Talanta 81 (2010) 505–509

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fluorescent quantum dot-labeled aptamer bioprobes specifically targeting mouse liver cancer cells

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ARTICLE INFO

Article history:
Received 4 September 2009
Received in revised form
17 December 2009
Accepted 20 December 2009
Available online 29 December 2009

Keywords:
Quantum dot
Aptamer
Probe
Label
Cell

ABSTRACT

Fluorophore-labeled bioprobes are the key for fluorescent-labeled imaging technology. In the present work, mouse liver hepatoma cell line BNL 1ME A.7R.1 (MEAR)-specific ssDNA aptamer TLS9a was used to fabricate quantum dot-labeled aptamer bioprobe (QD-Apt), which was obtained by conjugating streptavidin-modified quantum dots (SA-QDs) with biotin-derived aptamer via the interaction between biotin and streptavidin. The QD-Apt was of monodispersity and excellent fluorescence properties. When the optimum ratio of SA-QDs to aptamer, which is 1:16, was used in the preparation of the QD-Apt, the resultant QD-Apt was of satisfactory bioactivity. They could specifically recognize MEAR cells and could not recognize BNL cells and Hela cells. Particularly, the growth and viability of QD-Apt bound MEAR cells were not affected by QD-Apt within 84 h compared to control cells, indicating that the probe was biocompatible and suitable for live cell imaging.

1. Introduction

Fluorescent-labeled imaging has been an important technique for cell biology research. Fluorophore-labeled bioprobes are integrated both with biomolecules capable of specific recognition and fluorophores. Aptamers, screened out by systematic evolution of ligand by exponential enrichment (SELEX) [1,2], can almost meet the needs for recognizing various components of cells even the whole cells. Hence, aptamer-based bioprobes are powerful for cell imaging and discriminating, and discovery of biomarkers for cancer cells and so on [3–17]. Unique fluorescence properties of quantum dots (QDs) make them an excellent biolabel. With the development of preparation and surface modification of QDs, cellular molecule tracking and *in vivo* imaging based on QD-labeled fluorescent bioprobes are attracting more and more attention. In 2004, Dwarakanath et al. first reported a QD-labeled aptamer bioprobe for recognition of *E. coli* O111:B4 bacteria [12]. Ikanovic et al. screened out a DNA aptamer for bacillus thuringiensis (BT) spores and then semiquantitatively determined BT spores using a QD-labeled aptamer probe [18]. Chu et al. conjugated A9 aptamer of prostate-specific membrane antigen (PSMA) with QDs and realized the labeling of fixed cells, live cells or prostate tumor cells (LNCaP) in mimicked collagen matrix [19]. In our group a DNA aptamer GBI-10 specific for tenascin-C was conjugated with CdSe/ZnS QDs to produce a bioprobe, which could specifically recognize glioma cells with massively surface-expressed tenascin-C [20]. Bagalkot et al. constructed QD-Apt-doxorubicin tertiary complex. Such a multifunctional nanosystem could deliver doxorubicin specifically to target tumor cells and at the same time monitor the process. The tertiary complex was of specificity and sensitivity, being promising in cancer imaging and therapy [21]. Ko et al. used two QD-labeled probes to realize synchronous imaging of nucleolin and integrin avb3 in cancer cells at a subcellular level [22]. It can be seen that fluorescent QD-labeled aptamer bioprobes are of good prospects in cell imaging, especially caner cell imaging and detection. However, the methods for construction of the bioprobes need to be developed, and more systematic researches are highly necessary.

In the present work, one simple and universalizable method for construction of the QD-Apt was developed. The fluorescent QD-labeled TLS9a aptamer bioprobe (QD-Apt) was constructed by interaction of biotin with streptavidin. TLS9a is one of the DNA aptamers of mouse liver hepatoma cell line BNL 1ME A.7R.1 (MEAR) cells. It has a sequence of only 39 nucleotides whereas its K_d value is as low as 7.38 nM [3]. The very strong and specific affinity between streptavidin and biotin makes the probe obtainable under mild reaction conditions. The preparation is facile and repeatable, and the reaction can be finished within 1 h at room temperature. The QD-Apt can specifically recognize MEAR cells. The bioprobe is so biocompatible that it does not affect the cell growth or cell viabil-

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doi:10.1016/j.talanta.2009.12.031

ity after its recognition of cells, indicating that its performance is excellent and it is promising.

