PEG-750-Cer-C8: N-Octanoyl-Sphingosine-1-[Succinyl (Methoxy (Polyethylene Glycol) 750)] (fusogenic lipid).

4. Biotin-PEG-DSPE: 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N [Biotinyl (Polyethylene Glycol) 2000] (Ammonium Salt) (biotinylated lipid).
5. Chloroform (HPLC quality).
6. OGP: 1-o-n-octyl-β-D-glucopyranoside (detergent for dialysis).
7. ITK-carboxyl QD_{655} (CdSe core and ZnS shell) with maximum fluorescence emission peak at 655 nm. Hereafter referred to as QD_{655}.
8. Streptavidin-coated QD_{525}, maximum fluorescence emission peak at 525 nm, hereafter referred to as QD_{525}. Due to narrow emission spectra, alternative two non-overlapping QDs colors can be used.
9. Biotin-EGF (or appropriate biotinylated ligand).
10. Dialysis Cassette, Slide-A-Lyzer of 10 kDa cut-off for 0.5–3 mL sample (Pierce, Rockford, IL, USA).
11. SM-2 Biobeads with adsorbent capacity ~117 mg OGP/g beads (Bio-Rad, Hercules, CA, USA).

2.3 Preparing Discontinuous Sucrose Gradient

1. Prepare 20 % sucrose solution in HBS and filter-sterilize through a 0.22 μm pore size filter. Stock solutions of 20 % sucrose can be stored in frozen aliquots at −20 °C (*see* **Note 1**).
2. 2.5 % sucrose solution in HBS.
3. 10 % sucrose solution in HBS.
4. 2.5 mL thin-wall centrifuge tubes (*see* **Note 2**).

2.4 Measuring Biotin Incorporation in BLPs

1. Stock solution of 1 μM biocytin in sterile distilled water.
2. Freshly prepared mix containing 50 nM Alexa Fluor® 488-Streptavidin conjugates and 125 μM of 4-Hydroxyazobenzene-2-carboxylic acid (HABA) in HBS.

2.5 Cell Culture and Microscopy

1. Sterile Glass bottom cell culture chambers suitable for fluorescence microscopy.
2. Coverslips, acid washed and sterilized.
3. Cell culture medium and antibiotics appropriate for the particular cell line.

3 Methods

3.1 Preparation of BLPs with Encapsulated Quantum Dots

1. Prepare BLPs formulation by mixing 8.4 μmols DOPE, 0.6 μmols DOTAP, 0.024 μmols Biotin-PEG-DSPE, and 0.25 μmols of PEG-750-Cer-C8 in 2 mL glass vials (Fig. 2) (*see* **Note 3**).

