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Synthesis, Characterization and Applications of Selenocysteine Responsive Nanoprobe Based on Dinitrobenzene Sulfonyl-modified Poly(carbonate)s Micelles

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In trace amount, selenium (Se) participates in different physiological functions of human body. Its biological significance is manifested in its presence as selenocysteine (Sec) in genetically encoded selenoproteins that are important in redox regulation, anti-inflammation, and cancer treatment. Recently stimuli-responsive micelles were developed as biosensors but there are no nano micelle probes for the selective imaging of Sec under biological condition (pH = 7.4). Herein we report the design and preparation of 2,4-dinitrobenzenesulfonyl-decorated block poly(carbonate) copolymer, *viz.* PMPC-Dns, for Sec imaging. We found that the PMPC-Dns trapped with the fluorescence doxorubicin (DOX) drug selectively responds to Sec, while getting little interference from biological thiols, amines or alcohols. We applied the PMPC-Dns probe successfully to image endogenous Sec in cervical cancer tissues as well as in Hela cells. In the course of studies, we observed simultaneous release of the trapped DOX. Hence, besides Sec imaging, the probe can be used for controlled delivery of hydrophobic molecules for biomedical applications.

Introduction

Selenium (Se) is considered as an essential micronutrient and its participation in physiological functions is well recognized.[1] Various kinds of Se metabolites such as hydrogen selenide, selenocysteine (Sec), selenite, selenophosphate, selenodiglutathione, and charged Sec-tRNA[2,3] are biosynthesized in animals. The anticancer ability of Se was discovered in 1969.[2] Nonetheless, it is noted that a number of diseases are related to Se intakes of abnormal levels.[2,4] Selenium compounds of low molecular weight such as Sec and methylselenol[5,6] are key metabolites in cancer prevention.[7,8] Sec is a Se-containing amino acid encoded by a UGA stop codon[9] that locates in the active sites of selenoproteins (SePs). It functions in redox signalling and anti-inflammation as well as in the production of active thyroid hormones. It is hence essential to find out the Sec is a pathological roles of Sec because the majority of in vivo functions of the Se-containing entities are performed by it. For this kind of studies, a biocompatible tool is needed to monitor the metabolites in living systems.

In terms of stability, simplicity, sensitivity, portability, cost-effectiveness, and storage convenience, stimuli-responsive micelles have advantages, and were studied for the development of biosensors. The commonly used stimuli are pH, temperature, light, redox potential, ultrasound, charge, gases, biomolecules, and enzymes.[10–18] To the best of our knowledge, the use of nanomicelle probes for selective recognition of Sec under biological condition (pH = 7.4) has never been reported. Up to now, only a few fluorescence probes of small molecules were studied for the detection and imaging of Sec[19, 20] or SePs.[21] In 2006, Maeda et al.[22] reported the use of a fluorescence probe to make discrimination between Sec and cysteine (Cys) based on the fact that the selenol group of Sec is inclined to ionize rather than to stay as the thiol group of Cys at pH 5.8. In 2014, Zhang et al.[19] reported the design, synthesis, and biological evaluation of a series of potential Sec probes based on the same mechanism under physiological conditions. Recently, Kong et al.[20]

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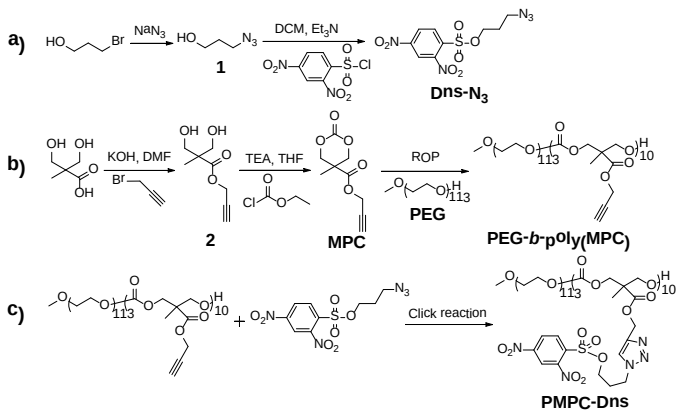
developed a fluorescence method to investigate the inhibitory mechanism of Se in tumor cells. But due to enhanced permeability and retention (EPR) effect,[23] the reported methods cannot be widely applied in biomedical researches as a result of poor biocompatibility and low photobleaching resistance.[24]

