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ORIGINAL PAPER

Folate Conjugated Chitosan Grafted Thiazole Orange Derivative with High Targeting for Early Breast Cancer Cells Diagnosis

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Abstract The folate receptor (FR) is over-expressed on many solid tumors and has been exploited for targeted delivery of folic acid linked liposomes to cancer cells in vitro. In the present study, we developed a novel folic acid (FA) conjugated chitosan (CTS) grafted thiazole orange (TO) complex (FA-CTS-TO), and the formation can be used to label tumor cells. The structure of TO derivatives was confirmed by ^{1}H NMR and MS, and the fluorescence probe of FA-CTS-TO complex was confirmed by Fourier transform infrared analysis and Differential thermal analysis. The in vitro and in vivo of FA-CTS-TO complex were tested in breast cancer cells and the results showed a high targeting specificity in tumor cells with FR over-expressed. Such prominent fluorescence properties demonstrate again that FA-CTS-TO complex as a tumor targeting fluorescence probe is appropriate for breast cancer cells.

Keywords Fluorescence · Folic acid · Chitosan · Thiazole orange · MCF-7 cells

Introduction

With the rapid development of molecular biology, considerable progress has been achieved in the research on the use of various types of fluorescence probes to identify cancer cells [1–7]. Owing to the advantage of its fastness and low consumption, fluorescence labeling technique has become one of the focuses in this field. Thiazole orange (TO), as a typical fluorescence probe molecule, possesses many excellent and distinctive characteristics: it has a good optical stability, a high molar absorption coefficient, and a high sensitivity. The dye has a weaker fluorescence in solution, but shows fluorescence enhancement over 1000 times when inserted into a DNA molecule, especially the double-helix of DNA, and over 3000 times when inserted into RNA [8, 9]. As a biological fluorescence probe, TO has been widely used in recognition of variation cancer cells and in vivo marker [10, 11].

Recently, with the development of molecular biology and new drug resources, polysaccharide has been widely paid attention to. Chitosan is a special kind of carbohydrate polymer and can be found widely in nature [12, 13]. It is with good characteristics, such as biocompatibility, biodegradability and no toxicity. In addition, it can penetrate the cell membrane, and react with biomolecule such as protein, DNA, antigen-antibody, and so on [14, 15]. Nam prepared the modified glycol chitosan (HGC) nanoparticle by conjugation of glycol chitosan (GC) and 5-β cholanic acid, and the results showed that HGC were not only of good compatibility and low toxicity, but also of good cell penetration and retention [16]. He synthesized chitosan modified CdTe/CdS quantum dots and used it to label MDCK cell [17]. Park synthesized glycol chitosan modified fluorescein to label cancer cells [18]. All of their imaging results were satisfying and proved chitosan of good advantages in biochemical marker.

Folic acid has a good affinity to the tumor cells with folate receptor (FR) over-expressed. FR has three major forms, namely FR-α, FR-β, FR-γ, and the FR-α form is over-expressed in various types of human carcinomas,

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including breast cancer, ovarian cancer, endometrial cancer, lung cancer, and so on [19, 20]. The FR has been exploited for targeted delivering of folic acid linked fluorescence probe and drugs to tumor cells in vitro. With this feature, many fluorescence substances, such as quantum dots (QDs), fluorescence dyes, and drug molecules can be grafted by folic acid (FA) to synthesize a fluorescence probe molecule, and then delivered it to cancer cells for providing molecule imaging [21–24]. Successful tumor selective FR-targeting has been reported. Setua synthesized folate-Y_2O_3 nanocrystals as a fluorescence probe to label cancer cells, and the fluorescence microscopy and flow-cytometry data showed highly targeted and imaged A549 cells in vivo with high selectivity [25]. Chen used folic acid conjugated CdHgTe quantum dots to get a high targeting fluorescence probe, and then used it to label Hela cell, the imaging results displayed a high targeting affinity and sensitivity for in vivo early tumor diagnosis. [26].

In the present study, for targeting purpose, we have designed and synthesized a conjugated probe based on folic acid, CTS and TO derivative. TO derivative was synthesized by a convenient chemical reaction (Scheme 1). The TO derivative with carboxyl group was modified by CTS to obtain a coupled probe TO-CTS, then the TO-CTS with some amine residues was modified by folic acid to obtain a coupled probe FA-CTS-TO complex (Scheme 2), which was applied to target and facilitate the recognition of breast cancer cells with over-expressed FR on the membrane cellular surface. The model of FA-CTS-TO complex was shown in Fig. 1, and optical characteristics of albumin and tumor cells labeling were also studied.

