

Fluorescent immunolabeling of cancer cells by quantum dots and antibody scFv fragment

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Abstract. Semiconductor quantum dots (QDs) coupled with cancer-specific targeting ligands are new promising agents for fluorescent visualization of cancer cells. Human epidermal growth factor receptor 2/neu (HER2/neu), overexpressed on the surface of many cancer cells, is an important target for cancer diagnostics. Antibody scFv fragments as a targeting agent for direct delivery of fluorophores offer significant advantages over full-size antibodies due to their small size, lower cross-reactivity, and immunogenicity. We have used quantum dots linked to anti-HER2/neu 4D5 scFv antibody to label HER2/neu-overexpressing live cells. Labeling of target cells was shown to have high brightness, photostability, and specificity. The results indicate that construction based on quantum dots and scFv antibody can be successfully used for cancer cell visualization. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3122775]

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1 Introduction

The biological application of nanoparticles is a rapidly developing area of nanotechnology that raises new possibilities in the diagnostics and treatment of human cancers.^{1,2}

Semiconductor quantum dots (QDs) are tiny light-emitting particles on the nanometer scale and represent a new class of fluorescent labels for biology and medicine. Compared with conventional fluorophores (organic dyes and fluorescent proteins), quantum dots have unique optical properties, offering advantages for biomedical application. These are size-tunable symmetric narrow emission spectrum, broad absorption spectrum, and high resistance to photobleaching.^{3,4} In cancer diagnostics, fluorescent nanoparticles such as QDs coupled with cancer-specific targeting carriers are highly promising agents for fluorescent labeling and determination of immune status of tumors, as well as for visualizing of peripheral metastases.⁵

The object of our study was human epidermal growth factor receptor 2/neu (HER2/neu) overexpressed cancer cells. HER2/neu overexpression correlates with poor prognosis for patient treatment and high chemotherapy resistance. Therefore, detection of HER2/neu-overexpressing cells is of great

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clinical importance.^{6,7} Antibodies against cancer markers located on the surface of tumor cells (e.g., cell surface antigens or receptors overexpressed in cancer cells) are excellent targeting agents ensuring directed delivery of fluorescent nanoparticles to the tumor. A number of studies have demonstrated the feasibility of coupling QDs to a variety of antibodies for tumor targeting. QDs coupled with full-size monoclonal anti-HER2/neu antibodies have been successfully used for fluorescent detection of HER2/neu-overexpressing cancer cells.^{8–11} The antibodies were bound to nanocrystals by cross-linking reagents or using a three-layer labeling strategy based on a biotin-streptavidin system (primary antibody followed by biotinylated secondary antibody, followed by streptavidin-QDs conjugate).

Recently, constructions based on antibody fragments are regarded to be promising as targeting agents for medicine. These small fragments of antibody, while retaining the same binding specificity, are more efficient at penetrating tumor masses because of their smaller size, do not interact with receptors of immune system cells and proteins of the complement system, and are more effectively cleared from the circulation. One of the variants of such constructions is single-chain Fv fragment (scFv)—recombinant polypeptide in which variable domains of heavy and light chains (VL and VH) are connected by a flexible linker.¹² Such small-size (25 to 30 kDa) antibody fragments show improved tumor penetration, more homogenous tissue distribution, and much more rapid blood clearance properties compared with intact antibody when used *in vivo*.^{12–14} These characteristics make them potentially more useful carriers of nanoparticles.