In this paper, we report the use of amphiphilic polymer micelles for the detection of Sec. The 2,4-dinitrobenzenesulfonyl-decorated block poly(carbonate) copolymer, *viz*. PMPC-Dns, was synthesized. Then by means of ring-opening polymerization (ROP) of cyclic carbonates, we generated amphiphilic block copolymers with poly(carbonate)s hydrophobic chains. Finally by means of copper-catalysed azide-alkyne Huisgen 1,3-dipolar cycloaddition (a coupling reaction), we produced the Sec-responsive micelles in which the fluorescence doxorubicin (DOX) drug was trapped. The copolymer that self-assembles into micelles consists of a hydrophobic core surrounded by a hydrophilic shell. It should be noted that the hydrophobic ends are the sites for Sec recognition. In the presence of Sec, 2,4-dinitrobenzenesulfonyl is activated and there is the generation of hydrophilic ends in the destabilized micelles. The consequence is the release of DOX for fluorescence imaging. In this study, we investigated the synthesis and cytotoxicity of the micelle probe, as well as the release of DOX as induced by Sec.

Results and discussion

Synthesis and self-assembling of amphiphilic copolymers

The monomers of azide-functional dinitrobenzenesulfonate(Dns-N_3) and 5-Methyl-5-propargyloxycarbonyl-1,3-dioxan-2-one(MPC) were synthesized according to the procedure shown in Scheme 1. Sec-responsive copolymer PMPC-Dns was prepared through a two-step method. Firstly, PEG-*b*-poly(MPC) copolymer was synthesized via ROP of MPC with poly(ethylene glycol) (PEG) being the macroinitiator (Scheme 1b). The copolymer was characterized using the ^{1}H NMR and Gel permeation chromatography (GPC) techniques. The ^{1}H NMR spectrum of PEG-*b*-poly(MPC) in $CDCl_3$ is shown in Figure S1c. The degree of polymerization (DP) of the polycarbonate backbone was determined to be 10 by comparing the integrals of peaks at δ =3.36 (CH_3O–, methyl protons of PEG end group) with



Scheme 1. Synthesis of Dns-N_3, MPC, PEG-*b*-poly(MPC) and amphiphilic copolymer PMPC-Dns.

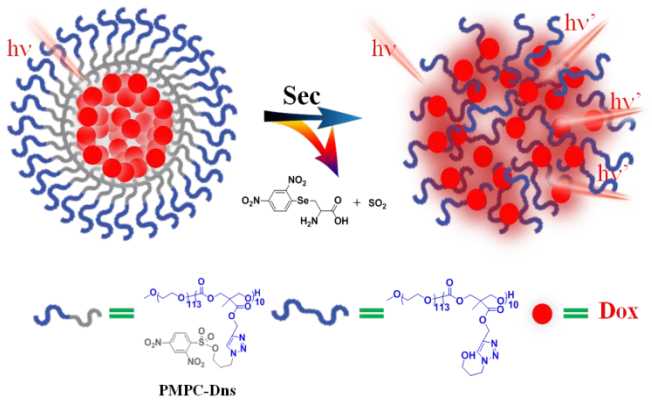


Figure 1. Working principle and response mechanism of the PMPC-Dns probe for Sec detection.

