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ORIGINAL PAPER

Fluorescent Biological Label for Recognizing Human Ovarian Tumor Cells Based on Fluorescent Nanoparticles

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Abstract In this paper, we report a method for recognizing human ovarian tumor(HOT) cells using fluorescent biological label based on core-shell nanoparticles. The luminescent nanoparticles were synthesized with a water-in-oil (W/O)micromulsion technique. The fluorescent silica core-shell nanoparticles modified with anti-HER2 antibody using bifunctional cross-linker glutaraldedhyde targeted the corresponding tumor antigen in the cell surface of the SKOV3 ovarian cancer cells. The specific immunoreactivity of antibody-nanoparticles with cells was characterized by laser scanning microscopy (LSM) and scanning electron microscope (SEM). The results showed that the method offered potential advantages of sensitivity and simplicity due to high binding efficiency between nanoparticles and cells and provided an alternative method for the detection of HOT.

Keywords Fluorescent nanoparticles · Biological label · Human ovarian carcinoma SKOV-3 cells · Recognition · Detection

Introduction

Nanoparticles present some unique advantages such as high reactivity and beneficial physical properties (electrical, electrochemical, optical, and magnetic characteristics). Silica-coated fluorescent nanoparticles, with high photostability, ultra-small size, biocompatibility and non-toxicity are widely used in biological analysis and immunoassay [1–5]. The fluorescent silica nanoparticles can be used as biomarkers in biochemical and biomedical field because the fluorescent dye can be effectively entrapped inside the silica shell and the surface of silica particles can be easily modified by biomolecules such as proteins, antibodies, DNA etc. Tan et al [6, 7] prepared silica-coated fluorescent nanoparticles encapsulated with different dyes which can be readily bioconjugated with specific antibody and aptamer and used those fluorescent nanoparticles as the sensing system to detect leukaemia and lymphocyte disease. Wiesner and his coworkers [8] have accomplished biomolecular optics imaging and detection of rat basophilic leukemia (RBL) mast cells based on the physical adsorption between nanoparticles and specific antibody.

Human epidermal growth factor receptor 2 (HER2/neu, also known as ErbB-2) is a member of the epidermal growth factor receptor (ErbB) family and plays an important role in the pathogenesis of ovarian and breast cancer [9]. The amount of HER2 on the cells will be amplified at 20–30% incidence in human breast tumor [10, 11]. And the amplification also occurs in ovarian, lung and gastric tumor. However, the normal expression of HER2 in adult tissue is very weak. Human ovarian carcinoma cells SKOV-3[12] are HER2—overexpressing tumor cells. The antigen of this tumor cell can be selectively targeted using anti-HER2 monoclonal antibody easily. Hapca et al [13] reported the thiolated poly (DL-lactic acid) nanoparticles (PLA NPs) covalently coupling to anti-HER2 antibodies via a bifunctional cross-linker, *m*-maleimidobenzoyl- *N*-hydroxy-sulfosuccinimide ester (sulfo-MBS).

Nowadays, determination methods of the tumor cells have received a great development. But all the previous assays each were not so perfect. [14]. Radioimmunoassay (RIA) requires radioisotopes, so precautions against hazards

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of their use must be taken. Enzyme immunoassay (EIA) needs active enzymes, which require special storage. In recent years, fluorescent detection methods have achieved major improvements in bioanalytical applications because of their extraordinary sensitivity and selectivity [15]. Fluorescence immunoassays employ a fluorescent signal for analytical detection and have become a common clinical chemistry procedure for the analysis of a wide range of analytes, such as drugs, hormones, and proteins [16]. Brewer et al [17] used fluorescence spectroscopy to characterized ovarian tissue in vivo and concluded that fluorescence spectroscopy could be adapted to improve early diagnosis of ovarian neoplasia. Their research showed that fluorescence imaging and spectroscopy can be used to detect the differences among the normal ovary, endosalpingiosis, benign neoplasm, and cancer using a system for the measurement of fluorescence excitation emission matrices.

In this work, we synthesized the fluorescent silica nanoparticles doped with an organic dye rhodamine B isothiocyanat (TRITC), and detected the ovarian carcinoma cells SKOV-3 based on this nanoparticles. The nanoparticles, prepared by water in oil microemulsion method [18–20], with higher dispersive behavior and light stability, were clearly characterized by laser scanning confocal microscopy and scanning electron microscope. The results demonstrated that the fluorescent nanoparticles as labels in tumor cell showed a great improvement in sensitivity, selectivity, and multiplexing capacity. This method, using the designed antibody-functionalized fluorescent nanoparticles specifically targets specific antigen on ovarian carcinoma cells SKOV-3 for realizing the aim of detecting tumor cells, can be used as a new measure technique in biological assay- antigen-antibody affinity assays.

