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Thiazole Orange derivatives: Synthesis, fluorescence properties, and labeling cancer cells

Xuening Fei *, Yingchun Gu, Ying Ban, Zhijun Liu, Baolian Zhang

Department of Material Science and Engineering, Tianjin Institute of Urban Construction, Tianjin 300384, China

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ABSTRACT

A series of Thiazole Orange (TO) derivatives were synthesized and modified by introducing different substitutional groups on benzothiazole and 4-methylquinoline All the TO derivatives were confirmed by ^{1}HNMR and MS. TO derivative bearing NH_2– was modified by folic acid and used to label breast cancer cells. The phenomenon of fluorescence enhancement was shown by the fluorescence spectrums of TO derivatives and micrographs of the labeled breast cancer cells. It offered a new try in the aspect of labeling cells by the embedded dyes.

1. Introduction

The 4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinoliniumiodide fluorophore, better known as cyanine dye Thiazole Orange (TO), possesses many distinctive and desirable properties: it has very low intrinsic fluorescence, chemical stabilities, and a high molar absorption coefficient.[1] The dye is virtually non-fluorescent when not combined with nucleic acids, but shows fluorescence enhancement over 1000-fold when inserted into DNA, especially into double-helix of DNA, and over 3000-fold when inserted into RNA.[2–8] This fluorescence character can be used to improve the detection sensitivity, which reduces background interference that is difficult to remove from intracellular environments or tissues.[9,10] Moreover, the dye has higher affinity to tumor than to the normal cells, which will be widely used in early-stage labeling of cancer cells.[11]

Recently, considerable efforts have been devoted to the modification of TO derivatives because of the important roles that they play in various chemical and biological processes. Chitosan oligosaccharide (CTS), a typical bioactive polysaccharide,[12,13] is easy to be modified selectively by appropriate biological molecules based on special object, which provides a new method for traditional detection in biomedicine investigation. As a non-viral vector, galactosylated chitosan showed good targeting effect when used in gene therapy for hepatocellular carcinoma.[14] In 2005, Kim et al.[15] used fluorescent dyes modified by galactosylated chitosanthiocyanate to get targeted image for hepatocytes. It was found that optical images were mainly concentrated in hepatocytes at 10, 60, 120 min after injection respectively. CTS acts as a bridge between targeted cancer cells and fluorescent dyes, which can enhance the cellular compatibility with dyes and reduce toxicity.

A variety of different targeting strategies are currently under investigation to enhance the specificity of labeling tumor. Folic acid has shown potential as a targeting device because the folate receptor is highly overexpressed in a number of human tumors including breast cancer, ovarian cancer, cervical cancer, colorectal cancer, nasopharyngeal cancer, lung cancer, whereas in normal tissue, its expression is significantly lower, which may be ideally suited for the study of diagnosis and therapy of cancers.[16–25]

Kennedy et al.[26] used cyanine dyes modified by folic acid to study the optical imaging of tumor cells. It was found that tumor cells with folate receptor showed bright signs but normal cells hardly. This method can distinguish tumor cells from normal cells.

In the present study, we have prepared a series of designed molecules of which different substitutional groups have been introduced to benzothiazole and 4-methylquinoline. TO and its derivatives were synthesized using the reaction scheme as shown in Scheme 1. These TO derivatives share a common core structure. TO derivatives with an amine residue can be modified by folic acid easily for the study of identifying cancer cells as targets that have an extra folacin acceptor on the cellular surface. By connecting CTS to TO derivative with carboxyl residue (TO–COOH) to gain TO derivative (TO–COOH–CTS), a new labeling strategy also has been developed.

* Corresponding author. Tel.: +86 22 23085033; fax: +86 22 23085032.
E-mail address: xueningfei@126.com (X. Fei).

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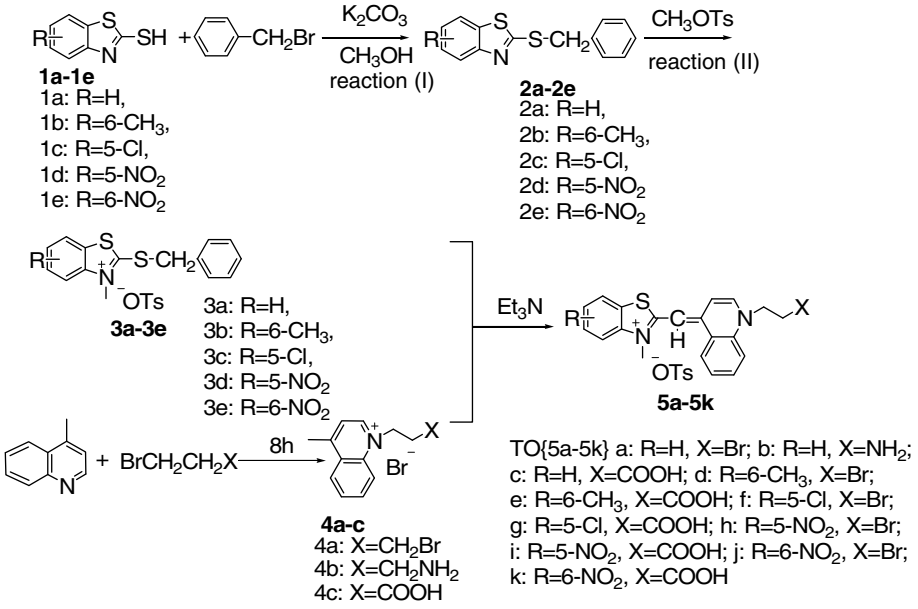

Scheme 1. Synthetic routes of the TO derivates.

