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Short communication

Rapid and sensitive detection of cancer cells by coupling with quantum dots and immunomagnetic separation at low concentrations

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ABSTRACT

This work presents a rapid and sensitive method for detecting cancer cells at low concentration. In this method, two biomarkers of T-help cancer cells are detected simultaneously. One biomarker is conjugated with magnetic beads to separate T-help cell from the mixed cells and the other biomarker, associated with quantum dots, is used to detect fluorescence. The specific T-help cells can be quantified using the relationship between the QD fluorescence intensity and the cell frequency following magnetic separation. The intensity of fluorescence increases linearly with the frequency of T-help cells from 10^{-7} to 10^{-3}, and neither B cells nor red blood cells interfere with the detection of T-help cells. Moreover, the total detection time is under 15 min, even though the frequency of specific T-help cells is as low as 5×10^{-7}. The numerous advantages of detecting specific cells at low concentration using the presented method include ease of preparation, low cost, fast detection, and high sensitivity.

1. Introduction

The rapid and sensitive detection of low-frequency cancer cells is great importance, because it has a drastic positive effect on diagnosis and prognosis (Ishii et al., 2004; Li et al., 2003). In blood, the number of circulating tumor cells is correlated very sensitively with the recurrence of cancer and relapse. In the early stage of a tumor, disseminated cells circulate in the blood at extremely low concentrations, making the detection of low-frequency cancer cells difficult (Pantel and Otte, 2001). Conventional methods for detecting trace cells include culture-based techniques and enzyme-linked immunospots (ELISPOT). However, increasing the number of cells by culture or ELISPOT to a number that suffices for measurement is labor-intensive and time-consuming (Pahar et al., 2003; Alix-Panabières et al., 2005). Two other approaches, flow cytometry (FCM) and polymerase chain reaction (PCR), have been established for detecting low-frequency cells in childhood acute lymphoblastic leukemia and circulating endothelial cells associated with cancer. However, the detection sensitivities of FCM and PCR are approximately 0.01% and 0.001%, respectively (Neale et al., 2004; Steurer et al., 2008). Therefore, the development of highly sensitive, speedy and specific methods of detecting specific cancer cells at low frequency is important for prognosis.

Quantum dots (QDs) have been developed as a new class of fluorescent probes with several important advantages over conventional dyes (Eggeling et al., 1998; Hoebe et al., 2007). These include brightness, low photobleaching, broad excitation spectra and narrow emission bandwidth (Chan and Nie, 1998; Dubertret et al., 2002). QDs have been applied in fluorescence labeling for cellular imaging, DNA mutations, and tumor cell identification (Wu et al., 2003; Wang et al., 2010; Kantelhardt et al., 2010). Additionally, immunomagnetic separation is a simple and efficient method that has been utilized to isolate specific micrometastatic cells from colorectal cancer and stem cells (Flatmark et al., 2002; Kekarainen et al., 2006).

This work demonstrates a high-throughput and sensitive method for detecting cells at low concentration, based on the coupling of immunomagnetic-bead-captured cells with QDs as markers for fluorescence analysis. Human Jurkat cells (T-help cells) are used as a model for circulating tumor cells, and the membrane of T-help cells is labeled with QDs and magnetic beads using CD3 and CD4 markers, respectively. B cells and red blood cells are used as mixed cells to interfere with the detection of T-help cells. Experimental results demonstrate that the period required to detect specific cells was approxi-

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mately 15 min at a low cell frequency of 5×10^{-7}. The proposed method provides a simple, rapid and sensitive means of real-time measurement of the concentration of specific cells at low frequency.

2. Materials and methods

2.1. Materials

Human Jurkat cells (T-help cells) (ATCC TIB-152) and Human C1R cells (B cells) (ATCC CRL-1993) were obtained from American Type Culture Collection (USA). Quantum dot-625 streptavidin conjugates (QD625-streptavidin) with a maximum emission wavelength of 625 nm were obtained from Invitrogen (USA). Magnetic beads labeled with anti-human CD4 (anti-CD4-MB) (catalog no. 18052) and magnet (catalog no. 1800) were manufactured by StemCell Technologies. Biotinylated anti-human CD3 (anti-CD3-biotin) (13-0038) was purchased from eBioscience Inc. Ficoll-Paque PLUS reagent was obtained from GE Healthcare Amersham Biosciences (Sweden). All chemicals were used as received. Ultra-pure water (18 MΩ cm) was used to prepare all solutions.