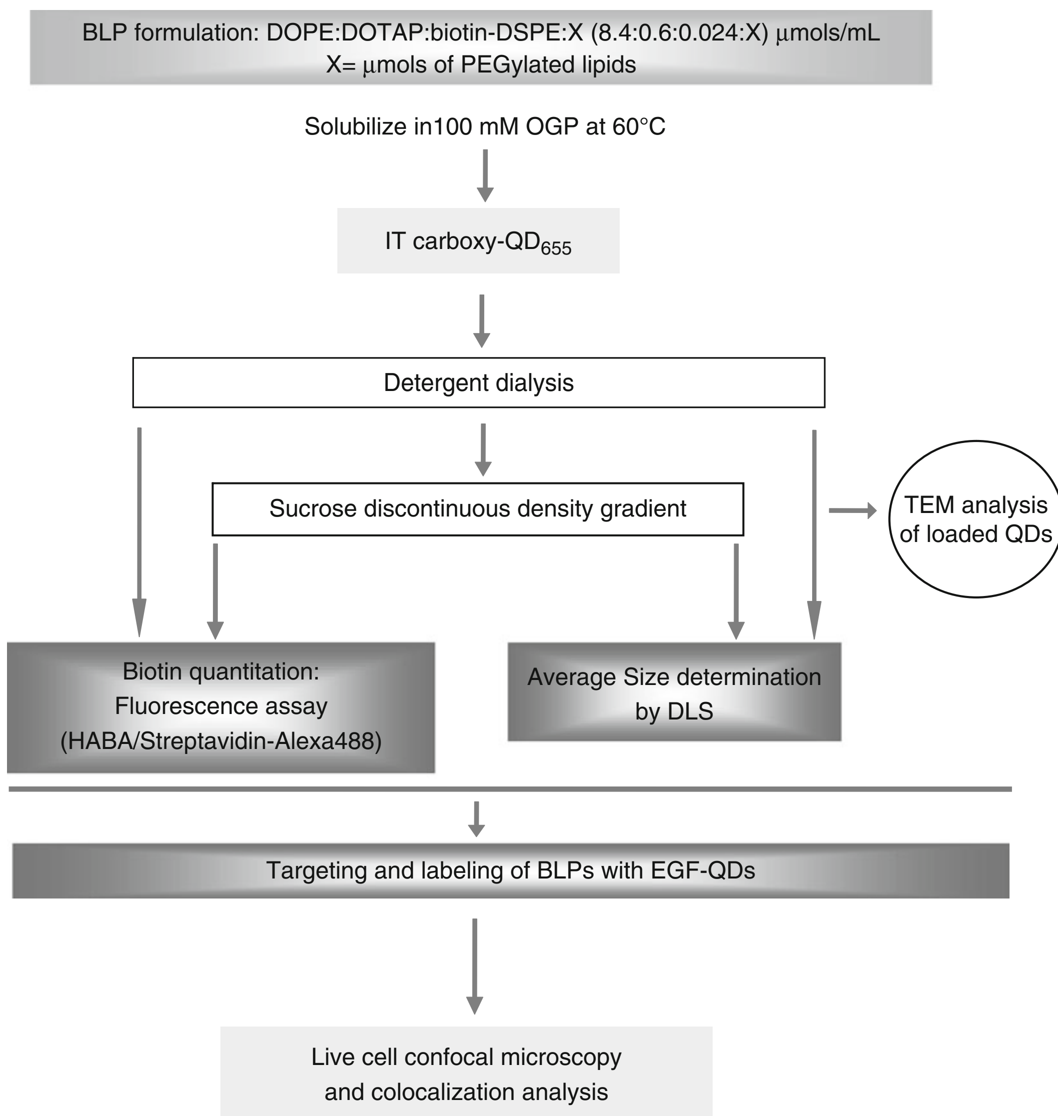

Fig. 2 Flow diagram of BLPs preparation, characterization, and targeting

2. Dry the solution with a stream of argon under a fume hood and remove residual chloroform under vacuum overnight.
3. Solubilize the opaque dry lipid film by adding 0.5 mL of 100 mM OGP in HBS with continuous stirring at 60 °C. Let the transparent lipid mix cool to room temperature (20–25 °C).
4. Prepare 0.5 mL of a 50 nM ITK-carboxyl QD_{655} in HBS (*see* **Note 4**) and add to the solubilized lipid–detergent mixture at room temperature (20–25 °C), mix well with micropipette and transfer the final 1 mL volume into the Dialysis Cassette.

In this step, and for any lipid variation in the original formulation, prepare a control of BLPs without QDs to check for vesicle formation during detergent dialysis.

5. Dialyze lipids-detergent-QDs solution and control formulation without QDs against 1 L HBS for 3 h (*see* **Note 5**).
6. Change buffer (1 L of HBS) and continue dialysis in a cold room (4–8 °C).
7. Carry out the last buffer change (third) adding polystyrene SM-2 Biobeads in the HBS buffer to minimize residual OGP content.
8. Recover the solution (~1 mL) from the dialysis cassette; this should not change at the end of dialysis. Keep on ice protected from light or in refrigerator while preparing the discontinuous sucrose density gradient (*see* **Note 6**).