In this work, we describe a fluorescent complex for specific visualization of HER2/neu overexpressing cells using CdSe/CdS core-shell QDs and anti-HER2/neu 4D5 scFv, which are characterized by serum stability and ability of highly effective coupling with its antigen-target—extracellular domain of HER2/neu.¹⁵ QDs were bound to 4D5 scFv by a previously described barnase-barstar system analogous to the streptavidin-biotin system.¹⁶

2 Materials and Methods

2.1 Synthesis and Solubilization of Nanocrystals

CdSe QD cores capped with oleic acid were synthesized by a high-temperature method using high-boiling organic solvent starting from cadmium oleate and trioctylphosphine selenide, as we described earlier.¹⁷ Core sizes were obtained by luminescent spectroscopy using luminescence wavelength dependence on CdSe QD size.¹⁸

An analogous method with trioctylphosphine sulfide was used for CdS shell growth. CdSe QD cores (50 mg in 1 ml hexane) were added to cadmium oleate solution (0.5 mmol in 5 ml hexadecane pure, made as described for diphenylether), and the mixture was rapidly heated under argon atmosphere. When reaction mixture temperature was near 250 °C, 0.5 ml 1 M trioctylphosphine sulfide [(C₈H₁₇)₃PS] solution in trioctylphosphine was injected in the reaction mixture with vigorous stirring. The temperature was stabilized at 250 °C for 15 min for growth of CdS shell. Isolation and purification of the obtained CdSe/CdS heterostructures were analogous for CdSe cores.

QDs were treated with mercaptoacetic acid (MAA, HSCH₂COOH), which allows transferring them into water solution. For ligand exchange, MAA was slowly added to hexane solution of CdSe/CdS with vigorous stirring at room temperature. When QD coagulation was detected, MAA addition was finished, and the reaction mixture was stirred about 30 min. Twofold MAA quantity was typically required to exchange oleate CCO⁻ groups on QDs surfaces. MAA-modified QDs were initially water insoluble, but low ammonia addition resulted in solubility of QDs in water.

The composition and optical properties of synthesized QDs were examined by UV-VIS absorbance, IR, and photoluminescence spectroscopy.

The optical absorption spectra were obtained with Perkin-Elmer Lambda-35 for UV-VIS and SpectrumOne for IR range spectrometers.

For IR measurement, the QDs were precipitated by ethyl ether and dried under vacuum. IR spectra were registered using a solid-state sampler for SpectrumOne with ZnSe/diamond optics.

The fluorescence spectra of water solutions of unconjugated QDs and barstar-QDs were obtained with Cary Eclipse (Varian) fluorescence spectrophotometer. QDs were excited at 350 nm.

2.2 Isolation and Purification of Proteins

Isolation and purification of barstar (monoalanine mutant C40A containing the cysteine residue at position 82 only) were performed by the method described in Ref. 19, with modifications. To produce barstar, *E. coli* HB101 was transformed with pMT641 plasmid.²⁰ Transformed cells were grown in YTPS broth (1% yeastrel, 1% trypton, 0.5% NaCl, 80 mM K₂HPO₄, 20 mM KH₂PO₄, 2 mM MgCl₂, 0.1 g/l ampicillin, pH 7.4) at 37 °C until a stationary growth phase was attained, and then the cells were harvested by centrifugation at 7000 g for 10 min at 4 °C.

Further, the cells were resuspended in lysis buffer (20 mM Tris, 10 mM KH₂PO₄, 10 mM EDTA, 10 mM 1,4-dithiothreitol, 100 mM NaCl, pH 8.0) and sonicated on ice. The obtained lysate was clarified by centrifugation at 18,500 g, nucleic acids were precipitated by 0.04% polyethyleneimine, and proteins were fractionated by step salting-out of ammonium sulphate. A 40 to 80% fraction was solved in 0.1 M tris-HCl, 10 mM EDTA, 10 mM 1,4-dithiothreitol (pH 8.0) buffer and applied on a C16/100 column with 180 ml superfine sephadex G-100 balanced by TSDT buffer (20 mM Tris, 20 mM NaCl, 2 mM 1,4-dithiothreitol, 0.05% Tween-20, pH 8.5). 115 to 140 ml eluate fraction was applied on a Hi-Trap with FastFlow Qsepharose 1 ml (GE Healthcare), washed subsequently by TSDT and TDG buffer (20 mM Tris, 4 mM 1,4-dithiothreitol, 10% glycerin, pH 8.5) and eluted by NaCl concentration gradient from 0 to 1 M in TDG buffer. Homogeneous barstar was eluted with 100 mM NaCl.