2.2. Cell culture

T-help cells and B cells were maintained according to the methods in Supplementary material. Red blood cells (RBC) were obtained the informed consent from healthy donors and were separated by Ficoll-Paque PLUS reagent.

2.3. Scanning electron microscopy and fluorescence microscopy images

The preparation of the cell samples for scanning electron microscopy (SEM) and the fluorescence image system were described in Supplementary material.

2.4. Experimental protocols

The total analytical procedure comprised three steps. The first was QD labeling: 4 μl of 0.5 mg/ml anti-CD3-biotin was immuno-incubated with a total of 10^8 mixed T, B and RBC, including 0–10^5 T-help cells and re-suspended in 200 μl phosphate buffer saline solution (PBS) at 4 °C for 15 min, before being centrifuged and rinsed, forming a mixture of biotin-conjugated T-help cells. Then, 1 μl of 1 μM QD625-streptavidin was incubated with a mixture of biotin-conjugated cells and re-suspended in 200 μl PBS at 4 °C for 30 min, before being centrifuged and rinsed, generating QD-labeled T (QD-T) mixed cells.

The second step was immuno-magnetic separation. A 4 μl of human CD4 positive selection cocktail was added to the QD-T mixed cells for 15 min, and 2 μl of the magnetic beads was incubated with the mixed cells for another 15 min at room temperature, yielding QD-T-MB mixed cells. The suspension of the QD-T-MB mixed cells was diluted to 2.0 ml with PBS solution and separated by the magnet. The supernatant fraction was poured off, leaving immuno-magnetically labeled specific T-help cells that contained QDs.

The third step was measurement of fluorescence. Following the final immuno-magnetic separation, the specific T-help cells that contained QDs and magnetic nanoparticles were re-suspended in 200 μl of borate buffer solution for fluorescent measurement. To obtain stable fluorescence from QD-labeled T cells for quantification, these cells were photo-activated for 10 min before detection. The conditions, a 10 min of photo-activation of the QD-labeled T cells before detection and the use of a borate buffer solution at pH 11.0 for the last re-suspension, were optimized, according to our previous investigation (Hsieh et al., 2010).

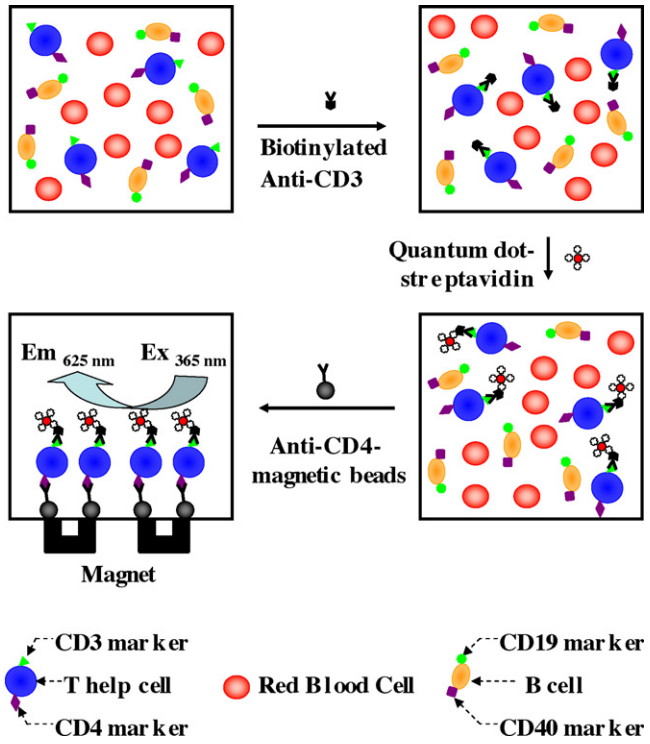

Fig. 1. Principle of detecting the specific T-help cells using combination of two biomarkers with quantum dots as fluorescence probe and magnetic beads for immuno-separation. The excitation wavelength of 365 nm was used and the emission wavelength of 625 nm was recorded.