3.2 Ultracentrifugation in Discontinuous Sucrose Density Gradient

In order to separate QDs loaded BLPs from less dense empty BLPs and free non-encapsulated QDs, load the samples obtained after the detergent dialysis on top of a discontinuous sucrose density gradient and perform ultracentrifugation as described:

1. Cool the ultracentrifuge with swinging buckets to 10 °C and set speed to 160,000 × *g*.
2. Prepare a discontinuous sucrose gradient in 2.5 mL ultraclear thin-wall centrifuge tubes by carefully applying with a tip or syringe, layers of 0.5 mL sterile sucrose solutions in HBS [12]. Two centrifuge tubes are required for loading the 1 mL sample recovered after detergent dialysis.
3. Add first 0.5 mL of 2.5 % w/v sucrose solution, under this fraction pipette 0.5 mL of 10 % sucrose fraction and under this, the 20 % sucrose (*see* **Note 7**). Carefully equilibrate the ultracentrifuge tubes by weight before adding dialysate containing the BLPs.
4. Add carefully 0.5 mL of the dialysate on top of the gradient by touching the internal wall of the tube over the 2.5 % sucrose band and place the ultracentrifuges tubes in the rotor with swinging buckets with the help of forceps. Carry out ultracentrifugation for 5 h at 10 °C.
5. After ultracentrifugation put the tubes in a transparent holder and briefly illuminate with UV light to excite QD_{655} fluorescence. Three well-defined fluorescent bands should be observed (Fig. 3, panel a). Mark with an indelible pen the QDs containing fractions.
6. Back in the bench, carefully recover sucrose fraction I (*see* **Note 8**) then, recover the broader, turbid, and less intense fluorescent band underneath (fraction II) enriched in BLP-QD_{655} and finally the pellet with free non-encapsulated QD_{655} (fraction III).