Materials and methods

Instrumentation

Fluorescence spectra were obtained using a Hitachi F-2500 luminescence spectrometer (Japan). The picture of fluorescent nanoparticles was taken with a JEOL-2100 transmission electron microscopy (TEM, JEOL, Japan). The results of the incubation of nanoparticles with cells were characterized with a JSM-6380LV scanning electron microscope (JEOL,Japan) at an acceleration voltage of 20 KeV and a LSM5 PASCAL Laser scanning microscopy(Carl Zeiss, GERMANY). The MTT measurement was carried out using a spectrophotometer(Bio-RAD Model 680,USA). All optical measurements were carried out at room temperature under ambient conditions.

Reagents and materials

Rhodamine B isothiocyanate (TRITC) and N-(β-amimoethy1)-γ-amino-propyl- triethoxy -silane (AEAPS) were purchased from Sigma-Aldrich (St Louis, MO, USA) and Yudeheng Chemical Plant(Nanjing, China), respectively. Tetraethoxysilane (TEOS), cyclohexane, n-hexanol, Glutaraldehyde, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) and TritonX-100 were obtained from Shanghai Sangon Biological Reagent Company (Shanghai, China). $NaBH_4$, ethanol, acetone were obtained from Sinopham Chemical Reagent Co.,Ltd (Shanghai, China). The SKOV-3 cell line was supplied by Shanghai Cell Bank (Shanghai, China). The McCoy'5a medium, trypsinase and 3-(4,5-dimethyl-thiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. The fetal bovine serum was obtained from HyClone (FBS, Utah, USA). Penicillin and streptomycin from Shanghai Sangon and anti-HER2 monoclonal antibody from Chemicon International Inc(Temecula CA, USA) were used in this experiment.

Phosphate buffer (PBS) solutions were prepared according to standard laboratory procedure by 0.1 mol/L Na_2HPO_4 and 0.1 mol/L NaH_2PO_4. Other chemicals were analytical grade and used as received. Distilled water was further purified using the Milli-Q reagent grade water system (HFSuper PW, Shanghai Kanglei, China).

Synthesis of fluorescent nanoparticles

The fluorescent silica core-shell nanoparticles were synthesized according to the method proposed by Tan et al [18, 21]. The W/O microemulsion was prepared at room temperature first by mixing cyclohexane, n-hexanol and TritonX-100 according to the volume ratio (V/V) of 4:1:1, 0.4 mL TRITC dye aqueous solution was then added. The mixture was stirred for $1h$ prior to the addition of TEOS and AEAPS (5:3) into the microemulsion. Then the microemulsion was left to stir for $24h$. After the reaction was completed, acetone was added to break the microemulsion and recovered the nanoparticles. The contents were centrifuged and washed with ethanol and water for several times. Then the prepared nanoparticles were suspended in super-pure water until use.

Preparation of the antibody-labeled nanoparticle

According to the method proposed by Yang et al [22, 23], a mixture containing amino functionalized nanoparticles (2.0 mg) and 0.1 mL of 5% glutaraldehyde was added to 1.0 mL 4 mg/mL BSA solution in 0.1 mol/L PBS (pH5.0). After stirring at 4°C for $24h$, the excess BSA was removed from the mixture by separation using a centrifugal, and the BSA-modified nanoparticles (NPs) were dispersed in PBS

after washed with PBS for several times. Then the resulting NPs were coupled to 20 μL anti-HER2 antibody (0.1 mg/mL in PBS) in the presence of 0.1 mL of 5% bifunctional crosslinker glutaraldehyde. To the suspension 2.0 mg of $NaBH_4$ was added after the solution was gently shaken at 4°C for another 24*h*., incubated at room temperature for 2*h*. The antibody-conjugated nanoparticles were centrifuged, washed, suspended in 0.1 mol/L PBS buffer of pH7.4, and stored in suspension at 4°C before use. In this paper, anti-HER2 antibody modified-NPs were used to denote the anti-HER2 antibody-modified fluorescent nanoparticles.