Experimental Section

General

IR spectra were recorded on FT-IR instrument, NICOLET380 FT-IR, American. DTA thermograms were recorded on LCT-2 differential thermal balance, EXSTAR6000TG/DTA, American. UV absorbance spectra were scanned on a 2550 UV-Vision analysis instrument, Shimadzu, Japan. Fluorescence spectra were scanned on a fluorescence analysis instrument, Cary Eclipse, American. Mass spectral analysis data were obtained using an electrospray ionization (ESI) mass spectrometer. Melting points were taken on a Yanaco apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC-P300 (300 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS), using DMSO-d_6 as a solvent. All the reagents are analytically pure.

Synthesis of 2-Benzylmercapto-N-Methylbenzothiazole

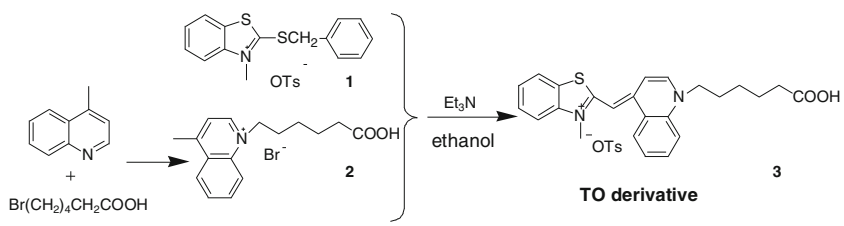
2-benzylmercapto-N-methylbenzothiazole was prepared according to traditional method [11] which served as our starting materials for further synthetic studies. 2-Benzylmercapto-N-methylbenzothiazole was performed by refluxing 2-benzylmercaotobenzothiazole (3.50 mmol) and methyl p-toluenesulfonate (1.30 g, 6.98 mmol) in xylene (20 mL), followed by TLC method until the reaction completely. After dumping the upper solution of the mixture, the residue was separated by column chromatography via silica gel with methanol to yield compound 1.

Synthesis of TO Derivative

TO derivative was synthesized by mixing compound 1 (1.20 mmol) and compound 2 (1.80 mmol) in 30 mL ethanol. Meanwhile, triethylamine (Et_3N, 0.5 mL) was added causing the reaction mixture to immediately turn a salmon pink color. After stirring for 24 h, the products were precipitated by addition of diethyl ether, filtrated, washed and dried to give salmon pink compound 3. [27]. The synthetic route of TO derivative was shown in Scheme 1.