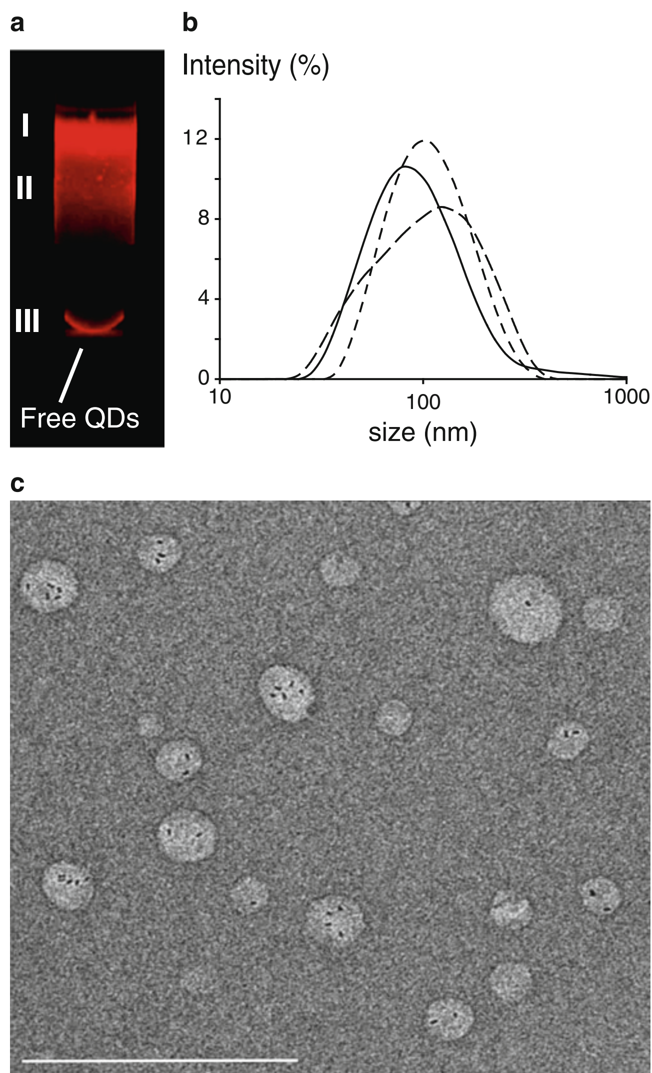

Fig. 3 Size characterization of BLP-QD_{655} after purification in discontinuous sucrose density gradient. (**a**) BLP-QD_{655} recovered in fluorescent fraction II, whereas free QDs appeared mainly in the pellet (fraction III). (**b**) Size distribution of three independent BLP-QD_{655} preparations measured in fraction II by DLS (**c**) TEM analysis of QD_{655} encapsulated in BLPs. Scale bar 500 nm. Reproduced from Sigot et al. (2010) [15], with permission from American Chemical Society

7. Take an aliquot of fraction II of the gradient and analyze for particle size, biotin incorporation, and QDs encapsulation efficiency (*see* **Note 9**).
8. Sterilize the BLP-QD_{655} samples through 0.22 μm filter, separate an aliquot for further characterization of the particles (Subheadings 3.3–3.5) and continue working under the laminar flow hood for BLPs surface labeling and cell targeting (Subheadings 3.6–3.9) (*see* **Note 10**).

3.3 BLPs-QDs Size Analysis

1. Measure average hydrodynamic diameter (Fig. 3, panel b) of recovered BLP-QD_{655} as well as control BLPs without QDs by Dynamic Light Scattering (DLS) (*see* **Note 11**).
2. Select an operating protocol for spherical particles; applying 12–15 runs per measurement, depending on the instrument number of runs is automatically selected according to the concentration of particles in the sample.
3. Perform three measurements on each sample and use the obtained intensity correlation data to calculate size distribution and average hydrodynamic diameter of the obtained BLPs. This can be achieved using the CONTIN algorithm [13] to fit the autocorrelation data, this mathematical approach is recommended for hetero- and polydisperse systems (*see* **Notes 12** and **13**).

3.4 QD_{655} Encapsulation Efficiency

Transmission Electron Microscopy (TEM) analysis of sucrose fraction II reveals an average of 2–5 QDs encapsulated in BLPs ranging from 100 to 130 nm in size (Fig. 3, panel c). Particle dimensions estimated from TEM are in agreement with the mean hydrodynamic diameter of ~110 nm as determined by DLS in the same fraction (Fig. 3, panel b).

3.5 Biotin Incorporation into BLPs

Biotin content was quantified using a fluorometric assay with nanomolar sensitivity based on Foster resonance energy transfer (FRET) [14]. Briefly, in the absence of biotin, HABA quenches the fluorescence emission of the Alexa Fluor® 488 dye via FRET. When biotin or a biotinylated molecule is added, HABA is displaced from the biotin binding sites resulting in an increase in the donor fluorescence intensity proportional to the amount of biotin present in the sample. Biotin concentration allows an estimation of biotinylated lipid recovery relative to the molar amounts of biotinylated lipid initially added to the formulation; and has to be checked for each liposomal preparation after detergent dialysis and after purification from sucrose gradient.

1. Prepare a standard curve (eight points in duplicate) of biocytin, a water-soluble biotin analogue, to give final concentrations between 0–200 nM.
2. Prepare before use, Mix-1 containing 25 nM Streptavidin-Alexa Fluor® 488 and 125 μM HABA in HBS.
3. Add 5 μL of standards or BLPs samples to 45 μL of Mix-1 in cuvettes suitable for fluorescence measurements. Excite Alexa Fluor® 488 with $\lambda = 485$ nm and collect spectra between 500 and 700 nm (*see* **Note 14**).
4. Plot fluorescence intensity at the emission maximum (519 nm) as a function of biocytin concentration (nM) and fit the data to a sigmoid curve to obtain the corresponding values for the unknown samples (Fig. 4) (*see* **Note 15**).