2. Results and discussion

2.1. Synthesis

As a traditional alkylating agent, iodomethane is expensive and of high toxicity, dimethyl sulfate is also virulent. Benzyl bromide was used as the alkylating agent to decrease the cost, toxicity, and reduce the damage to our environment in this paper. The influence factors of the alkylation were investigated, such as solvent, temperature, and reaction time. A series of solvents used in the experiment was studied separately and the results were shown in Table 1. It was found that the higher data of dielectric constant and lower data of dipole moment were useful factors for the increasing yield from entries 1 to 5. Based on the optimum reaction condition from Table 1, entry 5, the suitable reaction time was also studied, which was shown in Table 2. The desirable result was displayed in entry 2 (1 h, 93%). The yield hardly increased by prolonging reaction time. Moreover, the conditions of quaterization of 2-benzylmercaptobenzothiazole were also optimized. The appropriate reaction time, reaction temperature and solvent were concluded from data shown in Table 3–5, and the highest yield (78%) was obtained in refluxing xylene for 24 h. Solvent polarity is one of the major factor affecting the reaction process. The result revealed that the reaction temperature was the more important factor when the solvent data of the dielectric moment was similar to the other ones.

2.2. Chemical structure identification of modified TO derivates

The structures of modified TO derivates were determined by IR spectrum and differential thermal analysis (DTA) spectrum. The IR spectrum of TO bearing $-NH_2$ (TO–NH_2) and its derivates were shown in Figure 1 and TO–COOH derivates in Figure 2. From the IR curves of TO–NH_2 and TO–NH_2 derivative (folate–TO–NH_2) modified by *N*-hydroxysuccinimide eater of folic acid (NHS–folate), obvious differences of the absorbing peaks position between them can be found.

As shown in Figure 1, the spectrum peaks at 1695 cm^{-1}, 1297 cm^{-1}, 1188 cm^{-1}, 839 cm^{-1}, 820 cm^{-1}, 450 cm^{-1} were new ones in folate–TO–NH_2 compared to the spectroscopy curve of TO–NH_2 but they were found in the curve of folic acid at 1694 cm^{-1}, 1299 cm^{-1}, 1192 cm^{-1}, 839 cm^{-1}, 819 cm^{-1}, 445 cm^{-1}, respectively. Thus TO–NH_2 is modified by NHS–folate to gain folate–TO–NH_2. From Figure 2, it was found that the curves of folate–TO–COOH–CTS and TO–COOH–CTS were very similar, but new spectrum peaks appeared at 1608 cm^{-1}, 952 cm^{-1}, 848 cm^{-1}, 618 cm^{-1}, whose relevant peaks could be found in the curve of folic acid at 1606 cm^{-1}, 973 cm^{-1}, 839 cm^{-1}, 593 cm^{-1}, respectively. It is shown that folic acid is combined with TO–COOH–CTS to afford folate–TO–COOH–CTS.

According to DTA thermograms results, a endothermic peak at 124 °C and a exothermic peak at 195 °C was on the DTA curve of folic acid, while there was a exothermic peak at 181 °C on the DTA curve of TO–NH_2, but a exothermic peak at 282 °C on that of

Table 2
The effect of reaction time on the synthesis of **2a**

Entry	Time (h)	Yield (%)
1	0.5	83
2	1	93
3	2	93

Table 1
The effect of solvent categories on the synthesis of **2a**

Entry	Solvent	Dielectric constant[27] (25 °C)	Dipole moment[27] (D)	Temp. (°C)	Time (h)	Yield (%)
1	CH_3COCH_3	20.70	2.86	Reflux/56	4	62
2	$CHCl_3$	4.81	1.15	Reflux/61	1	71
3	$PhCH_3$	2.38	1.33	Reflux/111	1	70
4	EtOAc	6.02	1.89	Reflux/77	1	82
5	C_2H_5OH	24.55	1.73	Reflux/78	1	88
6	C_2H_5OH	24.55	1.73	rt	1	23
7	CH_3OH	32.70	1.70	rt	1	93

Table 3
The effect of solvent categories on the synthesis of **3a**

Entry	Solvent	Dielectric constant[27] (25 °C)	Dipole moment[27] (D)	Time (h)	Temp. (°C)	Yield (%)
1	$PhCH_3$	2.38	1.33	24	Reflux/111	32
2	$Ph(CH_3)_2$	—	—	24	Reflux/140	78
3	CH_3COCH_3	20.70	2.86	24	Reflux/56	0
4	EtOAc	6.02	1.89	24	Reflux/77	0
5	CH_3CH_2OH	24.55	1.73	24	Reflux/78	0
6	$CHCl_3$	4.81	1.15	24	Reflux/61	2