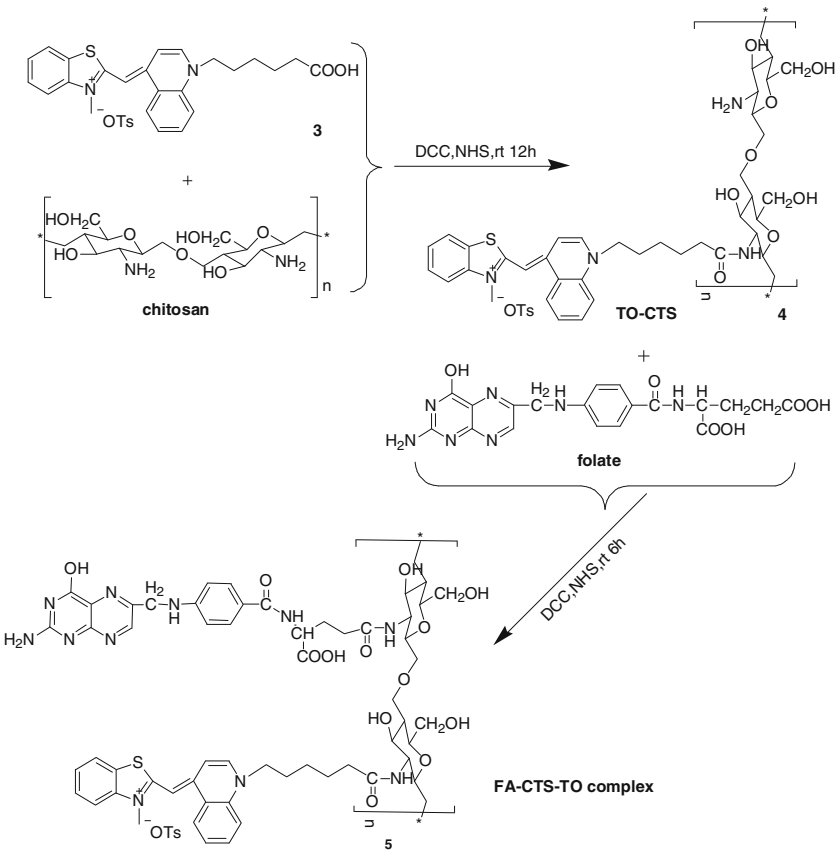
Synthesis of TO Derivative with CTS

Briefly, H_2O (20 mL) solution containing CTS (1000 mg, Molecular weight=3000) was dropped in to a 30 mL DMF solution containing NHS, DCC and TO derivative (100 mg). The reaction mixture was stirred at room temperature for 12 h, and then extracted with ether and ethyl acetate in turn. After that, the aqueous phase was washed with acetone to give a floccule, which was isolated by filtration, washed with methanol and dried to afford compound 4 (TO-CTS).



Scheme 1 Synthetic routes of the TO derivative

Scheme 2 Synthetic routes of fluorescence probe FA-CTS-TO complex



Synthesis of TO-CTS with Folic Acid

Briefly, H_2O (10 mL) solution containing TO-CTS (800 mg) synthesized above were dropped in to a 15 mL DMF solution containing folic acid (40 mg), DCC and NHS. The reaction mixture was stirred at room temperature for 6 h to give a floccule, then washed with HAC-NaAc buffer solution (pH=5.8, 0.2 mol/L) and acetone, and dried to afford product FA-CTS-TO complex 5. The synthetic routes of fluorescence probe FA-CTS-TO complex were shown in Scheme 2.

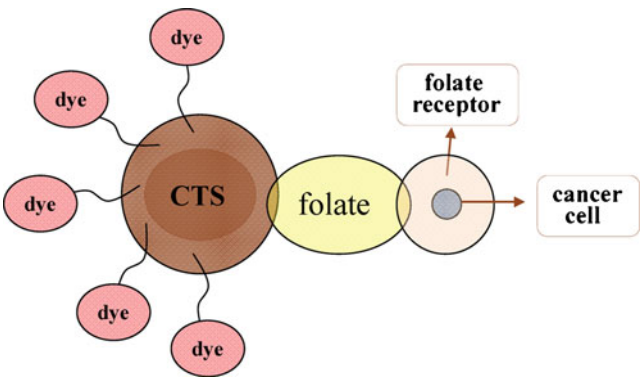


Fig.1 Model of fluorescence probe FA-CTS-TO complex

Label of Bovine Serum Albumin

Bovine serum albumin (50 mg) was dissolved in NaH_2PO_4-Na_2HPO_4 (100 mL, pH=7.7) buffer solution to afford a stock solution. 5 mL of the solution was diluted by NaH_2PO_4-Na_2HPO_4 buffer solution or albumin for fluorescence studies. Different probes were dissolved in H_2O and saved as the stock solutions respectively.

Label of Breast Cancer Cells

The specific method of fluorescence probe labeling breast cancer MCF-7 cell line was shown as above. MCF-7 cells were cultivated by routine method and the culture medium were minimum essential medium (MEM) supplemented with 10 % (*v/v*) of fetal bovine serum (FBS) and DulbeccÓs modified Eaglé-s medium (DMEM) supplemented with 10 % (*v/v*) of FBS. The cells were transplanted to a 24-well plate and the cell concentration was about 1×10^5/mL for each well. After 24 h, the old culture media was replaced with Hank's balanced salt solution (HBSS). TO-COOH was added to continue to be incubated for 8 h at 37 °C under a

5 % CO_2 atmosphere. The cells were washed by HBSS three times and fluorescence photos were performed using an inverted fluorescence microscope.