Incubation procedure of nanoparticles and tumor cells

SKOV-3 cell line were grown on glass coverslips in McCoy'5a medium supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin in an atmosphere of 95% humidified air and 5% CO_2 at 37°C. Cells were cultured until exponential phase, the growth medium was replaced by a fresh McCoy'5a medium supplemented with nanoparticles and incubated at 37°C for 6*h*, shake up, characterized by the LSM.

Cell viability experiment

The cells were harvested from the tissue culture flask using trypsinase and suspended in McCoy'5a medium at a concentration of 10^4~10^5 cells/mL. Then an amount of 10^3~10^4 cells/well(100 μL) was seeded in 96-well culture plate and allowed it to adhere overnight in an incubator at 37°C with 5% CO_2. Media was exchanged with fresh medium containing fluorescent nanoparticles with different concentrations (10 μL aqueous dispersion per well) and mixed gently. The plates were then set for 44*h*, 20 μL of MTT dissolved in sterile PBS at 5 mg/mL was added to each well. The cells were incubated for 4*h* again. After incubation, 100 μL SDS was added to each well and incubated continuously for another 12*h* in order to dissolve any purple MTT formazan crystals. Finally, the absorbance was measured at a wavelength of 570 nm using a model 680 microplate reader.

SEM analysis

After incubation of anti-HER2 antibody modified-NPs with cells on glass coverslips for 24*h*, cells were washed with 0.1 mol/L PBS buffer (pH7.4) for three times, fixed with 2.5% gluteraldehyde(V/V) in PBS for 2*h*. After front-fixation, cells were fixed in 1% osmium tetroxide for 1*h* at 4°C again for post-fixation, washed with PBS for 20 min. Then the samples were dehydrated (15 min/time) using alcohol of a series concentrations (30%, 50%, 70%, 80%, 90%, 95%, and 100% alcohol), air-drying. After the samples were sputtered with a film of gold, the nanoparticles were analyzed by the SEM [24].

Results and discussion

Characterization of nanoparticles

Figure 1 shows the TEM image of the nanoparticles synthesized here. As shown in Fig. 1, the size and shape of the TRITC-doped core-shell fluorescent nanoparticles are homogeneous, with a diameter of approximately 75 nm.

Under the condition of voltage of 400 eV and slit width of 2.5 nm, the fluorescent spectrum showed that the TRITC-doped core-shell fluorescent nanoparticles exhibited a maximum emission at 586 nm wavelength when excited at 558 nm in aqueous solution, which is in agreement with those reported by Yuan et al [25].

Modification of antibody on the fluorescent nanoparticle

Figure 2 is the schematic illustration of the antibody coupled to fluorescent nanoparticles surface. After the copolymerization of TEOS and AEAPS, the amino groups were introduced to the surface of nanoparticles. The BSA-coated nanoparticles, obtained by coupling BSA to nanoparticles through the bifunctional crosslinker glutaraldehyde[26], were conjugated to anti-HER2 antibody using glutaraldehyde again. According to the report [21], the reaction between rigid nanoparticle-conjugated antibody and the cell antigen on the solid-phase surface has a higher steric hindrance, addition of BSA can decrease the strong steric hindrance because BSA-conjucted-antibodies were spread in every direction of the nanoparticles and decreased the steric hindrance and ensured the reaction between antibody-modified nanoparticles and the cell antigen. On