2. Experimental

2.1. Reagents

Streptavidin-modified QDs solution (SA-QDs, $\lambda_{em} = 605 \pm 5$ nm) was purchased from Wuhan Jiayuan Quantum Dots Co., Ltd, China. Biotinylated TLS9a aptamer, with an ssDNA sequence of 5′-biotin-ttttttttttAGTCCATTTTATTCCTGAATATTTGTTAA CCTCATGGAC, was synthesized by Sangon Biochemistry Company, China. The BNL 1ME A.7R.1 (MEAR) mouse liver hepatoma cell line and its normal BNL CL2 (BNL) cell line as the control were friendly provided by Prof. W. Tan at the University of Florida (USA).

2.2. Preparation of the QD-aptamer bioprobe

The 5′-biotin-TLS9a aptamer was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) at given concentrations. Then, certain amount of aptamer solution was added into the SA-QDs solution followed by gently shaking for 1 h at room temperature. Then the solution was super-filtrated (100000 MWCO, 4 mL, Millipore) to remove the redundant aptamer and purified through a D-Salt™ Dextran Desalting Column (5 mL, Thermo), with the QD-Apt bioprobe being obtained. The probe was dissolved in PBS (20.8 mM NaH_2PO_4, 84 mM Na_2HPO_4, pH 7.4) and stored at 4 °C.

2.3. Agarose gel electrophoresis

The 1× TAE buffer (dilution of 50× TAE buffer: 40 mM Tris, 20 mM Acetic acid, 2 mM EDTA, pH 8.0) was used as the electrophoretic buffer. The concentration of agarose gel was 0.5%. 6 μL of QD-Apt bioprobe solution was injected into the loading hole with 6 μL of SA-QDs in the first hole as the control. Bromophenol Blue was used as an indicator in the electrophoresis. After electrophoresis for 1.5 h (5 V cm^{-1}), the gel was illuminated with an ultraviolet transilluminator (Bio-Print, Vilber Lourmat) and imaged with a CCD with BiocaptMw software.

2.4. Dynamic light scattering measurement

The QD-Apt bioprobe was filtered with 0.22 μm filtration membrane to remove aggregated particles. Then the SA-QDs in PBS and the QD-Apt solutions prepared in different ratios of QDs to the aptamer were respectively measured with a Nano-ZS 90 Zetasizer (Malvern Instruments Ltd) at 20 °C. The data of Z-Average diameter and Polydispersity Index (PdI) were given.

2.5. Spectroscopic characterization

A U-3900 UV–vis spectrophotometer (U-3900, Hitach, Japan) was used to acquire the UV–vis absorption spectra. A Fluoromax-4 luminescence spectrophotometer (JOBIN YVON, USA) was used to provide the photoluminescence spectra. The conditions for fluorescence measurements were as follows: 388 nm excitation wavelength, 450–700 nm scan range and 300 nm min^{-1} scan rate.

2.6. Determination of the optimum ratio of the QDs to the aptamer

A 5′-Cy5 and 3′-biotin co-modified TLS9a aptamer (Cy5-TLS9a-biotin) was employed in the current assay. After preparation of the QD-Apt as described in Section 2.2, unconjugated Cy5-TLS9a-biotin was collected through ultra-filtrating for three times. The ultrafiltrate was collected respectively. The absorption at 650 nm of each ultrafiltrate was measured respectively by a UV–vis spectrophotometer (U-3900, Hitach, Japan). All the processes were kept away from light.

2.7. Assay of bioactivity and specificity of the QD-Apt

MEAR cells, BNL cells and Hela cells were routinely cultured at 37 °C in a flask containing Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS, heat inactivated, GIBCO) and 100U/mL penicillin–streptomycin in a humidified atmosphere with 5% CO_2.

For cell labeling experiments, cells were first cultured for 24 h in a 35 mm glass culture dish (Mat Tek Corp.) at a density of about 5×10^4/mL. Then the cells were washed three times with wash buffer (4.5 g/L glucose and 5 mM $MgCl_2$ in Dulbecco's phosphate-buffered saline). Subsequently, the QD-Apt bioprobe or SA-QD in binding buffer (prepared by adding 20% FBS and 0.4 mg/mL yeast tRNA into wash buffer) at a final concentration of 40 nM was added and incubated for 1 h at 37 °C. The cells were washed three times with wash buffer to remove superfluous probes or SA-QDs. Finally, the cells were imaged with a confocal microscope (Leica SP5, Germany) with a 40× 1.25 oil-immersion objective equipped with an argon laser for excitation using an RSP500 filter (488 nm).