Table 4
The effect of reaction temperature on the synthesis of **3a**

Entry	Temp. (°C)	Time (h)	Yield (%)
1	80	24	0
2	111	24	32
3	140	24	78

Table 5
The effect of reaction time on the synthesis of **3a**

Entry	Time (h)	Yield (%)
1	24	77
2	36	78
3	48	78

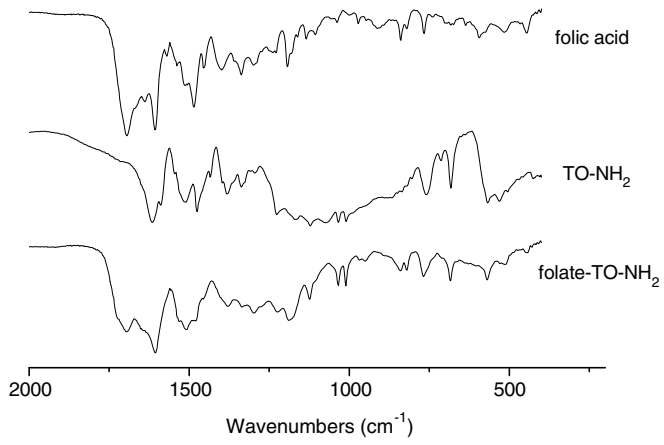

Figure 1. IR spectrums of TO–NH_2 derivatives.

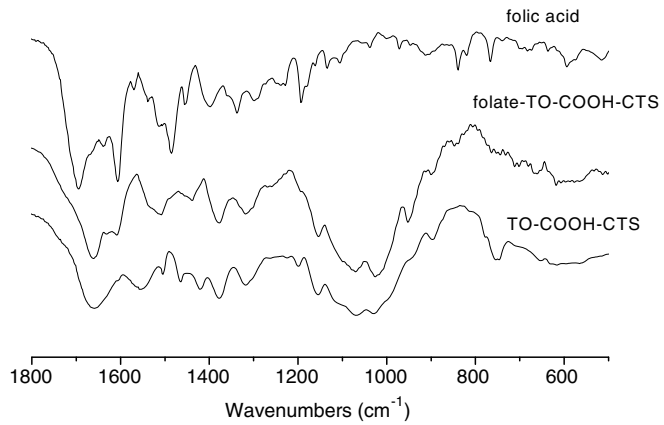

Figure 2. IR spectrums of TO–COOH derivatives.

folate–TO–NH_2. It is further illustrated that folate–TO–NH_2 is bonded by folic acid and TO–NH_2. To summarize, folate–TO–NH_2 was proved to be a new compound reacted by NHS–folate and TO–NH_2 with infrared spectroscopy and the DTA thermograms.

There was only a slow weightloss on the DTA curve of TO–COOH–CTS and there was a endothermic peak at 124 °C and a exothermic peak at 195 °C on the DTA curve of folic acid with no weightloss curve on it, but there were two exothermic peaks at 262 °C and 239 °C on the DTA curve of folate- TO–COOH–CTS with a slow weightloss. As shown above, folate–TO–COOH–CTS is proved to be a new compound bonded by folic acid and TO–COOH and not a simple mixture of them by infrared spectroscopy and the DTA thermograms.

2.3. Fluorescence properties

The fluorescence spectrums of TO derivatives were measured and the results were shown in Figure 3. It was shown that the values of maximal fluorescence emission (λ_{ex}) shift to a certain extent when TO was modified by different substitutional group. TO derivatives containing the nitro group had a higher data of λ_{ex}; and intensity. The special characteristics of their fluorescence didn't change obviously although the nitro group was at different position on the aryl ring.

The fluorescence spectrums of TO–COOH and TO–COOH–CTS were measured for comparing the differences of relevant fluorescence. The λ_{ex} of TO–COOH–CTS was close to that of TO–COOH with greatly increased fluorescence intensity and the phenomenon of enhancement of fluorescence was shown obviously in Figure 4. Based on this result, it may be possible to design and prepare a kind of fluorescence probe of Thiazole Orange with high sensitivity, good biological intermiscibility, and excellent water solubility. The interaction relationship between TO–COOH and CTS are being studied in our group.

The fluorescence spectrums of TO–NH_2 and TO–COOH marked by BSA were also measured and the results were shown in Figure 5. The λ_{ex} of TO–COOH appeared a red shift with decreased inten-

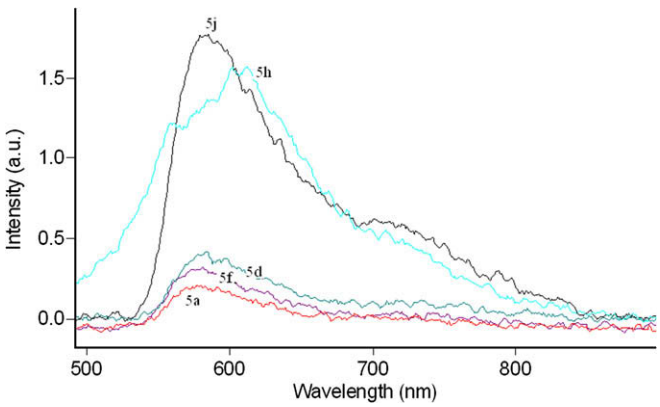

Figure 3. Fluorescence spectrums of TO derivatives.