3. Results and discussion

3.1. Proof of concept

Fig. 1 depicts the principles of the rapid and sensitive detection of low-frequency cancer cells using fluorescent analysis. All T-help cells (target) are designed to be detected simultaneously using their two biomarkers of CD3 and CD4 from the mixed B cells (with CD19 and CD40 markers) and RBC (without a CD marker). The natural bridge of the biotin–streptavidin molecule with remarkably high binding affinity (Aslan et al., 2005) is adopted to link the T-help cells to the QDs using anti-CD3-biotin antibody and streptavidin-functionalized QDs. Hence, firstly, the membrane of the T-help cell was conjugated with anti-CD3-biotin by the antigen-antibody reaction, yielding the biotinylated T-help cell, and then coupled with quantum dot-streptavidin via the biotin–streptavidin coupling reaction, forming the QD-labeled T-help cells (QD-T cells). Secondly, the membrane of the QD-T cells was associated with anti-CD4-MB, producing the QD-T cells that were bound to the magnetic beads (QD-T-MB). Thereafter, QD-T-MB was attracted by applying a magnet, and thus separated from the mixed cells. The isolated T-help cells (QD-T-MB) were quantified from the intensity of fluorescence when the QDs were excited at the appropriate energy. Fig. S1 displays the setup of fluorescent measurements.

3.2. Separation of specific cancer cells from mixed cells

To isolate the specific QD-T cells from the mixed cells, anti-CD4-MBs were adopted to interact with the QD-T cells by immuno-reaction with the CD4 biomarker on their membranes. A magnet was then used to isolate the QD-T cells. Fig. S2 displays

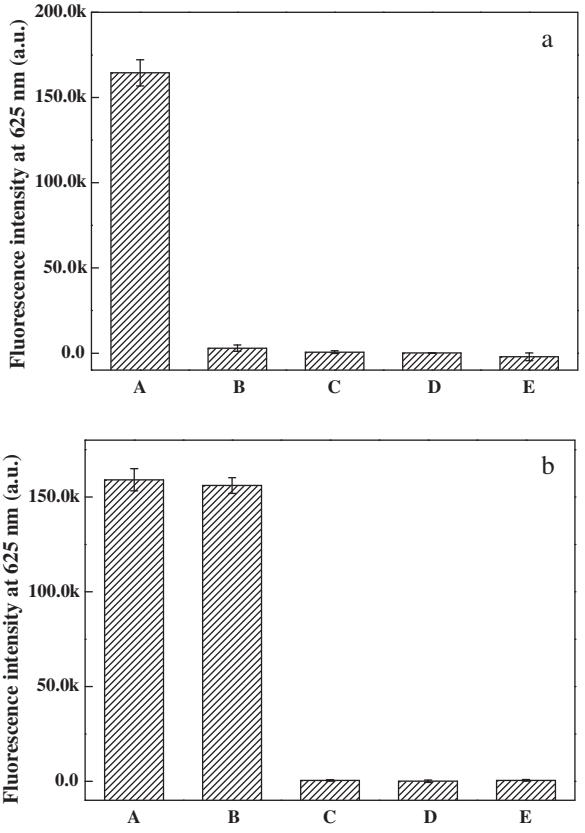

Fig. 2. Specific and cross-reaction tests of T-help cells after magnetic isolation. (a) Specific binding test of T-help cells. Columns A, B and C represent fluorescent intensity obtained from 10^5 T-help cells, 10^6 B cells and 10^6 RBC, respectively, incubated with anti-CD3-biotin, followed by QD625-streptavidin and then anti-CD4-MBs. Column D presents fluorescent intensity from 10^5 T cells incubated only with QD625-streptavidin and then anti-CD4-MBs. Column E presents intensity of auto-fluorescence from 10^6 T-help cells. (b) Cross-reaction test of T-help cells in 10^6 mixed cells. Columns A and B present fluorescent intensity of 10% T-help cells in 90% RBC mixed cells, and in 45% RBC with 45% B mixed cells, respectively, incubated with anti-CD3-biotin, followed by QD625-streptavidin and then anti-CD4-MBs. Columns C, D and E present the fluorescent intensity from 10% T-help cells in 90% B mixed cells, in 90% RBC mixed cells, and in 45% RBC with 45% B mixed cells, respectively, incubated without anti-CD3-biotin but with QD625-streptavidin and then anti-CD4-MBs. Error bar represents standard deviation of at least three measurements.