that at δ = 4.28 (–C(O)OCH_2CCH_2O–, the methylene protons of the carbonate units), which is close to the theoretical value of the protons of carbonate units as shown in Table S1. The results of GPC investigation reveal that the Sec-responsive copolymer has a narrow polydispersity index (PDI) of 1.04 and number-average molecular weight(M_n) of 7263 g moL^{-1}, which are in good agreement with the results of ^{1}H NMR end group analysis. Secondly, we modified the PEG-*b*-poly(MPC) to prepare the PMPC-Dns copolymer via click reaction (Scheme 1c), and purified the product by dialysis. We compared the ^{1}H NMR spectra of Dns-N_3, PMPC-Dns, and PEG-*b*-poly(MPC) (Figure S1). The signal at δ = 2.54 in Figure S1cassignable to the protons of the alkynyl groups of PEG-*b*-poly(MPC) disappears after click chemistry modification (Figure S1b), indicating that there is complete reaction of the propargyl groups of PEG-*b*-poly(MPC) with Dns-N_3. With reference to Figure S1a and c, Figure S1b reveals that over the PMPC-Dns copolymer, the Dns-N_3signals are at δ2.21, 3.47, 4.24-4.43, 8.49, 8.74 and 9.07, while signals owing to PEG-*b*-poly(MPC) are at δ 1.22, 3.37, 3.65 and 4.26. Moreover, a weak signal at δ7.72 that is attributable to the proton of triazole ring provides evidence for the attachment of Dns-N_3 to the polyester backbone. The GPC results reveal that the PMPC-Dns copolymer has a narrow PDI of 1.07 and M_n of 11353 g mol^{-1}, close to that calculated by ^{1}H NMR end group analysis (Table S1). The GPC trace of the graft copolymer shows a slight shift to lower retention time while maintaining narrow distribution with dispersity similar to that of the unmodified copolymer PEG-*b*-poly(MPC) (Figure S2). It is apparent that the PMPC-Dns graft copolymer can be readily prepared from propargyl-functionalized polycarbonate via the click reaction.

The amphiphilic copolymers PEG-*b*-poly(MPC) and PMPC-Dns self-assemble into micelles of hydrophobic core that are stabilized with hydrophilic PEG coronae (Figure 1), and their self-assembly behaviours were investigated in detail by fluorescence spectroscopy, as well as by dynamic light scattering (DLS) and scanning electron microscopy (SEM) techniques. Using hydrophobic Nile Red (NR) as fluorescence dye, the use of fluorescence spectroscopy can conveniently monitor the self-assembly of micelles and determine the critical micelle concentration (CMC) of amphiphiles.[30] As shown in Figure S3, the emission fluorescence intensity gradually increases with increasing amphiphile concentration, suggesting the spontaneous self-assembly of micelles. PMPC-Dns shows a CMC

value similar to that of PEG-*b*-poly(MPC) (0.01245 mg mL^{-1} vs. 0.01159 mg mL^{-1}), suggesting that the self-assembled micelles are thermodynamically stable. These values are consistent with literature data reported for graft copolymers.[30-32] Then SEM (Figure 2a and b) and DLS (Figure S4a and b) were applied to measure the size and morphology of the self-assembled micelles. In the SEM images, spherical morphology was observed with an average size of 60 and 90 nm for PEG-*b*-poly(MPC) and PMPC-Dns micelles, respectively. Despite being common in morphology, the PMPC-Dns micelles are larger than the PEG-*b*-poly(MPC) micelles in DLS-determined diameter, which can be attributed to the expanded hydrophobic nucleus of the former. In the presence of Sec, the PMPC-Dns micelles with the recognition site of 2,4-dinitrobenzenesulfonyl is activated, generating intertwining hydrophilic ends in the destabilized micelles. One can see that there are micellesthat are not activated in Figure 2d, and upon destabilization of micelles there is an obvious change of DLS average size from 118 (Figure S4b) to 13 nm (Figure S4d). After Sec activation, the product dialyzes in aqueous solution and lyophilizes. In ^{1}H NMR analysis, one can see that the signal at δ 8.49, 8.74, and 9.07 disappears and a weak signal at δ 7.72 appears (Figure S5), meaning the reaction goes along with the release of 2,4-dinitrobenzenesulfonyl. However the PEG-*b*-poly(MPC) micelles with no recognition sites show unchanged of morphology (Figure 2c) and DLS average size (Figure S4c) compared to the Figure 2a and Figure S4a, respectively.