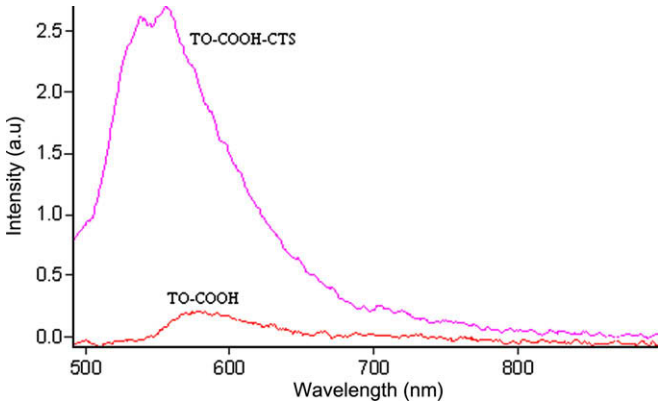

Figure 4. Fluorescence spectrums of TO–COOH and TO–COOH–CTS.

sity compared to that of TO–NH_2 and the intensity both increased after marked with BSA. We also studied the fluorescence spectrums of folate–TO–NH_2 and folate–TO–NH_2–BSA shown in Figure 6. It revealed that the λ_{ex} of folate–TO–NH_2–BSA was close to that of folate–TO–NH_2 with increased fluorescence intensity.

2.4. Label of breast cancer cells

Embedded fluorescence dyes can combine with nucleic acid and show fluorescence, such as TO, propidium iodide (PI). It is reported that PI can combine with double stranded DNA but cannot pass through the cell membrane.[28] In this paper, we observed an interesting phenomenon that TO and its derivatives can penetrate through the cell membrane into the nucleus and show strong fluorescence. TO–COOH and TO–COOH–CTS were used to study in labeling breast cancer cells. The photomicrographs of breast cancer cells incubated at 37 °C under the invert microscope were shown in Figures 7 and 8, which offered a new try in the aspect of labeling cells by the embedded dyes. Experimental results demonstrated that green fluorescence was observed in cell nucleus but not in the cytoplasm of the cells labeled by TO–COOH. However, in the photos of cells labeled by TO–COOH–CTS, both of cell nucleus and cytoplasm showed strong green fluorescence. Also, the cells labeled by TO–COOH–CTS showed stronger fluorescence than the cells labeled by TO–COOH, which exhibited noticeable enhancement of fluorescence. The result of cancer cells labeled by folate–TO–COOH–CTS was shown in Figure 9. In the photo green fluorescence was observed in cell nucleus but not in the cytoplasm, which appeared a similar case labeled by TO–COOH.

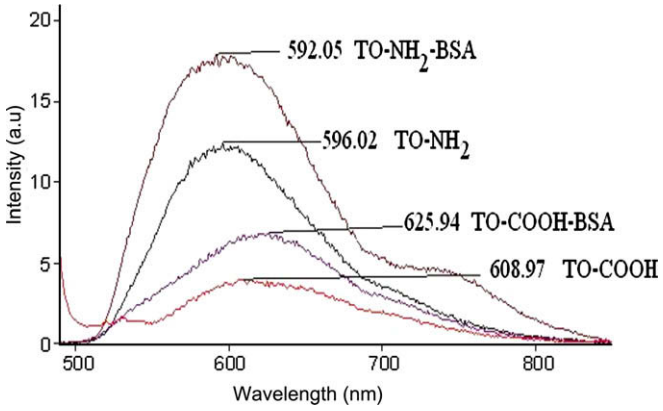

Figure 5. Fluorescence spectrums of TO derivatives marked by BSA.

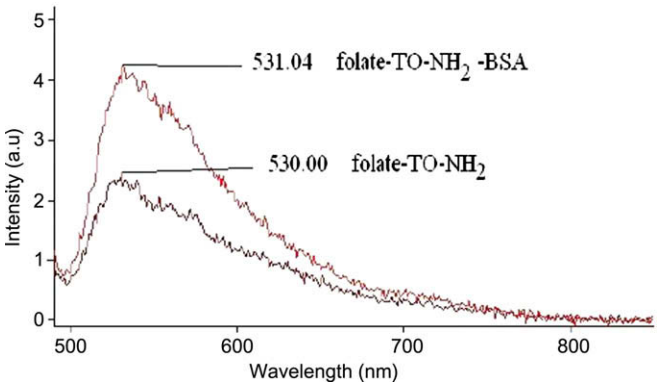

Figure 6. Fluorescence spectrums of folate–TO–NH_2 and derivative marked by BSA.

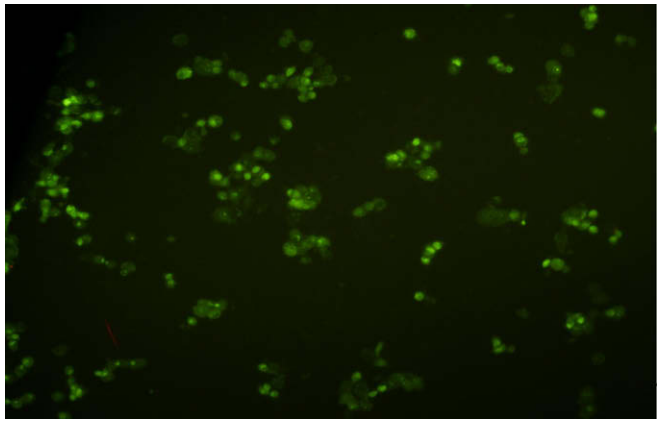

Figure 7. Micrographs of breast cancer cells labeled by TO–COOH.