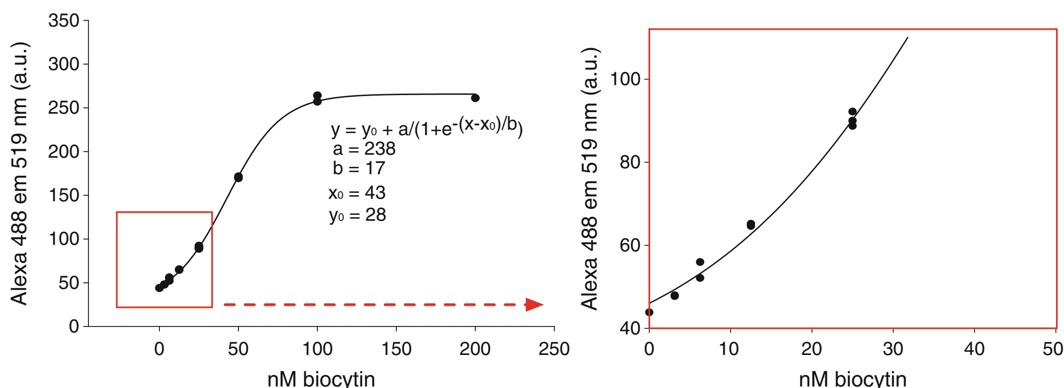

Fig. 4 Fluorometric assay for biotin quantitation. Biocytin standard curve concentrations range between 0 and 200 nM. Excitation of Alexa488 at 485 nm and emission collected from 500 to 700 nm. The data illustrate a ~6-fold increase in fluorescence signal upon complete displacement of HABA. The detection limit achieved was approximately 3 nM biocytin

3.6 Preparation of Ligand-Biotin-Streptavidin-QDs Complexes

The employed formulation of the streptavidin conjugated QDs contained an average of 6–8 covalently linked streptavidin molecules per QD molecule (*see* **Note 16**). Coupling Biotin-EGF to Streptavidin-QD_{525} at a ratio of 4:1 leaves free streptavidin in QDs for subsequent coupling to BLPs (*see* Subheading 3.7).

1. Dilute biotin-EGF (or biotinylated ligand of interest) to 400 nM in HBS + 0.2 % BSA.
2. Dilute Streptavidin-QD_{525} to 100 nM in HBS + 0.2 % BSA.
3. Mix equal volumes of Streptavidin-QD_{525} and biotinylated ligand with a micropipette.
4. Incubate at 4 °C for at least 30 min with gentle agitation or rotation before coupling to BLPs. This mix should be used within 5 days to prevent deterioration of the peptidic ligand.

3.7 Surface Modification of BLP-QD_{655} with Preformed Complexes of EGF-QD_{525}

1. Adjust biotin concentration of BLPs-QD_{655} to 1 μM and separate two aliquots of 150 μL.
2. Add 100 μL of preformed complexes of EGF-QD_{525} to 150 μL BLPs-QD_{655} and incubate for at least 2 h at 15 °C with continuous shaking (*see* **Note 17**).
3. In parallel, add 100 μL of HBS + 0.2 % BSA to 150 μL BLPs-QD_{655} and incubate for at least 2 h at 15 °C with continuous shaking.

3.8 Cell Targeting of EGF-QD_{525}-BLP-QD_{655} Particles

1. Plate cells in culture dishes containing sterile glass coverslips or in glass bottom culture chambers suitable for fluorescence confocal microscopy.
2. Grow A431 cells or the selected cells expressing the receptor of interest in complete culture medium with antibiotics at the appropriate densities 1 or 2 days prior to the experiment.