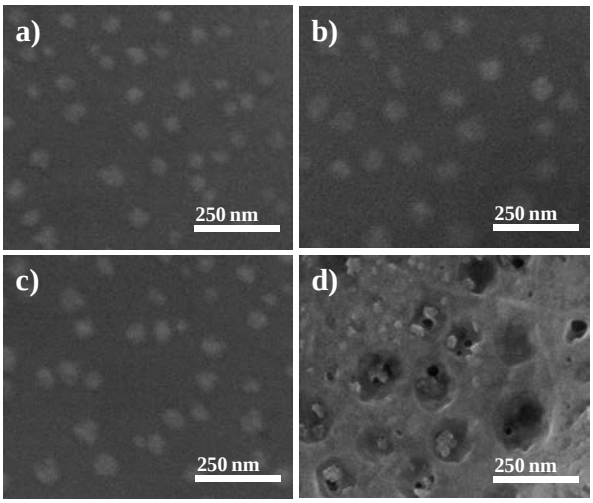


Figure2. SEM photographs of the micelles: (a) PEG-*b*-poly(MPC), (b) PMPC-Dns, (c) PEG-*b*-poly(MPC) treated by Sec, and (d) PMPC-Dns treated by Sec.

Working principle and feasibility of PMPC-Dns micelles for Sec Detection

The working principle of the Sec-responsive PMPC-Dns copolymer is schematically shown in Figure 1. The Sec recognition site 2,4-dinitrobenzenesulfonate is copolymerized with the polycarbonate backbone at the hydrophobic end. When the PMPC-Dns copolymer and the DOX drug are put in the aqueous solution, the micelles assemble and the hydrophobic DOX is wrapped inside. In the absence of Sec, the Sec recognition sites are stable under physiological conditions. In the presence of Sec, however, the sites react with Sec and there is the cleavage of 2,4-dinitrobenzenesulfonate. In the process, the hydrophobic ends change into hydroxyl ends that are hydrophilic, leading to the breaking up of micelles and unwrapping of DOX. Since the intensity of fluorescence reflects the amount of released DOX, the concentration of Sec can be measured as a degree of fluorescence intensity enhancement.

To validate the feasibility of the copolymer micelles for Sec detection, the Sec-responsive PMPC-Dns micelles were prepared with DOX trapped inside. The drug loading efficiency (DLE) and drug loading content (DLC) of the DOX-loaded PMPC-Dns micelles are found to be 6.5% and 7.63%, respectively. The fluorescence response of PMPC-Dns micelles towards Sec was examined (Figure S6a). Compared to that of Sec absence, there is a 2.78-fold enhancement of fluorescence intensity at 590 nm when Sec (1 mM) is introduced to the solution of micelles (0.5 mg mL^{-1}). To further evaluate the feasibility, time-dependent fluorescence changes of the micelles at 590 nm were monitored in the absence and presence of Sec (Figure S6b). Without Sec, no fluorescence changes at 590 nm are observed within 10 min, which indicates that there is no release of DOX and the micelles are stable. On the contrary, in the presence of Sec, there is obvious enhancement of fluorescence intensity that reaches a plateau after 1.6 h, indicating the release of DOX. The results clearly prove that the PMPC-Dns micelles respond to Sec.

Sensitive and specific detection of Sec

To demonstrate the applicability of the approach for qualitative identification of Sec, we measured the fluorescence spectra of PMPC-Dns micelles in phosphate-buffered saline (PBS) solution treated with different concentrations of Sec. As shown in Figure 3, with increasing concentration of Sec, there is increase of fluorescence intensity, displaying a concentration-dependent trend. A linear range is obtained between 0 and 25 μM. With further increase in Sec concentration, the fluorescence intensity reaches a plateau. Based on the 3σ rule, the detection limit is calculated to be 0.05 μM. The results reflect the cleavage of 2,4-dinitrobenzenesulfonateand destabilization of micelles in the presence of Sec. With higher concentration of Sec, more DOX is released and hence the higher fluorescence intensity.