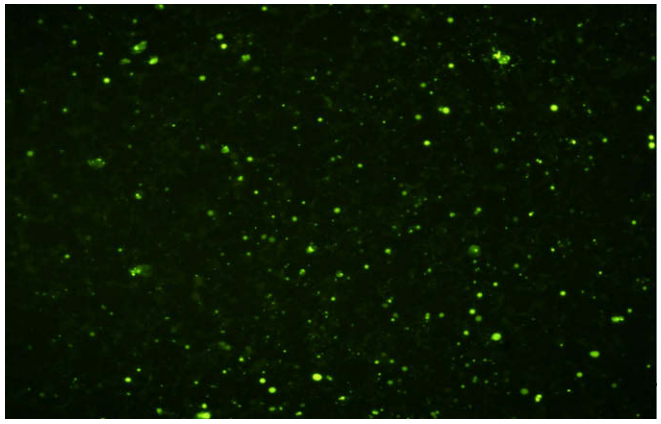

Figure 8. Micrographs of breast cancer cells labeled by TO–COOH–CTS.

TO–NH_2 and folate–TO–NH_2 were also used in the labeling cells and the results were shown in Figures 10 and 11. The strong bright fluorescence signals were seen in the distribution of the cell nucleus and not in the cell membrane of breast cancer cells labeled by TO–NH_2 and folate–TO–NH_2. It is observed that TO–NH_2 can penetrate through the cell membrane and exist stably in the nuclear with no toxicity.

Over repeated experiment, the labeled samples showed very little photobleaching, far less than with conventional dye molecules. So the structures of TO and its derivatives are not degraded and the dyes could penetrate through the cell membrane into the nucleus with no toxicity and show fluorescence and sense information.

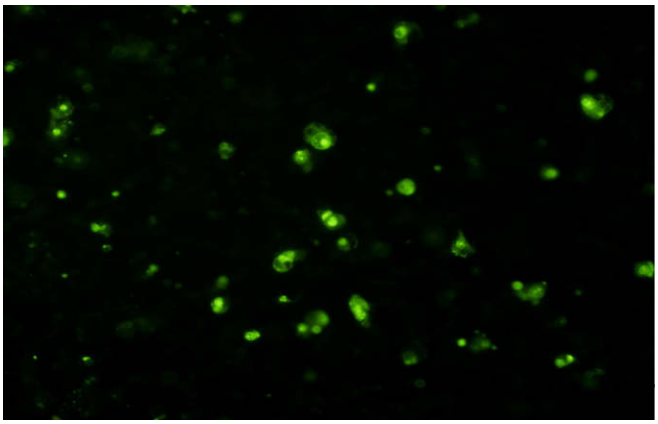

Figure 9. Micrographs of breast cancer cells labeled by folate–TO–COOH–CTS.

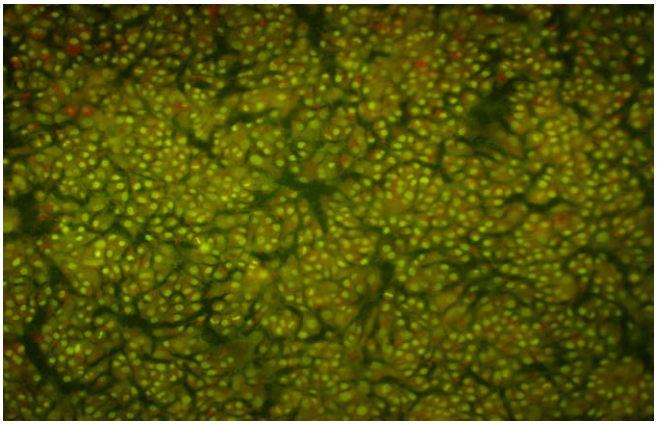

Figure 10. Micrographs of breast cancer cells labeled by TO–NH_2.

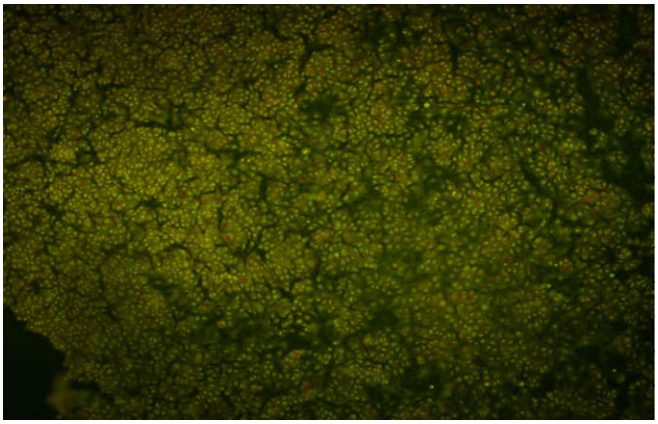

Figure 11. Micrographs of breast cancer cells labeled by folate–TO–NH_2.