3. Prior to the incubation with BLPs starve cells in serum free medium for 16 h to reduce signaling induced by EGF present in the serum.
4. Set the time lapse acquisition parameters in a confocal microscope (*see* Subheading 3.9).
5. Wash cells once with Tyrode's plus and maintain in this buffer for the experiment.
6. Dilute EGF-QD_{525}-BLP-QD_{655} in Tyrode buffer plus to obtain final concentration of 4 nM EGF.
7. Follow identical dilutions for the non-targeted BLPs-QD_{655} as performed with the targeted particles and use this solution as control to test for unspecific uptake.
8. Pre-incubate starved A431 cells with 200 μL of targeted EGF-QD_{525}-BLP-QD_{655} or non-targeted BLP-QD_{655} at 15 °C for 5 min to concentrate BLPs on the cells surface, at this temperature EGF receptor mediated endocytosis can be reversibly blocked.
9. Place the cell chamber on the microscope stage pre-equilibrated to 37 °C.
10. After initial binding (within 5 min) at 37 °C, take a snapshot; carefully remove excess of BLPs solution, BLPs in suspension will contribute to background fluorescence.
11. Add 500 μL of warm Tyrode's plus buffer and start a time lapse imaging acquiring every 5 min during 30 min or until endocytic uptake of the BLPs is evident (*see* **Note 18**).

When employing a different biotinylated ligand specific receptor targeting should be tested in the presence of soluble excess ligand and/or using cells devoid of the receptor of interest [15] and optimal concentrations of ligands has to be checked according to the binding constant for stimulated uptake of specific receptors. Residual free preformed complexes (EGF-QD_{525}) were expected to bind to EGF receptors (Fig. 5).

3.9 Simultaneous Detection of Two Different Colors of QDs and Colocalization Analysis

1. Begin acquisition of a time series with single or multiple focal planes (z-stack). Typically, a 63× or 40× 1.2 numerical aperture (NA) water immersion objective is recommended.
2. Simultaneous excitation of QD_{525} and QD_{655} can be achieved at 488 nm employing an imaging system with two detectors, e.g., Zeiss LSM 510 META, with appropriate filters (in this case, 520/20 band-pass and 585 long-pass, respectively (*see* **Note 19**)).
3. For visual analysis of BLPs internalization, subtract background from each channel and to every single focal plane of a z-stack at each time point. Then, reconstruct the 3D image and merge both channels to look for colocalization.