4D5 scFv-dibarnase fusion protein was extracted using the procedure described in Ref. 16.

2.3 Synthesis of QD-Barstar Conjugate

The resultant MAA-modified QDs were conjugated with barstar using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma) as a cross-linker. QDs in 20 mM

borate buffer pH 6.5 were first activated with EDC at room temperature for 15 min. The mixture was then purified through Sephadex G-25 column eluted with 20 mM borate buffer pH 7.4. Subsequently, barstar in the same buffer was added to the solution and reacted for 1 h at room temperature. Unbound protein was separated by Sephadex G-25 column eluted with PBS buffer pH 7.4. QDs, barstar, and EDC were mixed in molar ratio of 1:20:20, respectively.

2.4 Cell Cultures and Immunofluorescent Labeling

Human breast cancer SKBR-3 cells (HTB 30, ATCC) were seeded in 96-well plates (Corning, New York) containing RPMI-1640 medium (PanEco, Russia) with 10% fetal calf serum (HyClone, Belgium) and 2 mM L-glutamine, at density up to 2×10^3 cell per well and cultured at 37 °C with 5% CO₂ overnight.

The ingredients used for incubation were dissolved in 100 μ l PBS (pH 7.4) with 1% fetal calf serum, 25 mM 2-deoxyglucose, and 0.01% sodium azide.

Unfixed live cells were gently washed twice with PBS (pH 7.4). The cells were then incubated on ice sequentially with 10 μ g/ml 4D5 scFv-dibarnase that binds to the external domain of HER2/neu, and 80 nM barstar-conjugated QDs for 40 min each. Barstar-QD conjugates without antibody were incubated with the cells and served as a control.

Cells were imaged live using a Zeiss Axiovert 200 inverted epifluorescence microscope with a 40 \times lens and an AxioCam HRc CCD camera (Zeiss). Bright-field images were collected using phase contrast mode. QDs were excited at 332 to 382 nm with a mercury lamp as a light source. QD fluorescent images were collected using a 575 to 640-nm emission filter.

3 Results and Discussion

CdSe/CdS core-shell, 3.5-nm-diam QDs, synthesized by high-temperature method, were soluble only in nonpolar solvents (hexane, chloroform). Presence of $-\text{COO}^-$ vibration bands in the absence of $-\text{COOH}$ vibrational bands at IR spectrum (Fig. 1, dashed line) revealed that the as-synthesized QDs capped with oleate-ions, but not with the oleic acid itself.

Biocompatible water-soluble QDs were obtained by modifying the surface with mercaptoacetic acid (MAA)—a bifunctional compound capable of replacing oleate-anions on the surface of nanocrystals and containing hydrophilic groups that provide water solubility. This method of nanocrystal solubilization is widely used²¹ and was chosen because of its convenience and simplicity.

IR spectroscopy (Fig. 1, solidline) revealed successful replacing of hydrophobic oleate-anions by mercaptoacetic residues: C-H vibrational band intensity at around 3000 cm^{-1} significantly decreased, $-\text{COO}^-$ groups remained, and the C-S vibrational band at around 500 cm^{-1} appeared. The simultaneous presence of C-S vibrational band and absence of S-H vibrational band (Fig. 1, solidline) reveal that MAA molecule binds to QD surface using mercapto-group, but not carboxyl group. The latter group remains free (in $-\text{COO}^-$ ion form) for conjugation to proteins.