Results and Discussion

Chemical Structure Identification of TO Derivative

The structure of TO derivative was determined by ^{1}H NMR spectra. The reaction yield is 91 %. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.58 (t, J=7.50 Hz, 2H), 1.85 (t, J=7.20 Hz, 2H), 2.26–2.31 (m, 4H), 4.00 (s, 3H), 4.58 (t, J=7.20 Hz, 2H), 6.89 (s, 1H), 7.32 (d, J=6.90 Hz, 1H), 7.39 (t, J=7.65 Hz, 1H), 7.59 (t, J=7.95 Hz, 1H), 7.70–7.77 (m, 2H), 7.96 (t, J=7.65 Hz, 1H), 8.02 (d, J=7.80 Hz, 1H), 8.10 (d, J=8.70 Hz, 1H), 8.62 (d, J=7.20 Hz, 1H), 8.77 (d, J=8.10 Hz, 1H). ESI-MS: m/e 405.16 (M^+), 406.16 (M^++1).

Infrared Spectra and Differential Thermal Analysis of TO and its Derivatives

The structure of TO derivative was determined by infrared analysis spectrum (IR) and differential thermal analysis (DTA). The IR spectra of TO derivatives were shown in Fig. 2. It was found that the curves of FA-CTS-TO complex and TO-CTS were very similar, but new spectrum peaks appeared at 1644 cm^{-1}, 1560 cm^{-1} and 1355 cm^{-1}, whose relevant peaks could be found in the curve of folic acid at 1710 cm^{-1}, respectively. In addition, the spectrum peaks at 1560 cm^{-1}, 1644 cm^{-1} were new ones in FA-CTS-TO compared with the spectroscopy curves of TO and CTS. It was shown that folic acid and CTS are combined with TO derivative to afford FA-CTS-TO complex.

According to differential thermal analysis (DTA), two endothermic peaks at 124 °C, 156 °C and an exothermic peak at 195 °C was on the DTA curve of folic acid, while there was an endothermic peak at 165 °C and an exothermic peak at 300 °C on the DTA curve of TO, but an exothermic peak at 218 °C on the curve of FA-CTS-TO. There was an endothermic peak at 213 °C on the DTA curve of TO-CTS, and there was an endothermic peak at 221 °C and an exothermic peak at 283 °C on the DTA curve of CTS. As shown above, FA-CTS-TO is proved to be a new compound bonded by folic acid, CTS and TO and not a simple mixture of them by infrared spectroscopy and DTA thermograms.

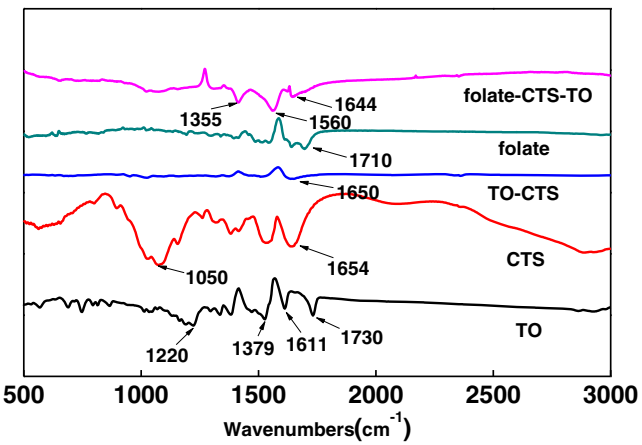


Fig. 2 IR spectra of TO and its derivatives

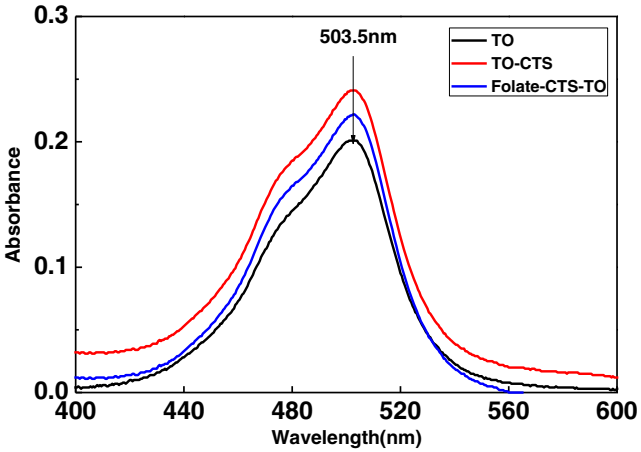


Fig. 3 UV spectra of TO and its derivatives, spectra of aqueous solution with the concentration of 2 mg·L^{-1}

UV–Vis Spectra of TO and its Derivatives

From Fig. 3, the absorption spectra of the studied TO monomer and its derivatives were observed. The maximal absorption wavelength of folic acid conjugated CTS grafted TO derivative (FA-CTS-TO) changed little compared with the TO monomer. Carboxyl-group on TO monomer is