3. Conclusions

In conclusion, eleven TO derivatives were synthesized in optimal conditions and confirmed by ^{1}HNMR and MS. The fluorescence spectrums of TO derivatives were studied preliminarily and the phenomenon of the enhancement of fluorescence was depicted. Breast cancer cells labeled by TO derivative bearing –COOH or –NH_2 and their derivatives were also studied preliminarily, which offered a new try in the aspect of labeling cells by the embedded dyes. The multiple relationships between TO derivatives and cells are being investigated.

4. Experimental

4.1. General experimental conditions

Fluorescence spectra were scanned on a fluorescence analysis instrument, Cary Eclipse, American. Mass spectral analyses 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 TMS (tetramethylsilane), using DMSO-d_6 as a solvent. DTA thermograms were recorded on LCT-2 differential thermal balance (China). All the reagents are analytically pure.

4.2. General procedure for the preparation of 2-benzylmercaptobenzothiazole derivatives 2a–2e

2-Mercaptobenzothiazole derivatives **1** were prepared according to traditional method[29] which served as our starting materials for further synthetic studies. 2-mercaptobenzothiazole derivatives (8.97 mmol) were added to anhydrous methanol (30 mL) and stirred to dissolve, then K_2CO_3 (2.00 g , 11.30 mmol) and benzyl bromide (2.00 mL, 10.00 mmol) were added to the mixture and stirred at room temperature. TLC method followed the track of reaction until the reaction completed.

Reaction mixture was filtered hot, and the filtrate was dried in vacuo. The residue was separated on a flash chromatography column (silica gel, solvent ethyl acetate:hexane 50:1(v/v)) to afford compounds **2**. The results are shown in Table 6.

4.3. General procedure for the preparation of 2-benzylmercapto-*N*-methylbenzothiazole derivatives 3a–3e

2-Benzylmercapto-*N*-methylbenzothiazole derivatives were performed by refluxing 2-benzylmercaptobenzothiazole derivatives **2** (3.50 mmol) and methyl *p*-toluenesulfonate (1.30 g, 6.98 mmol) in xylene (20 mL), followed by TLC method until the reaction completed. After dumping the upper solution of the mixture, the residue was separated by column chromatography via silica gel with methanol to yield compounds **3**. The data are shown in Table 7.

4.4. General procedure for the preparation of TO derivates 5a–5k

TO and its derivates were synthesized by mixing compounds **3** (2.00 mmol) and compounds **4** (2.72 mmol)[30] in 30 mL ethanol. To this solution, triethylamine (Et_3N, 0.80 mL) was added causing the reaction mixture to immediately turn a salmon pink color. After stirring for 60 min, the products were precipitated by addition of diethyl ether, filtrated, washed and dried to give salmon pink solid **5**.

4.5. TO derivative (5a)

Yield: 97%, mp: 202–204 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.42 (t, J = 6.75 Hz, 2H), 3.66 (t, J = 6.30 Hz, 2H), 4.03 (s, 3H), 4.69 (t, J = 6.75 Hz, 2H), 6.94 (s, 1H), 7.36–7.46 (m, 2H), 7.62 (t,

Table 6
Reaction time and physical properties of compound **2a**–**2e**

Entry	Yield (%)	Time (h)	Mp (°C)
2a	89	1	39
2b	92	4	Liquid
2c	98	2	67–69
2d	95	3	74–76
2e	78	3	105–108

Table 7
Reaction time and yields of compound **3a–3e**

Entry	Time (h)	Yield (%)
3a	24	81
3b	24	92
3c	24	33
3d	48	53
3e	96	21

J = 8.10 Hz, 1H), 7.75 (d, *J* = 7.80 Hz, 1H), 7.80 (d, *J* = 8.70 Hz, 1H), 8.00 (t, *J* = 7.35 Hz, 1H), 8.07 (d, *J* = 7.80 Hz, 1H), 8.14 (d, *J* = 8.70 Hz, 1H), 8.60 (d, *J* = 7.20 Hz, 1H), 8.81 (d, *J* = 8.40 Hz, 1H). ESI-MS: *m*/*e* 411.66 (M^+–1), 413.66 (M^++1), 414.36 (M^++2).

4.6. TO derivative (5b)

Yield: 95%. mp: 191–193 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.35 (t, *J* = 5.85 Hz, 2H), 2.98 (s, 3H), 3.12(t, *J* = 5.40 Hz, 2H), 5.18 (t, *J* = 6.75 Hz, 2H), 7.01 (t, *J* = 7.65 Hz, 1H), 7.10 (d, *J* = 9.00 Hz, 1H), 7.29 (t, *J* = 7.80 Hz, 1H), 7.52 (d, *J* = 9.00 Hz, 1H), 8.03 (t, *J* = 7.65 Hz, 2H), 8.26 (t, *J* = 7.95 Hz, 1H), 854 (d, *J* = 9.00 Hz, 1H), 8.67 (d, *J* = 9.00 Hz, 1H), 9.40 (d, *J* = 6.00 Hz, 1H). ESI-MS: *m*/*e* 348.33 (M^+), 349.26 (M^++1), 350.25 (M^++2).