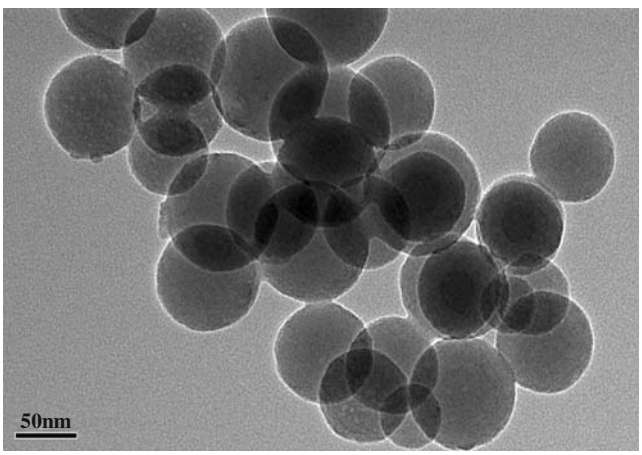

Fig. 1 TEM image of TRITC fluorescent nanoparticles

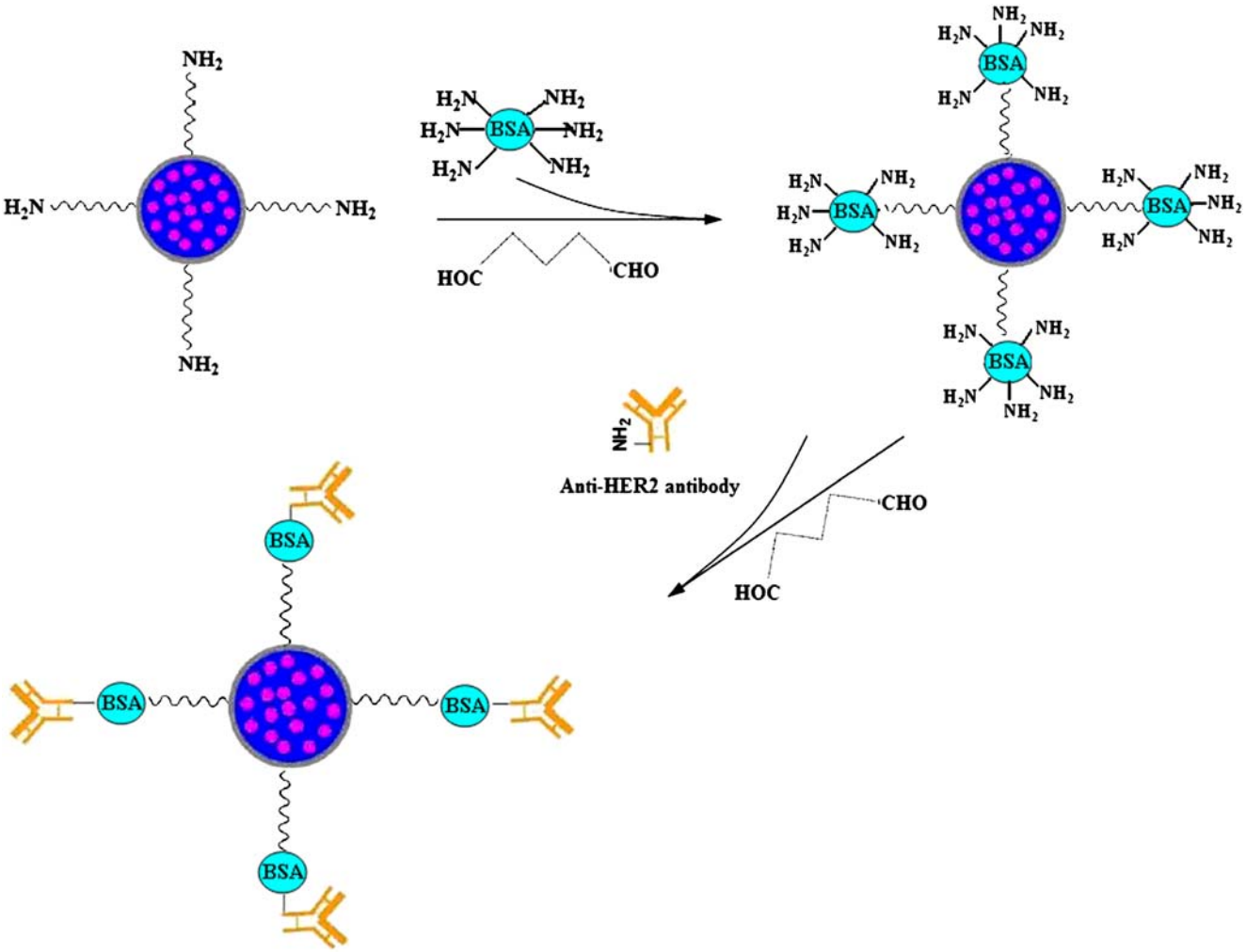

Fig. 2 Schematic representation of antibody immobilization process onto functionalized fluorescent core-shell nanoparticles

the other hand, the amino group amount of the surface of NPs and BSA, respectively, was compared by the fluorescamine method [27]. The data showed that the amino amount on the surface of BSA was higher than that of NPs here, which was helpful for the antibody linking.

The Fig. 3 shows the emission spectrum of the anti-HER2 antibody at 340 nm when excited at 280 nm in PBS (pH7.0, 0.1 mol/L). From Fig. 3, it can be seen that when the NPs coated with anti-HER2 antibody, the fluorescence intensity of antibody was lower than that of pure anti-HER2 antibody obviously. According to the report [28], we can calculate the binding efficiency (I) as follows:

$$I = (F_a - F_b - F_c)/F_a$$

Where, F_a is the reference fluorescence intensity of 20 μL anti-HER2 antibody, F_b is the fluorescence intensity of antibody in reaction solution after the conjugation reaction between antibody and nanparticles has happened and F_c is the fluorescence intensity of antibody in eluate of anti-HER2 antibody modified-NPs washed with PBS.