Due to the high selectivity of 2,4-dinitrobenzenesulfonate interaction with Sec as described by Zhang et el.,[19] the detection of Sec using the PMPC-Dns micelles is highly selective. In the presence of high concentration of various reducing reagents including thiols, beta-mercaptoethanol (b-ME), thioredoxinreductase (TrxR), dithiothreitol (DTT), N-acetyl-L-cysteine (NAC), Vitamin C, hydrogen sulfide (H_2S), Na_2SeO_3, and a mixture of Na_2SeO_3, Cys, and GSH, there is no detectable fluorescence changes as shown in Figure 4. The selectivity was further verified by ^{1}H NMR analysis as shown in Figure S7. There is no obvious change in ^{1}H NMR features of PMPC-Dns after the PMPC-Dns micelles was treated with the thiols and sulfur compounds. The results confirm that the PMPC-Dns probe shows no response toward the reducing reagents. It is apparent that H_2S and the reducing reagents with a sulfhydryl group do not response to the probe. It is noted that vitamin C also shows negative response while DTT (50 μM) only exhibits slight response. One

probable reason for TrxR not responding is that due to steric effect, it is hard for the Sec in SePs to get close to the recognition sites.

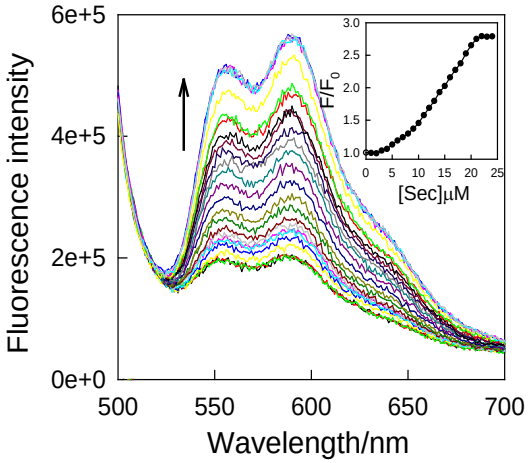


Figure3. Fluorescence spectra of PMPC-Dns micelles (0.5μg mL^{-1}) in PBS (pH 7.4, 20 mM) solution treated with increasing amounts of Sec. The arrow indicates the signal changes with increase of Sec concentration (0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 10.0, 12.0, 13.0, 14.0, 15.0, 16.0, 17.0, 18.0, 19.0, 20.0, 21.0, 22.0, 23.0 and 24.0μM). Inset: The F/F_0 ratio of micelles as a function of Sec concentration, where F_0 and F are fluorescence intensity of PMPC-Dns micelles at 595 nm in the absence and presence of Sec, respectively.

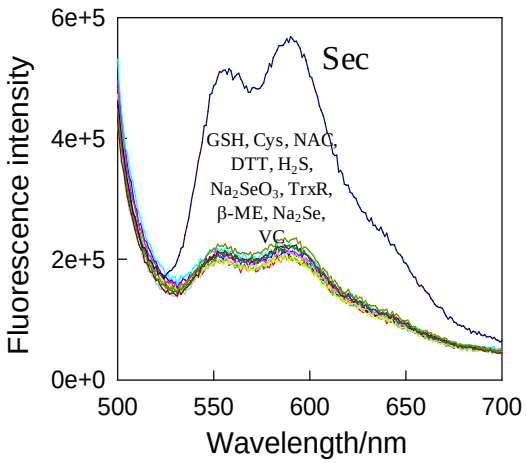


Figure4. The fluorescence of PMPC-Dns micelles (0.5μg mL^{-1}) in PBS (pH 7.4, 20 mM) solution treated with thiols and other sulfur compounds for 90 min (1μM).

PMPC-Dns micelles detection of Sec in cells and tissues

For suitability of biological applications, the probe has to be nontoxic. The long-term cellular toxicity of PEG-*b*-poly(MPC) and PMPC-Dns micelles towards the Hela cell lines was determined by means of a standard MTT (methyl thiazolyl tetrazolium) assay. As shown in Figure S8a, when the micelles concentration is 1 mg mL^{-1}, the cell viability still remains at *ca.* 93%, demonstrating its low cytotoxicity. The result can be attributed to the excellent biocompatibility of PEG and poly(carbonate)s. Moreover, the stability of PMPC-Dns micelles under condition of high concentration of Fetal Bovine Serum (FBS) (Figure S8b) is also of importance, especially in the detection of Se in tissues. We recorded