Water-soluble nanocrystals retained their nonaggregate state and fluorescence ability. Figure 2 (solidline) presents the

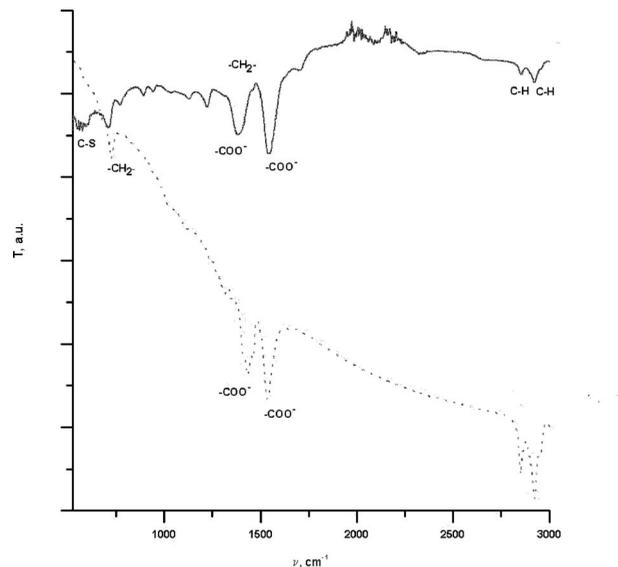


Fig. 1 IR spectra of QDs capped with oleic acid (dashed line) and mercaptoacetic acid (solid line).

fluorescence spectrum of water-soluble QDs with emission centered at 605 nm.

Immunofluorescent labeling, cell imaging, and other biological applications required creating of target QD-antibody constructions. Couples of specific molecules that are not sticky themselves but can stick only to each other provide a good opportunity for QD-based construction design. In this work, the previously described barnase-barstar module¹⁶ was used for binding QDs to scFv antibody. Bacterial ribonuclease from *Bacillus amyloliquefaciens* barnase and barstar, its natural inhibitor, are small-size proteins (12 and 10 kDa, respectively) that are highly affine to each other ($K_d \sim 10^{-14}$ M).

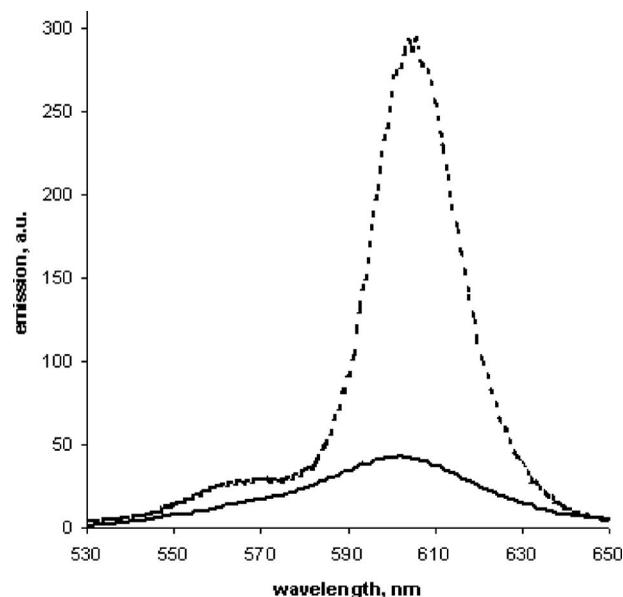


Fig. 2 Fluorescence spectra with absorption normalized at the excitation ($\lambda_{\text{exc}}=350$) of water-soluble MAA-coated quantum dots (solid line) and QD-barstar conjugates (dashed line).

Conjugation of one of these proteins to some antibody and the other to QDs provides a directed delivery of QDs to the target of interest. This construction can be used as molecular “LEGO bricks,”²² allowing us to obtain complex of once conjugated to barstar or barnase QDs and different antibody. In this context, the barnase-barstar module can be compared only to the biotin-streptavidin system but, in contrast to it, has some advantages.²²

To perform subsequent studies, MAA-coated QDs were conjugated to the barstar protein, a component of the barnase-barstar module.