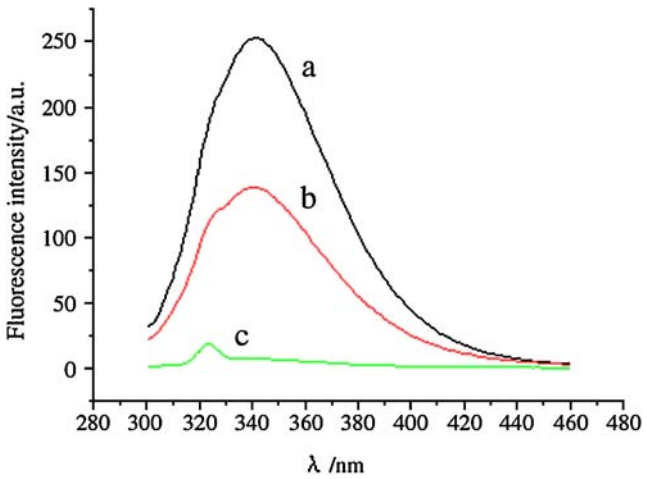

Fig. 3 Fluorescent spectra of anti-HER2 antibody (**a**), anti-HER2 antibody after connected on the NPs (**b**) and eluate (**c**)

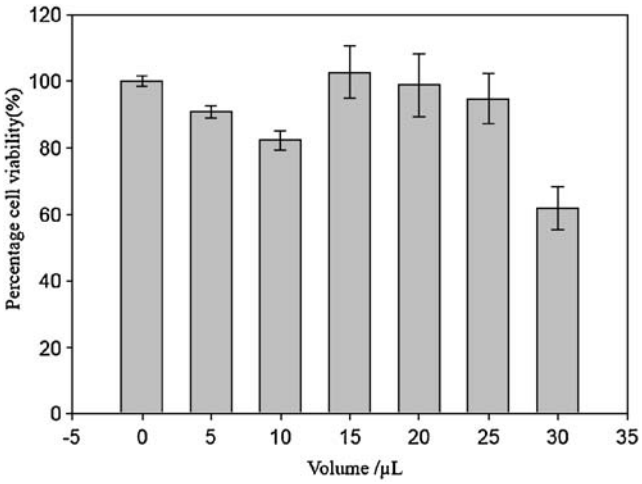

Fig. 4 The MTT experiment of nanoparticles culture with cells

According to the experimental data, we can get the relative efficiency of anti-HER2 antibody to NPs is 45.3%, which shows that a higher binding efficiency and larger amount of antibody coupled to NPs, This is the foundation for the detection of HOT which based on the anti-HER2 antibody modified-NPs reacted to the cancer cell antigen.

Recognition of SKOV-3 cell by nanoparticle

The anti-HER2 antibody modified-NPs can specifically bind to the antigen of the SKOV-3 human ovarian carcinoma cells easily. In this paper, the colorimetric MTT method was used for measuring the cell proliferation and viability according to the literature [29]. In the MTT assay, the absorbance of formazan produced by the MTT cleavage caused by dehydrogenases in living cells at 570 nm reflects the amount of alive cells. The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The control cells absorbance values were related to control wells containing cell culture medium. The sample without nanoparticles was taken as a reference, and its absorbance was adjusted as 100%. We could adopt following expression to calculate the relative cell viability (%)

$$\text{Relative cell viability}(\%) = \left([\text{A}]_{\text{test}}/[\text{A}]_{\text{control}}\right) \times 100\%$$

The higher the cell viability existed, the smaller the cell toxicity induced by nanoparticles. Figure 4 shows the MTT assay result of the culture of nanoparticles and cells.

As expected, the TRITC-doped fluorescent nanoparticles showed very low or no toxicity on the cells. The relative cell viability was as high as 82% when adding 325 μg or less than 325 μg of nanoparticles to culture cell. While the relative cell viability fell to 60% when the addition amount of nanoparticles was larger than 325 μg .The results suggests excessive nanoparticles affect the growth and metabolism of cells and lead to the decrease of the alive cell number. Perhaps the reason for the result is that nanoparticles were metabolized as a foreign by the cells when smaller amounts of nanoparticles were added into cells. However, if the addition amounts of nanoparticles was too much, the cell metabolism became difficult, i.e. the cell life activity and metabolism were affected obviously.