4.7. TO derivative (5c)

Yield: 97%. mp: 171–173 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.74 (t, *J* = 6.45 Hz, 2H), 3.99 (s, 3H), 4.74 (t, *J* = 6.10 Hz, 2H,), 6.88 (s, 1H), 7.32 (d, *J* = 6.90 Hz, 1H), 7.40 (t, *J* = 7.65 Hz, 1H), 7.60 (t, *J* = 7.80 Hz, 1H), 7.75–7.78 (m, 2H), 7.96 (t, J = 7.8 Hz, 1H), 8.00 (d, J = 7.8 Hz, 1H), 8.12 (d, *J* = 9.00 Hz, 1H), 8.69 (d, *J* = 9.00 Hz, 1H), 8.77 (d, *J* = 9.00 Hz, 1H). ESI-MS: *m*/*e* 363.25 (M^+), 364.25 (M^++1).

4.8. TO derivative (5d)

Yield: 96%. mp: 150–152 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.39 (t, *J* = 6.70 Hz, 2H), 2.42 (s, 3H), 3.66 (t, *J* = 6.45 Hz, 2H), 4.00 (s, 3H), 4.66 (t, *J* = 7.20 Hz, 2H), 6.87 (s, 1H), 7.28 (d, *J* = 7.20 Hz, 1H), 7.42 (d, *J* = 8.70 Hz, 1H), 7.74 (d, *J* = 7.50 Hz, 1H), 7.68 (d, *J* = 8.70 Hz, 1H), 7.84 (s, 1H), 7.98 (t, *J* = 7.65 Hz, 1H), 8.09(d, *J* = 8.40 Hz, 1H), 8.54 (d, *J* = 9.00 Hz, 1H), 8.76 (d, *J* = 9.00 Hz, 1H). ESI-MS: *m*/*e* 445.86 (M^+−1), 447.48 (M^++1), 449.42 (M^++3).

4.9. TO derivative (5e)

Yield: 95%. mp: 207–210 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.47 (s, 3H), 2.65 (t, *J* = 6.90 Hz, 2H), 3.99 (s, 3H), 4.80 (t, *J* = 5.10 Hz, 2H), 6.85 (s, 1H), 7.33 (d, *J* = 7.80 Hz, 2H), 7.37 (d, *J* = 8.70 Hz, 1H), 7.57 (d, *J* = 8.10 Hz, 1H), 7.73 (d, *J* = 9.00 Hz, 2H), 7.96 (d, *J* = 7.80 Hz, 1H), 8.08 (d, *J* = 8.40 Hz, 1H), 8.70 (t, *J* = 6.00 Hz, 2H). ESI-MS: *m*/*e* 397.41 (M^+), 398.41 (M^++1), 399.41 (M^++2), 400.51 (M^++3).

4.10. TO derivative (5f)

Yield: 96%. mp: 190–192 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.43 (t, *J* = 6.90 Hz, 2H), 3.66 (t, *J* = 6.30 Hz, 2H), 3.99 (s, 3H), 4.73 (t, *J* = 6.75 Hz, 2H), 6.92 (s, 1H), 7.37–7.47 (m, 2H), 7.78 (t, *J* = 7.65 Hz, 1H), 7.93 (s, 1H), 8.04 (d, *J* = 9.00 Hz, 2H), 8.17 (d, *J* = 9.00 Hz, 1H), 8.68 (d, *J* = 9.00 Hz, 1H), 8.82 (d, *J* = 9.00Hz, 1H). ESI-MS: *m*/*e* 427.22 (M^++1), 428.24 (M^++2), 429.27 (M^++3).

4.11. TO derivative (5g)

Yield: 95%. mp: 209–211 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.30 (t, *J* = 6.50 Hz, 1H), 3.93 (s, 1H), 4.74 (t, *J* = 5.85 Hz, 2H), 6.84 (s, 1H), 7.31 (d, *J* = 6.90 Hz, 1H), 7.39 (d, *J* = 8.7 Hz, 1H), 7.73 (t, *J* = 7.35 Hz, 1H), 7.77 (d, *J* = 9.00 Hz, 1H), 7.87 (s, 1H), 7.98 (d, *J* = 9.00 Hz, 1H), 8.14 (d, *J* = 9.30 Hz, 1H), 8.77 (t, *J* = 7.95 Hz, 2H). ESI-MS: *m*/*e* 377.33 (M^+), 378.35 (M^++1), 379.36(M^++2).

4.12. TO derivative (5h)

Yield: 92%. mp: 206–208 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.44 (t, *J* = 6.60 Hz, 2H), 3.68 (t, *J* = 6.45 Hz, 2H), 4.04 (s, 3H), 4.75 (t, *J* = 6.90 Hz, 2H), 6.94 (s, 1H), 7.11 (d, *J* = 7.80 Hz, 1H), 7.50 (d, *J* = 8.10 Hz, 1H), 7.80 (t, *J* = 7.05 Hz, 1H), 8.05 (t, *J* = 7.65 Hz, 1H), 8.18–8.22 (m, 2H), 8.50 (s, 1H), 8.78–8.86 (m, 2H). ESI-MS: *m*/*e* 456.42 (M^+), 458.33 (M^++2), 459.33 (M^++3), 460.31 (M^++4).