It was observed that MAA-modified QDs precipitated in 3 to 4 days. Barstar-conjugated QDs remained dissolved for at least 2 to 2.5 weeks in the same conditions. Figure 2 presents fluorescence spectra with absorption normalized at the excitation of water-soluble MAA-coated quantum dots and QD-barstar conjugates. The spectra at Fig. 2 indicate that quantum yield of fluorescence was enhanced about three times after QDs conjugation to barstar. A red shift of the fluorescence peak (608 nm), as compared with that of unconjugated QDs (605 nm) was also observed.

So, barstar-QD conjugates (as well as water-soluble QDs) retain their nonaggregate state and ability to fluoresce. The conjugation allows not only QDs binding to fusions of different antibodies with barnase, but also, enhanced quantum yield and stability of MAA-coated QDs in water solutions.

A low fluorescence quantum yield as well as insufficient stability of QDs solubilized using MAA were noted earlier by other authors.^{23–25} This is presumably determined by the dynamical character of MAA molecules bound with nanocrystal surface (S–S and/or Cd–S bonds). Stabilization of nanocrystals and increased quantum yield of fluorescence after formation of conjugates with small proteins (such as barstar) may be explained by the change of polarity of quantum dots in the environment and by neutralization of surface charge, as was demonstrated by other authors.²⁶ Another possible explanation in the case of cystein-containing barstar mutant used in our work is additional passivation due to protein SH-groups linking to the surface of a quantum dot.

The applicability of the barnase-barstar module for target delivery of QDs was examined by the example of HER2/neu-overexpressing cell imaging.

HER2/neu belong to the epidermal growth factor receptor (EGFR) family. Family members are found on the surface of eukaryote cells and generally play a role in the regulation of cellular growth and differentiation. Homodimerization and heterodimerization among family members promote signal transduction cascade and cellular proliferation.²⁷

Modular targeting molecules with 4D5 scFv antibody fusion to two barnase molecules in series were used as a carrier for QD-barstar. We have shown earlier that such molecules very efficiently bind to the external domain of HER2/neu cancer marker.^{16,28} The QD-barstar conjugates effectively stained HER2/neu on the surface of human breast cancer SKBR-3 cells after the cells were incubated with a 4D5 scFv-dibarnase. When the cells were incubated with QD-barstar alone, weak or no detectable signal was observed on the cell surface, indicating that the QD-barstar conjugates have very low nonspecific binding (Fig. 3).

To conclude, we have demonstrated effective application of CdSe/CdS semiconductor nanocrystals obtained in our re-

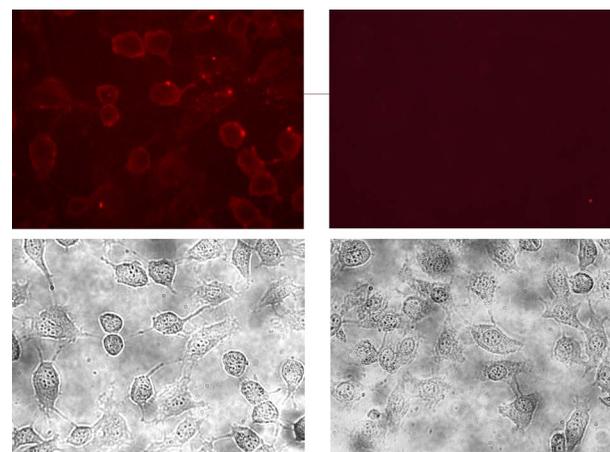


Fig. 3 Detection of HER2/neu-overexpressing SKBR-3 cells with QDs and 4D5 scFv. Top row: Fluorescence channel. Bottom row: bright-field images. Left: Live breast cancer SKBR-3 cells were incubated with 4D5 scFv-dibarnase and QD-barstar conjugate. Right: Cells were incubated with QD-barstar conjugates only.

search for visualization of HER2/neu-overexpressing cancer cells using 4D5 scFv antibodies and the barnase-barstar system. Such target fluorescent complexes may be useful tools for different applications in cellular biology, immunohistochemistry, and intravital cancer imaging.

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