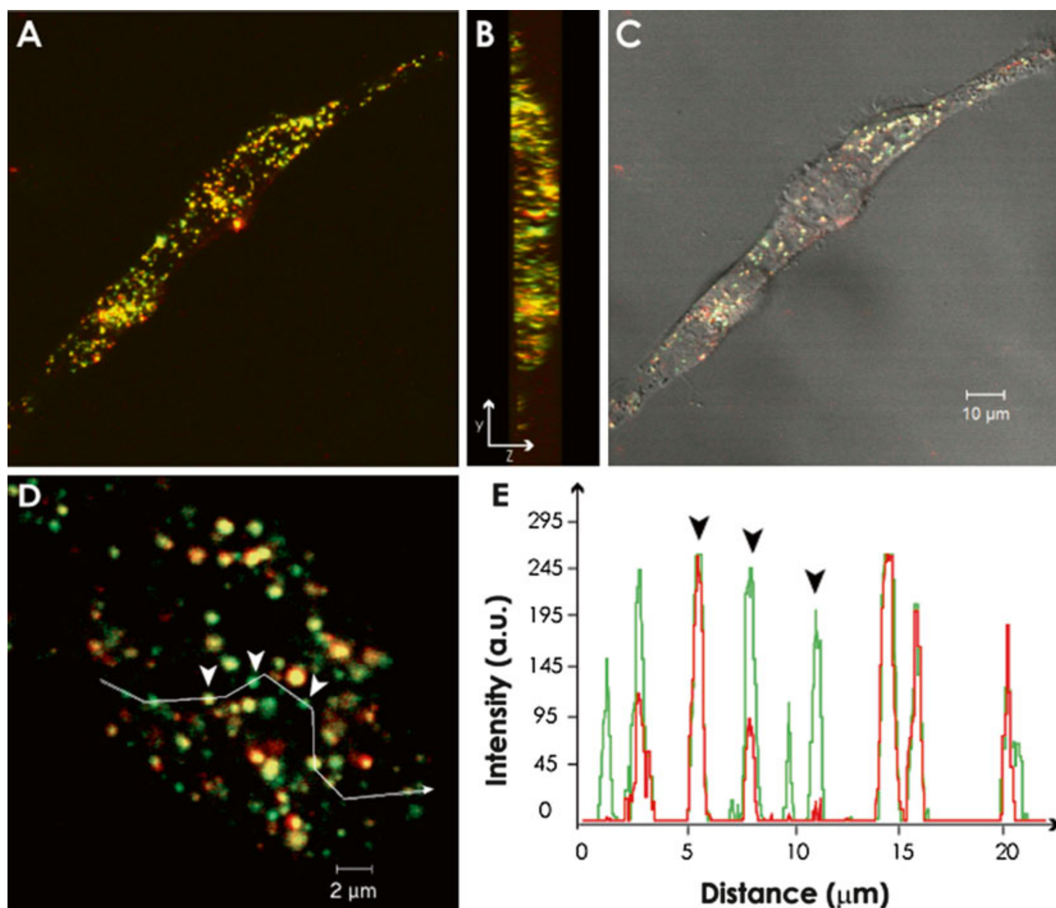

Fig. 5 Intracellular fate of EGF Receptor targeted and QDs labeled BLP. (**a**) 3D reconstruction of two A431 cells with internalized BLP after 10 min pulse incubation and 1 h chase at 37 °C. (**b**) *y–z* plane showing colocalized QDs inside cells and underneath the cell membrane. (**c**) Overlay of image (**a**) with DIC image. (**d**) Line-profile across endosomal vesicles. (**e**) Fluorescence intensity along the line-profile in (**d**) revealing different intensity levels for colocalized QDs as well as size variations among vesicles. Internalized two-color QDs labeled and targeted BLP were distinguished from independently internalized green EGF-QD_{525} complexes (*arrowheads* in panels *d* and *e*). Scale bar 2 μm. Reproduced from Sigot et al. (2010) [15], with permission from American Chemical Society

Select z-stack at $t = 0$ min, when BLPs are bound but not internalized and then select z-stacks after 5, 10, 15, 20, and 30 min (end point) imaging at room temperature (20–25 °C). Additionally, 2D representations of 3D cells can be created from maximum intensity projections of several slices in the z-dimension, excluding the top and bottom planes of all cells in the microscopic field.

4. Analyze colocalization of both QDs in background-corrected images of single focal intracellular planes (not projections) by presenting a plate of three images, two corresponding to the

red (loaded QD_{655}) and green (surface tagged QD_{525}) channels, and a third image where the channels are merged and the overlapping pixels displayed in yellow (Fig. 5).

5. Quantitate colocalization of QD_{525} and QD_{655} by calculating Manders' overlap coefficients [16] (*see* **Note 20**).

4 Notes

1. Thaw 20 % sterile sucrose solution in the refrigerator and mix well until the solution appears homogeneous before proceeding with subsequent dilutions. Is preferable to prepare multiple aliquots of the stock and use one for each gradient to prevent bacterial contamination.
2. For this purpose we employed Beckman ultraclear thin-wall centrifuge tubes in TL-100 Beckman ultracentrifuge and rotor TLS 55 with swinging buckets.
3. If lipids are obtained as lyophilized powders, dissolve first each lipid in chloroform and transfer volumes with Hamilton syringe or glass pipettes, avoid plastic tips during this step, contaminants could be extracted by chloroform and micropipette can be damaged as well.
4. Commercially obtained QDs solution should be homogeneous and free of aggregates. Number of streptavidins conjugated to QDs has to be check for each lot number of the product from the selected supplier.
5. Visible turbidity should be detected within the first hour of dialysis, indicating the formation of self-assembled lipid–QDs particles. The particles should remain in solution without forming aggregates. Empty self-assembled BLPs are smaller ~50–70 nm size and their true presence has to be checked by DLS.
6. BLP-QD_{655} cannot be frozen due to irreversibly QDs fluorescence quenching and destabilization of BLPs bilayer and should be stored refrigerated (4–8 °C), for storage periods longer than a week, BLPs solution should be filter-sterilized through 0.22 μm pore size. However, this step results in considerable sample loss.
7. Use a syringe with long needle to load the solutions at the bottom of the tubes. Clear lines between the layers should be seen, indicating that minimal mixing has occurred.
8. Sucrose fraction I, may contain QD loaded BLPs but it is enriched in free lipids and empty vesicles and is not further employed.
9. Sucrose does not interfere with DLS measurements, biotin quantitation, or subsequent BLPs labeling. However, is