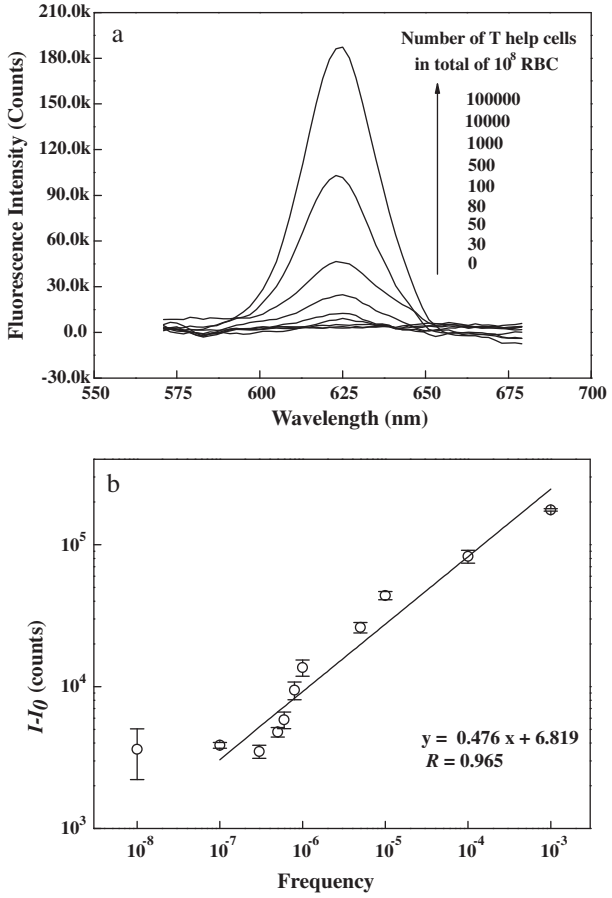

Fig. 3. (a) Fluorescent emission spectra obtained using QDs and magnetic beads to probe 0–10^5 specific T-help cells, in total of 10^8 RBC mixed cells, after magnetic separation. Data were obtained by subtracting measurements of boric buffer blank at pH 11. (b) Log–log calibration plot of fluorescent intensity versus T-help cell frequency. I_0 and I represent fluorescent intensity recorded at wavelength of 625 nm for borate buffer without and with specific T-help cells in 10^8 of RBC mixed cells, respectively. Error bar represents standard deviation of at least three measurements.

the fluorescent microscopic images of QD-T cells in B mixed cells before and after magnetic separation. The QD-T cells from B mixed cells can be distinguished by fluorescence microscopy. Moreover, the surface morphology of a T-help cell before and after binding to anti-CD4-MB was examined by SEM, as shown in Fig. S3. The surface of the T-help cell had surface microvilli before and after binding to anti-CD4-MB. Some of the magnetic beads on the surface of the T-help cell are aggregated, causing the size of magnetic beads is between 80 and 200 nm.

3.3. Specific binding and cross-reaction tests

The specificity following conjugation was verified by the antigen-antibody recognition reactions that occurred simultaneously with two markers – the CD3 marker of the T-help cell and anti-CD3-biotin, which reaction was followed by a coupling reaction between anti-CD3-biotin and QD625-streptavidin, and the CD4 marker of the T-help cell and anti-CD4-MBs. Therefore, QD-T-MB cells were formed by incubating T cells with anti-CD3-biotin, then QD625-streptavidin, and finally anti-CD4-MBs.