Fluorescent image of the conjunction of cell and nanoparticle

Binding specificity is critical for most bioanalysis, and specific binding is also especially crucial for nanotechnique application such as disease diagnostics by imaging and disease therapeutics by drug delivery using nanoparticles. It is well known that specific antibody and its corresponding cell surface receptor can form a reversible but tight complex via non-covalent bond function including hydrogen bond, van der Waals force and electric charge action, etc[30]. Therefore, we immobilized anti-HER2 antibody on the surface of fluorescent nanoparticles, then

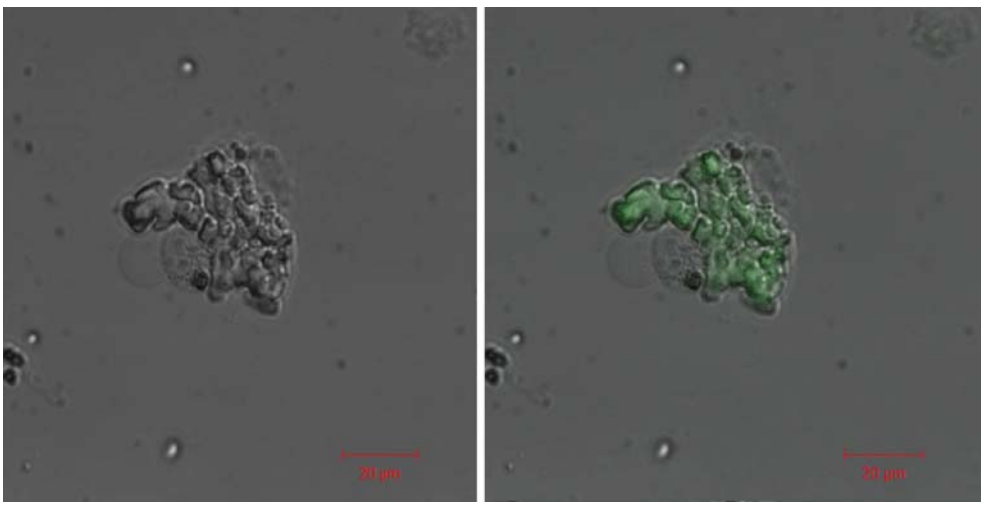

Fig. 5 Optical image (**a**) and fluorescent image of antibody-nanoparticles culture with cells (**b**)

based on its strong fluorescence characteristic, we used this functionalized NPs to detect HOT SKOV-3 cells.

Figure 5 indicates the fluorescence images of the SKOV-3 cells labeled with the anti-HER2 antibody modified-NPs. From Fig. 5, it can be seen that the surface of SKOV-3 cell was covered with nanoparticles almost completely due to the antibody bind specifically to the HER2 antigen. The result showed that the cells were successfully bound to the anti-HER2 antibody modified- nanoparticles with the higher combine efficiency, moreover, the bright fluorescence of the nanoparticle on the tumor cells surface makes it hopeful for early detecting HOT cells as a fluorescent probe for biological imaging. Compared with traditional fluorescence analysis, the special advantage of this method is its high stability and sensitivity due to photochemical stability of the core-shell nanoparticles.

Conclusions

In the present work, a new detection method of using anti-HER2 antibody modified fluorescent nanoparticles for a specific targeting of ovary cancer cells was developed. The biological modification of nanoparticles was prepared by connecting TRITC fluorescent nanoparticles and cells by the amino groups through crosslinker glutaraldehyde. A stable compound, formed after the anti-HER2 antibody conjugated-nanoparticles was cultured with SKOV-3 cells of overexpressing HER2 antigen, was characterized by SEM, LSM, etc. The result showed that the nanoparticle has a higher binding efficiency with cells. The MTT experiment showed that the fluorescent nanoparticles had little cell toxicity to HOT cells, which providing the foundation of the cell detection and the medication targeting-transportation using fluorescent nanoparticles. The SEM image of cells cultured with anti-HRE2 antibody-nanoparticles indicated the specifically reaction of cells and NPs linking with the anti-HER2 antibody. This method provides an alternative method of detection of HOT cells. It is possible that different cancer cells can be detected by modification of different antibodies on the NPs.

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