2.8. Cytotoxicity assay

To study the potential cytotoxicity of the QD-Apt we used, the MTT assay was performed with MARE cells. Cells were cultured in 96 wells at a density of 2.5×10^4/mL in a 5% CO_2 atmosphere at 37 °C for 24 h, washed three times with 180 μL of wash buffer, and then incubated with 100 μL of bioprobe solution (40 nM) or binding buffer. After 1 h incubation under 5% CO_2 at 37 °C, the cells were gently washed and kept growing in DMEM medium for hours. Then the cells were washed three times, and 180 μL of serum-free fresh medium supplemented with 20 μL of MTT solution (5 mg/mL) was added into each well. After 4 h of incubation at 37 °C, the MTT, reduced by the mitochondrial reductase of vital cells, formed a dark insoluble product. The dark pellet was dissolved in 100 μL of DMSO, leading to a violet solution, whose absorbance at 570 nm was determined.

3. Results and discussion

3.1. Preparation and characterization of QD-Apt

One of the superiorities of QD-labeled aptamer bioprobes is potentially diverse because a specific aptamer could essentially be screened out for any of target molecules or species. The suitability and simplicity of methods to prepare QD-labeled aptamer probes are important for their bioapplication. Hereby, we utilized the highly specific and strong interaction between biotin and streptavidin to fabricate CdSe/ZnS QD-labeled TLS9a aptamer bioprobe (QD-Apt).

Fig. 1 is for agarose gel electrophoresis of SA-QDs and QD-Apt. It can be seen that the electrophoresis speed of SA-QDs was relatively slow, suggesting that the ratio of charge to mass was relatively small. With increasing amount of bound aptamer from 0.5:1 (aptamer:SA-QDs) to 4:1, the band front gradually moved forward. This is due to relatively large ratio of charge to mass of the single-stranded oligodeoxyribonucleotide aptamer. The increase in the ratio of charge to mass after the biotin-derived aptamer binds to SA-QDs makes the bioprobe migrate faster than SA-QDs. As SA-QDs bind more biotin-derived aptamer molecules on their surface, the electrophoresis speed will increase. Keeping increasing the quantity of aptamer will make the bioprobe reach a basically constant ratio of charge to mass. The reason for this is mainly that the influ-

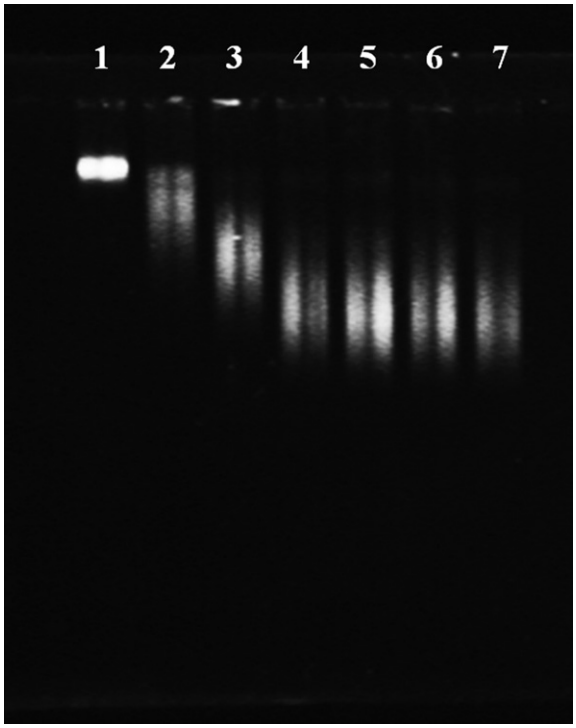

Fig. 1. Agarose gel electrophoresis of QD-labeled aptamer bioprobe produced in different ratios of biotin-derived aptamer to SA-QDs. Lanes 1–7: 1, SA-QDs; 2, QDs:aptamer = 1:0.5; 3, QDs:aptamer = 1:1; 4, QDs:aptamer = 1:2; 5, QDs:aptamer = 1:4; 6, QDs:aptamer = 1:8; 7, QDs:aptamer = 1:16.

ence from the ratio of charge to mass already becomes weak when the mass of the probe has been large because the increased number of negative charges of aptamer molecules and corresponding added mass can hardly change the ratio of charge to mass of the whole bioprobe. In addition, the fluorescence bands in lanes 1 and 2 overlapped a little, which should be attributed to incomplete binding between SA-QDs and biotin-derived aptamer due to a 0.5:1 ratio of aptamer to SA-QDs, with free SA-QDs. But from lane 3 on, the bands did not overlap the band in lane 1, suggesting that all the SA-QDs had already been conjugated with aptamer.