Fig. 2a shows the specificity of the T-help cells after magnetic separation, when anti-CD3-biotin, QD625-streptavidin and anti-CD4-MBs were sequentially incubated with the cells of 10^5 T-help cells, 10^6 B cells and, 10^6 RBC. Strong fluorescence was clearly observed from the T-help cells, but a dim fluorescence was obtained from B cells and RBC (without the CD3 marker), indicating that the QD-streptavidin and magnetic beads were successfully attached to the membrane of the T-help cells by indirect specificity of the antigen–antibody reaction. Moreover, in control experiments, incubation of 10^5 T-help cells only with QD-streptavidin, and then with anti-CD4-MBs yielded weak fluorescence, as did the self-fluorescence, verifying that the non-specific adsorption of QD625-streptavidin on T-help cells was negligible.

Only T-help cells were observed herein because two receptors of the CD3 and CD4 were investigated simultaneously to elucidate the coupling of QDs and magnetic beads. The cross-reaction of QD-T-MB cells was examined using B cells and RBC. As displayed in Fig. 2b, the cross-reaction was performed by mixing the T-help cells with the B cells and RBC, and then incubated with anti-CD3-biotin, QD625-streptavidin and anti-CD4-MBs, following magnetic separation. In each case, 10^6 mixed cells were used, and they comprised 10% T-help cells either in 90% RBC or in 45% B cells and 45% RBC. Significant fluorescence was clearly observed from the T-help cells not only when mixed with RBC but also when mixed with B cells and RBC. In the control experiments, only QD625-streptavidin and anti-CD4-MBs (without anti-CD3-biotin) were incubated with 10% T-help cells in 90% RBC, in 90% B cells, or in 45% B cells with

45% RBC, following magnetic separation. All experiments yielded weak fluorescence. This finding confirmed that the cross-reaction of QD625-streptavidin and anti-CD4-MBs on T-help cells with B cells and RBC is prevented. The experimental results demonstrate that anti-CD4-MBs functions as a magnetic sensor that separates specific T-help cells from the mixed cells, and QD625-streptavidin that is coupled with anti-CD3-biotin serves as a reliable optical sensor to detect specific T-help cells.

3.4. Detection of T-help cells in mixed cells

T-help cells in mixed B cells or RBC were detected by incubating all of the cells sequentially with anti-CD3-biotin, QD625-streptavidin fluorescent probe, and anti-CD4-MBs. Thereafter, a magnet was applied to separate out the specific QD- and MB-labeled T-help cells. The population of T-help cells, separated by the magnet, is quantified by measuring the intensity of fluorescence from the QD-labeling. Fig. 3a plots the fluorescent spectra of T-help cell populations of 0–10^5, in 10^8 RBC mixed cells after magnetic separation. Experimental results indicate that the fluorescent intensity of QD-T-MB cells rises with the T-help cell populations in mixed cells. The concentration of the specific T-help cells in the mixed cells can be presented as a frequency. Fig. 3b plots the log–log calibration graph of the fluorescent intensity versus T-help cell frequency in 10^8 RBC mixed cells. Fig. S4 plots the log–log calibration graph of the fluorescent intensity against T-help cell frequency in 10^8 B mixed cells. An approximately linear relationship was observed at T-help cell frequencies between 1×10^{-7} and 10^{-3}, not only in mixtures with RBC but also in mixtures with B cells. The detection limit, calculated from three standard deviations (3SD) of the blank, was a frequency around 5×10^{-7}. Notably, detecting the cells took under 15 min, including the photo-activation period of 10 min for the QD-T-MB cells. Detecting a rare-cell in 10^8 mixed cells has been reported by fiber-optic array scanning technology (FAST) (Krivacic et al., 2004). Even though the detection time is 5 min, their detection sensitivity is 1.5×10^{-5}. Therefore, compared with the FAST, our proposed method is more sensitive in the detection of cancer cells.

4. Conclusions

This work demonstrates an efficient, specific, and sensitive method for detecting cancer cells at low concentrations using quantum dots (QDs) and magnetic beads as fluorescent and separated probes, respectively. The intensity of fluorescence increases linearly with the frequency of T-help cells in the range of 1×10^{-7} to 10^{-3} and the detection limit is about 5×10^{-7}. Furthermore, the specific T-help cells can be detected in less than 15 min. Neither B cells nor red blood cells caused significant interference. In the future, the proposed method will be used to detect Epstein-Barr Virus (EBV)-specific memory T lymphocytes from nasopharyngeal carcinoma patients for diagnosis or prognosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.04.023.

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