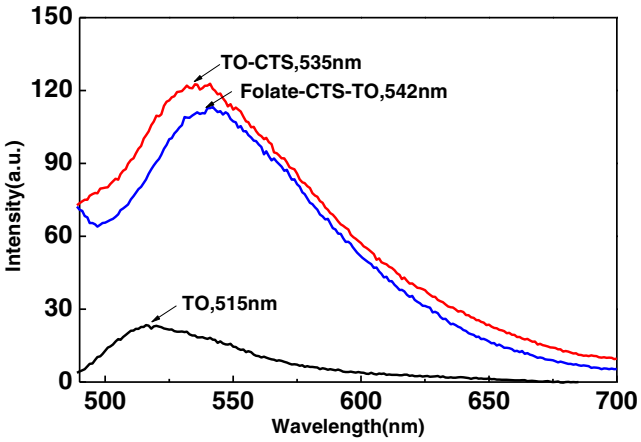


Fig. 4 Fluorescence spectra of TO and its derivatives, spectra of aqueous solution with the concentration of 3 mg·L^{-1}

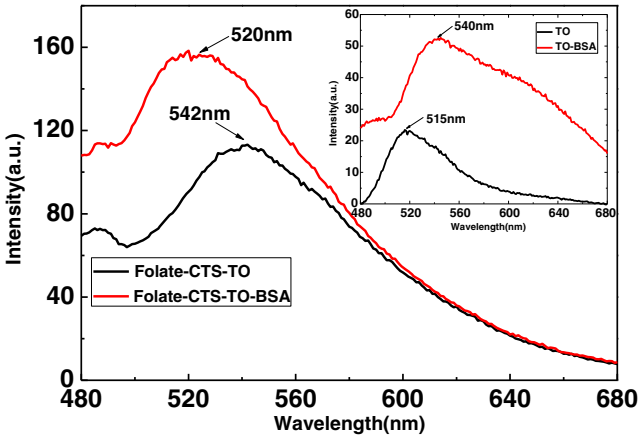


Fig. 5 Fluorescence spectra of BSA labeled by TO and FA-CTS-TO complex, spectra of aqueous solution with the concentration of 3 mg·L^{-1}

situated at the end of saturated chain, and could be fully extended in the reaction, so the maximal absorption wavelength was unaffected.

Fluorescence Spectra of TO and its Derivatives

The fluorescence spectra of TO and its derivatives (the excitation and emission slits widths are 5 nm) were recorded at room temperature, and the results were shown in Fig. 4. It was shown that the fluorescent intensity and water-soluble of FA-CTS-TO complex were enhanced greatly compared with that of TO monomer. The maximal emission wavelength of FA-CTS-TO complex were occurred to red-shift compared with TO, because the steric hindrance effect of CTS limited rotation of TO monomer, which changed the conformation of TO, and then enhanced the fluorescent intensity.

Label of Bovine Serum Albumin

TO monomer and FA-CTS-TO complex were used to study in labeling bovine serum albumin (BSA) and the fluorescence spectra results were shown in Fig. 5. The emission wavelength of TO monomer appeared a red-shift and the fluorescence intensity increased after labeled BSA, but the emission wavelength of FA-CTS-TO complex appeared a blue-shift and the intensity also increased after labeled BSA. Because cyanine dye TO, with embedded features, could insert into BSA structure by affinity. Accordingly, special structure can lead to the increasing of fluorescence intensity.

Label of Breast Cancer Cells

Embedded fluorescence dyes can combine with nucleic acid and show fluorescence, such as TO, propidium iodide (PI), and so on. It is reported that PI can combine with double stranded DNA but cannot pass through the cell membrane [28]. In this paper, fluorescence probe TO and its derivatives were used to label breast cancer cells, and the fluorescence behaviors of the MCF-7 cells were observed. The photomicrographs of breast cancer cells incubated at 37 °C under the invert microscope were shown in Fig. 6, which offered a new try in the aspect of labeling cells by the embedded dyes. In Fig. 6(a), TO monomer could penetrate cytoplasm into cell nucleus, and experimental results demonstrated that green fluorescence was observed in cell nucleus but not in