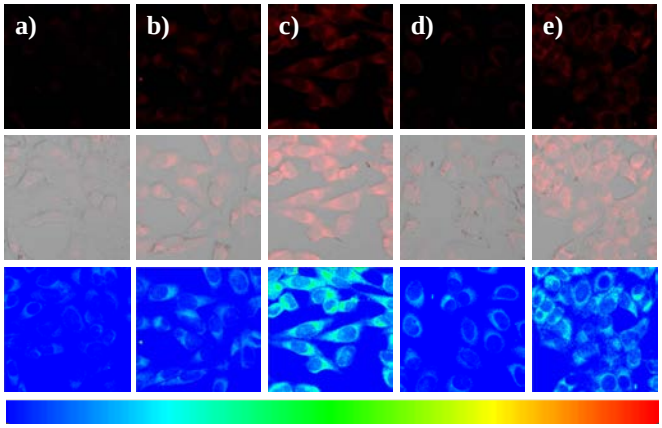


Figure5. The Hela cells were treated with Sec (5 μM) for 2 to 12 h, followed by incubation with PMPC-Dns micelles (0.5μg mL^{-1}), and there was the appearance of a bright red fluorescence signal (Figure 5b, 5c), whereas in the case of control cells there was no obviously fluorescence enhancement (Figure 5a). For the cells stimulated with sodium selenite for 12 h, there was notable appearance of fluorescence (Figure 5e). A short time treatment (2 h) resulted in weak but obviously enhanced signal (Figure 5d). The images of the last row were acquired by Image Pro Plus software. The colour strip shows the pseudo colour change with Sec.

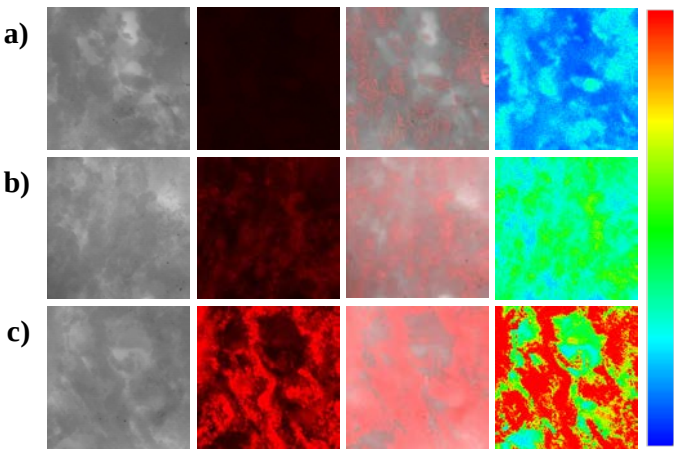


Figure6. The cervical tumor cells were treated with Sec (5 μM) for 12 h, followed by incubation with PMPC-Dns micelles (0.5 mg mL^{-1}), and there was the appearance of a bright red fluorescence signal (Figure 6c), whereas in the case of control cells there was no obvious fluorescence enhancement (Figure 6a). When the cells were stimulated with sodium selenite for 12 h, there was notable appearance of fluorescence (Figure 6b). The images of the last row were acquired by Image Pro Plus software. The colour strip shows the pseudo colour change with Sec.

the fluorescence spectra of the probe after different periods of storage time. The results show that the PMPC-Dns micelles are stable under biological condition for a long time. Hence, the PMPC-Dns micelles can serve as a selective and sensitive probe for qualitative analysis of Sec. In addition, the Sec can be used as a target agent for drug delivery in cancer therapy.