4.13. TO derivative (5i)

Yield: 92%. mp: 182–184 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.91 (t, *J* = 7.05 Hz, 2H), 4.04 (s, 3H), 4.89 (t, *J* = 6.90 Hz, 2H), 6.97 (s, 1H), 7.12 (d, *J* = 8.40 Hz, 1H), 7.48 (d, *J* = 8.10 Hz, 2H), 8.22–8.27 (m, 2H), 8.48 (s, 1H), 8.78–8.86 (m, 3H). ESI-MS: *m*/*e* 408.38 (M^+), 409.40 (M^++1), 410.39 (M^++2).

4.14. TO derivative (5j)

Yield: 94%. mp: 228–230 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.45 (t, *J* = 7.65 Hz, 2H), 3.68 (t, *J* = 6.6 Hz, 2H), 4.01 (s, 3H), 4.80 (t, *J* = 6.75 Hz, 2H), 7.02 (s, 1H), 7.12 (d, *J* = 7.8 Hz, 1H), 7.50 (d, *J* = 8.10 Hz, 2H), 7.83–7.87 (m, 2H), 8.08 (t, *J* = 7.35 Hz, 1H), 8.25 (d, *J* = 8.40 Hz, 1H), 8.86 (d, *J* = 7.20 Hz, 2H). ESI-MS: *m*/*e* 457.21 (M^+), 458.21 (M^++1), 459.22 (M^++2), 460.21 (M^++3).

4.15. TO derivative (5k)

Yield: 93%. mp: 238–240 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.97 (t, *J* = 6.15 Hz, 2H), 4.02 (s, 3H), 4.91 (t, *J* = 6.15 Hz, 2H), 7.04 (s, 1H), 7.52 (d, *J* = 6.90 Hz, 1H), 7.85 (t, *J* = 9.00 Hz, 2H), 8.08 (t, *J* = 7.40 Hz, 1H), 8.29 (d, *J* = 9.00 Hz, 1H), 8.42 (d, *J* = 8.70 Hz, 1H), 8.89 (t, *J* = 8.10 Hz, 2H), 9.00 (d, *J* = 8.10 Hz, 1H). ESI-MS: *m*/*e* 408.40 (M^+), 409.42 (M^++1), 410.39 (M^++2).

4.16. Modification of TO–NH_2 with folic acid

Folic acid (500 mg dissolved in 10 ml of dry dimethyl sulfoxide (DMSO) plus 0.25 ml of Et_3N was reacted with *N*-hydroxysuccinimine in the presence of dicyclohexylcarbodiimide overnight at room temperature. The byproduct, dicyclohexylurea, was removed by filtration. The filtrate was concentrated under reduced pressure and the residue was precipitated in diethyl ether. The product, *N*-hydroxysuccinimide eater of folic acid (NHS–folate) was washed several times with anhydrous diethyl ether, dried under vacuum, and stored as a yellow powder.[17,31]

Equal amount of NHS–folate and TO–NH_2 were dissolved in DMSO, adding Et_3N to adjust pH to be weak basic. The mixture was stirred at room temperature until the reaction was completed by TLC. Precipitation with diethyl ether was followed by filtration and yielded folate–TO–NH_2.

4.17. Modification of TO–COOH with CTS

H_2O (8 mL) solution containing chitosan oligosaccharide (600.00 mg, Mw < 3000) and DMSO (4.00 mL) solution containing *N*,*N*-diisopropylethylamine (DIEA) (56 mg, 0.40 mmol) were dropped into a 20 mL dimethylformamide (DMF) solution containing TO–COOH (20 mg, 0.037 mmol), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTu) (41.20 mg, 0.109 mmol) and *N*-hydroxybenzotriazole (HOBt) (14.72 mg, 0.109 mmol) which was

cooled at −5 °C, the reaction mixture was stirred at room temperature for 48 h, extracted with ether and ethyl acetate in turn after being washed with acetone to give a floccule, which was isolated by filtration followed by washing with acetone and drying to afford a rose solid TO–COOH–CTS (606.80 mg).

4.18. Modification of TO–COOH–CTS with folic acid

H_2O (8 mL) solution containing TO–COOH–CTS (624.20 mg) synthesized above and DMSO (1.00 mL) solution containing DIEA (14 mg, 0.10 mmol) were dropped into a 5 mL DMF solution containing folic acid (4.08 mg, 0.00925 mmol), HBTu (10.3 mg, 0.025 mmol) and HOBt (3.88 mg, 0.025 mmol) which was cooled at −5 °C, the reaction mixture was stirred at room temperature for 3 h. The mixture was extracted to give a floccule, and then washed and dried to afford a rose solid folate–TO–COOH–CTS (620.12 mg).

Folate–TO–COOH–CTS could also be synthesized by adding TO–COOH, CTS and folic acid simultaneity to react by the same method above.

4.19. Method of labeling cells

Different dye was dissolved in DMSO and saved as the stock solutions respectively. The specific method of dyes labeling breast cancer MCF-7 cell line was shown as below:

(1) 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 Eaglés medium (DMEM) supplemented with 10% (v/v) of FBS.
(2) 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 Hanks' 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.
(3) The cells were washed by HBSS three times and fluorescence microscopy was performed using an inverted fluorescence microscope.

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