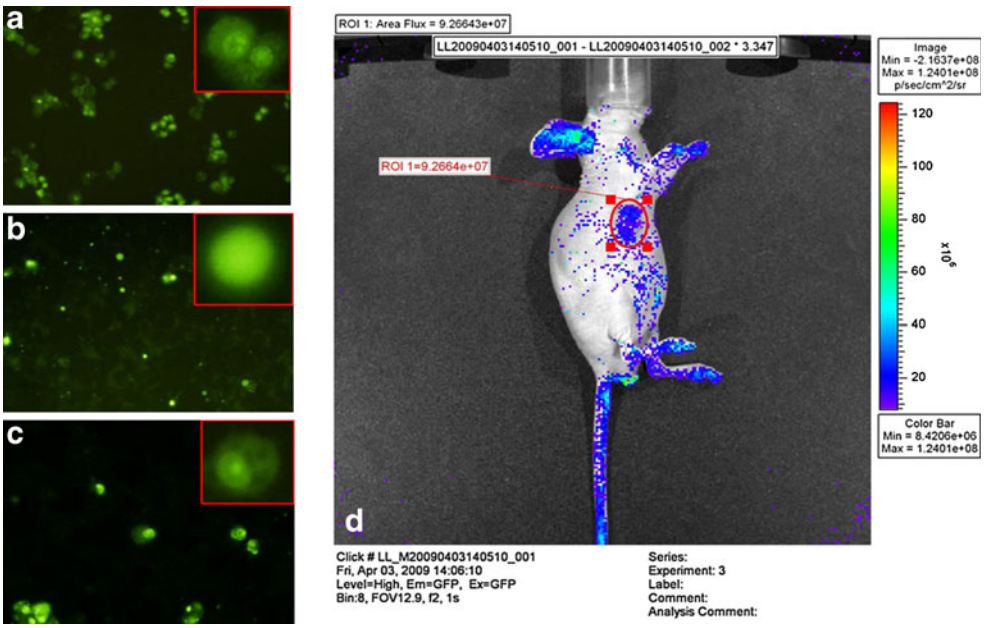


Fig. 6 Fluorescence microscopy imaging of breast cancer cells (MCF-7) labeled by (**a**) TO, (**b**) TO-CTS, (**c**) FA-CTS-TO. The photomicrographs of breast cancer cells incubated at 37 °C under the invert microscopy. (**d**) Fluorescence imaging of MCF-7 tumor mouse in 72 h postinjection of FA-CTS-TO complex

the cytoplasm of the cells. However, in the photograph of cells labeled by TO-CTS, both of cell nucleus and cytoplasm showed strong green fluorescence. Also, the cells labeled by TO-CTS showed stronger fluorescence than the cells labeled by TO, which exhibited noticeable enhancement of fluorescence. The result of cells labeled by FA-CTS-TO complex was shown in Fig. 6(c), and green fluorescence was observed in cell nucleus but not in the cytoplasm.

In order to confirm the targeting effect of fluorescence probe FA-CTS-TO complex, fluorescence imaging based on the specific marking of tumors was carried out. For imaging, a female 6 week old nude mouse suspended MCF-7 cells was inoculated into the breast tissues. Fluorescence probe of FA-CTS-TO complex was administered to mouse with cancer cells via tail veil injection and the fluorescence imaging was shown in Fig. 6(d). The fluorescence probe of FA-CTS-TO complex has a good targeted recognition property in label of breast cancer cells MCF-7, and the complex fluorescence probe has non-toxicity. Fluorescence signals were still emitted from the breast tumor at 72 h postinjection.

The experiment results proved that fluorescence probe FA-CTS-TO complex was able to penetrate tumor cells and target FR over-expressed ones in vivo selectively. The nude mouse was alive at 6 d postinjection. The folic acid conjugated compound can enter the intracellular cytoplasm via endocytosis and selectively deliver the fluorescence probe of FA-CTS-TO complex to breast cancer cells and to label them.

Conclusion

In summary, TO derivative and folic acid conjugated chitosan grafted thiazole orange (FA-CTS-TO) complex were prepared through a convenient synthetic method. Furthermore, the fluorescence properties of TO derivative and fluorescence probe FA-CTS-TO complex were studied. Also, the fluorescence imaging of breast cancer cells MCF-7 and mouse were studied and the results showed that fluorescence probe of FA-CTS-TO complex can be penetrate cytoplasm and target FR over-expressed ones in vivo selectively. Therefore, such prominent fluorescence properties demonstrate again that FA-CTS-TO complex as a tumor targeting fluorescence probe is appropriate for breast cancer cells.

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