In order to further optimize the ratio of aptamer to SA-QDs, an aptamer modified with both 5′-Cy5 and 3′-biotin, namely Cy5-aptamer-biotin, was conjugated with SA-QDs to obtain bifluorophore-labeled aptamer probe. The amount of Cy5-aptamer-biotin on SA-QDs surface was determined by UV–vis spectrophotometry.

An absorption peak of Cy5 appeared at 650 nm, while quantum confinement absorption peak of SA-QDs was at 598 nm. Both the absorption peaks basically do not overlap. After preparation of Cy5-aptamer-QD in different ratios of SA-QDs to Cy5-aptamer-biotin, the optical density at 650 nm (OD_{650}) of ultrafiltrate was determined and corresponding concentration of unbound Cy5-aptamer-biotin was obtained as listed in Table 1. It can be seen that the OD_{650} values of ultrafiltrates after the first ultrafiltration were very small and increased a little with increasing aptamer quantity when the ratio of SA-QDs to Cy5-aptamer-biotin was changed over a range of 1:4 to 1:16. For the second ultrafiltration almost no unbound Cy5-aptamer-biotin was found in ultrafiltrates. However, a relatively large amount of unbound Cy5-aptamer-biotin was detected in the ultrafiltrates when the ratio reached to 1:32 and 1:64. Besides, after three ultrafiltrations Cy5-aptamer-biotin still existed in the ultrafiltrates, indicating that aptamer was greatly superfluous at these ratios. Furthermore, a large number of flexible aptamer molecules on the surface might disturb aptamer to form

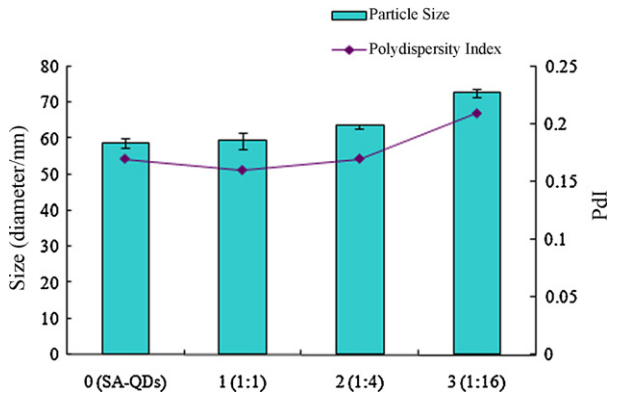

Fig. 2. Size distribution of SA-QDs and QD-Apt produced at different ratios of SA-QDs to biotin-derived aptamer.

the right conformation, affecting the bioactivity of the probe due to the steric hindrance and finite surface area of QDs.

Fig. 2 gives the size distribution of QD-Apt produced at different ratios of SA-QDs to biotin-derived aptamer by dynamic light scattering. Evidently, with increasing aptamer amount the average aquo particle size of QD-Apt bioprobe gradually increased from 58.775 to 72.712 nm, indicative of increasing aptamer molecules bound on the surface of SA-QDs. The average PdI value ranged from 0.1 to about 0.2, suggesting that the bioprobe had both good uniformity and dispersivity.

Based on above results, a 1:16 ratio of SA-QDs to aptamer was used to fabricate the bioprobe. In such a ratio a bioprobe with good fluorescence properties could be produced. Relative to that of SA-QDs, the spectrum of QDs after their conjugation of aptamer was scarcely changed (Fig. 3), suggesting that bound aptamer did not affect the surface structure of QDs mainly due to relatively large SA linker that can avoid the direct contact of aptamer with QDs. Hence, this is not the case for the preparation of QD-Apt by coupling with EDC and NHS [20].

3.2. Bioactivity and specificity of QD-Apt

Both MEAR and BNL cells are mouse liver cell lines, of which BNL cells are noncancerous while MEAR cells, chemically induced from BNL, are cancerous. Clearly, both the cells are closely kindred. When TLS9a aptamer was screened out by Cell-SELEX, counterselection was carried out with BNL cells in each screening round so as to improve its specificity [3]. Thus, the resulting TLS9a aptamer is highly selective between the two cell lines.