susceptible of bacterial contamination. Alternatively, samples can be dialyzed against 2 L of sterile HBS to minimize sucrose content before particle analysis.

10. Significant sample loss occurs during filtering. In our experience, using sterile solutions and labware during BLPs preparation and working under a laminar flow during cell targeting allowed us to perform BLPs binding and uptake experiments for up to 24 h in the presence of antibiotics with occasional contamination.
11. DLS measurements were performed with ZetaSizer Nano from Malvern Instruments. Samples were diluted in phosphate buffered saline (PBS) and measurements performed at room temperature (20–25 °C). The PBS used to dilute the samples was previously filtered through 0.02 μm pore size to eliminate potential interfering impurities.
12. The mean hydrodynamic diameter of a population of particles calculated by Dynamic light scattering involves the determination of how the intensity of the light scattered by a solution of moving particles varies with time. This variation is correlated with the speed at which particles move, which can be characterized by their diffusion coefficients [17]. The average hydrodynamic diameter of particles is obtained from the diffusion coefficients.
13. The average hydrodynamic diameter represents only an intensity-based average value and does not give any information on the prevailing size distribution. For this reason, the polydispersity index (pdi) is also stated to give information about the actual distortion of a monomodal distribution. The pdi can have values between 0 and 1 and is equivalent to the variance σ^2 of the size distribution. BLPs preparations with pdi <0.25 were considered as monodisperse solutions and further characterized.
14. It is convenient to test several dilutions in duplicate of BLPs to obtain an accurate biotin concentration, high concentration of BLPs particles may also interfere in biotin quantitation due light scattering.
15. In the conditions assayed a ~6-fold increase in fluorescence signal upon complete displacement of HABA was observed with a detection limit of approximately 3 nM biocytin. Standard colorimetric HABA assay can be performed for samples containing above 10 μM biotin.
16. Previous control experiments are necessary to establish that streptavidin-QDs does not interfere with the normal biological function of the selected biotin-ligand [18].
17. Although increasing the number of EGF ligands per particle should in principle improve the binding to cells and increase the

number of internalized particles, larger size of the carrier would probably interfere with the receptor-mediated endocytosis [19].

18. A431 cells are maintained healthy in Tyrode's plus for up to 30 min at 37 °C. However, for longer incubations Tyrode's buffer should be replaced by phenol free culture medium and imaging performed under oxygen-controlled atmosphere. The labeled and targeted BLPs can be continuously monitored using confocal microscopy during cell binding and uptake for periods longer than 1 h without significant photobleaching highlighting the convenience of QDs as labeling probes for long-term imaging of dynamic processes. However, cell phototoxicity cannot be overlooked.
19. Within the given settings, channel cross-talk was negligible as determined using single-labeled samples.
20. Manders' coefficients are proportional to the fraction of fluorescence of the colocalizing fluorophores in each channel of the composite image, i.e., relative to the total fluorescence in that channel. These coefficients are more appropriate when signal intensities in the two image channels have significant different levels.

Acknowledgements

This work was financially supported by the Max Planck Society, the DFG Research Center for Molecular Physiology of the Brain (CMPB)—Excellence Cluster EXC 171—FZT103 "Microscopy at the Nanometer Range," and the EU sixth framework (FP6) project FLUOROMAG, LHSB-CT-2006-037465.

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