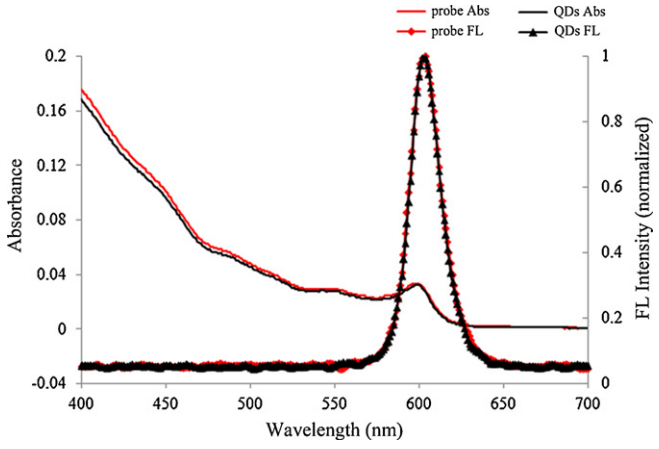

Fig. 3. Absorption and fluorescence spectra of SA-QDs and QD-Apt, prepared from a 1:16 ratio of SA-QDs to aptamer, at the same concentration.

Table 1
OD_{650} of ultrafiltrates versus corresponding concentration of Cy5-aptamer-biotin after the as-prepared Cy5-aptamer-QD was removed.

	Ultrafiltrate 1		Ultrafiltrate 2		Ultrafiltrate 3	
	OD_{650}	$C_{aptamer}$ (nM)	OD_{650}	$C_{aptamer}$ (nM)	OD_{650}	$C_{aptamer}$ (nM)
1:4	–	–	–	–	–	–
1:8	0.004	1.24	–	–	–	–
1:16	0.012	3.91	–	–	–	–
1:32	0.036	11.91	0.026	8.58	–	–
1:64	0.103	34.26	0.088	29.25	0.087	28.92

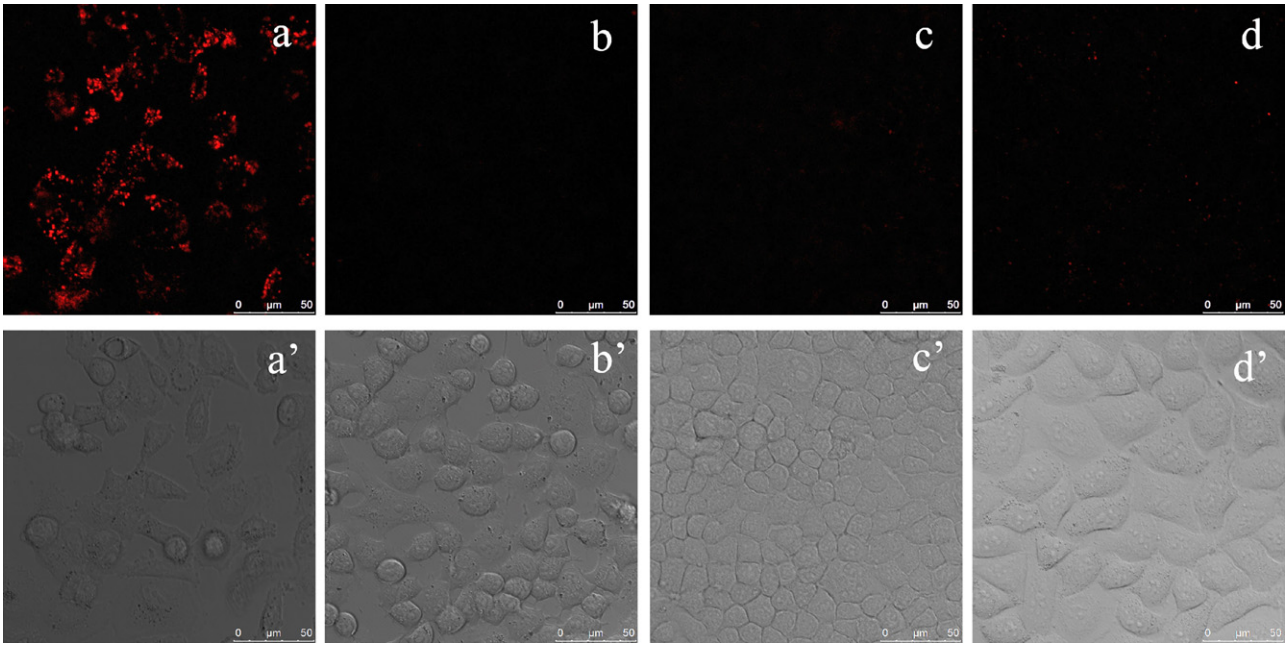

Fig. 4. Fluorescence microscopic images of QD-Apt bioprobe, prepared from a 1:16 ratio of SA-QDs to aptamer, and the cells. (a, a′) MEAR cells incubated with QD-Apt bioprobe; (b, b′) MEAR cells incubated with SA-QD nanoparticles; (c, c′) BNL cells incubated with QD-Apt bioprobe; (d, d′) Hela cells incubated with QD-Apt bioprobe; (a, b, c, d) fluorescence; (a′, b′, c′, d′) bright field.

Fig. 4 is the fluorescent images of MEAR cells and BNL cells recognized by the as-prepared QD-Apt. Clearly, the bioprobe could specifically recognize MEAR cells (Fig. 4a and a′), while it could not recognize BNL cells (Fig. 4c and c′) and other cancerous cells, such as Hela cells (Fig. 4d and d′). SA-QDs without aptamer on their surface also could not recognize MEAR cells (Fig. 4b and b′). These suggest that the specificity of the QDs-Apt results from the bioactivity of the aptamer immobilized on the surface of QDs to recognize some specific structure on the surface of MEAR cells. The success of the probe fabrication makes it feasible to recognize and detect target cells based on QD labeling and aptamer techniques.

3.3. Biocompatibility of QD-Apt

As for bioprobes, their biocompatibility is highly important for their application. As far as live cell imaging application is concerned, the effect of bioprobes on the natural physiological behaviors of cells should be emphasized. Accordingly, the effect of QD-Apt on the growth of MARE cells was examined by MTT method (Fig. 5). The data in Fig. 5 were obtained by averaging 3 determinations. It can be seen that the absorbance at 570 nm of the MTT was almost the same for the QD-Apt incubated cells and the control cells within 84 h, showing that the growth and viability of QD-Apt bound MEAR cells were not affected by QD-Apt. So it can be thought that the probe is biocompatible and suitable for live cell imaging. Such satisfactory biocompatibility should be attributed mainly to the use of biocompatible PEG coating on QDs for attachment of SA and aptamer, which could prevent QDs from Cd^{2+} release into the cells,

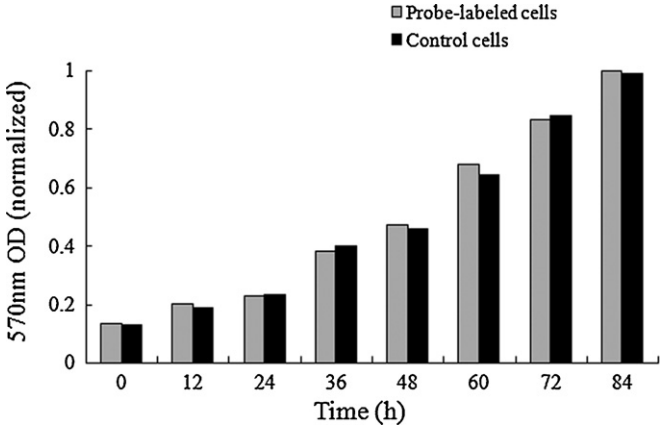

Fig. 5. Histogram for the viability of MEAR cells recognized by QD-Apt, prepared from a 1:16 ratio of SA-QDs to aptamer, versus incubation duration.

resulting in cytotoxicity. On the other hand, the results also suggest that the binding of aptamer to cells might not remarkably affect the cell life cycle.

4. Conclusions

A fluorescent bioprobe QD-Apt has been fabricated by biotin–streptavidin interaction. The fabrication is facile, fast and repeatable. And the resultant probe nanoparticles were uniform in

particle size and excellent in fluorescence properties. The aptamer on QD-Apt surface is bioactive and biocompatible, which can be used for specific detection of target MEAR cells. Within 84 h the probe scarcely had detectable cytotoxicity. Owing to the universality of the probe fabrication, it can be extended to prepare diverse fluorescent probes of QD-labeled aptamers for labeling of different cells and multi-color imaging.

Acknowledgements

This work was supported by the National Key Scientific Program (973)-Nanoscience and Nanotechnology (No. 2006CB933100), the Program for New Century Excellent Talents in University (No. NCET-08-0046), the National Natural Science Foundation of China (No. 20505001), the Excellent Young Scholars Research Fund of Beijing Institute of Technology (No. 2007YS0603) and the Ministry of Public Health (No. 2009ZX10004-107). The authors thank Professor Zhi-Ling Zhang, Dr. Zhi-Quan Tian and Dr. Min Xie for their helpful discussion.

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