The imaging of Sec in live cells was performed using Hela cells as a model. Since the physiological concentration of Sec is low in cells, we first determined whether the PMPC-Dns micelles could respond

to exogenous Sec. After culturing the Hela cells with Sec for 2 to 12 h, we detect a bright red fluorescence signal when the probe is applied (Figure 5b, 5c), while there is no obvious enhancement of fluorescence intensity in the case of control cells (Figure 5a). We further determined the endogenous Sec in the cells. Sodium selenite (Na_2SeO_3) is a precursor of Sec biosynthesis, and the supplement of cells with sodium selenite could significantly increase the Sec level in the cells.[33] After the cells were stimulated with sodium selenite for 12 h, there is notable appearance of fluorescence (Figure 5e). A short time treatment of 2 h gives weak enhancement of fluorescence signal (Figure 5d). The results demonstrate that the probe is suitable for imaging Sec in live cells.

To further investigate the response of PMPC-Dns micelles under biological condition, we selected cervical neoplasm for imaging. Figure 6 shows the micelles staining of frozen slices of the cervical tumor tissues. Strong fluorescence signals (Figure 6c) was observed over the cervical neoplasm slices that were treated with Sec for 12 h. The tissue slice (Figure 6b) treated with Na_2SeO_3 for the same time period also gives fluorescence signal but slightly weaker in intensity. As for the neoplasm slice not treated with Sec or Na_2SeO_3, there is no obvious fluorescence enhancement (Figure 6a).The results are in good agreement with those of Hela cells imaging. The confocal images clearly demonstrate that the PMPC-Dns micelles can be used for qualitative analysis of the Sec level of cervical cancer cells under biological condition.

Conclusions

We designed and prepared a novel 2,4-dinitrobenzenesulfonyl-decorated block poly(carbonate) copolymer, *viz*. PMPC-Dns, via a convenient click conjugation of Sec-responsive 2,4-dinitrobenzenesulfonyl molecules to propargyl-functionalized poly(carbonate)s. DLS and SEM measurements revealed that the polymer self-assembles in aqueous solution into spherical micelles with an average diameter of 100 nm. The CMC of the micelles was determined to be 0.01245 mg mL^{-1} by fluorescence spectroscopy using NR as fluorescence dye. The follow-up studies demonstrated that PMPC-Dns with the fluorescence drug DOX trapped inside could selectively respond to Sec and other selenols under biological conditions (pH = 7.4). It was found that there is little interference from the biological thiols, amines, or alcohols. The PMPC-Dns probe was successfully applied to image the endogenous Sec in Hela cells as well as that in live cervical neoplasm. It is noted that during Sec imaging, there is simultaneous release of the entrapped hydrophobic DOX molecules under physiological conditions. To the best of our knowledge, PMPC-Dns is the first Sec-induced probe selective for Sec imaging. The work opens up a way to study the role of Sec in biological, pathological as well as tumor xenograft model systems. Furthermore, the approach provides a methodology for controlled delivery of hydrophobic molecules in biomedical applications.

Experimental Section

1 Materials

Monomethoxypoly (ethyleneglycol) (PEG_{5k}, M_n=5000), ethyl chloroformate, 2,4-dinitrobenzenesulfonyl chloride, 3-bromo-1-propanol and 2,2-bis(hydroxyl methyl)propionic acid were purchased from Alfa Aesar and used as received. We dried 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), dichloromethane (DCM), triethylamine, dimethylformamide (DMF) over calcium hydride for 24 h at room temperature (RT) and distilled them under reduced pressure. The thiourea catalyst (TU) was synthesized as reported previously and recrystallized from dry methylene chloride.[25] We synthesized $(Sec)_2$ according to the procedure reported by Braga et al.[26] The Sec was generated from $(Sec)_2$ in the presence of DTT (100 μM, from 50 μM $(Sec)_2$ and 50 μM DTT). Tetrahydrofuran (THF) was dried by means of refluxing over a benzophenone-sodium mixture until a deep blue appearance, and then subject to distillation.

2 Characterization

UV-Vis absorption spectra were recorded over Hitachi U-4100 UV/Vis spectrophotometer using quartz cuvettes of 1 cm path length. The steady-state fluorescence emission spectra were recorded over an equipment of Photon Technology International. Fluorescence emission spectra were collected using a bandwidth of 5 nm and 0.2×1 cm^2 quartz cuvettes containing 500 μL of solution. ^1H and ^{13}C NMR spectra were recorded on an Invoa-400 (Invoa 400) spectrometer and referenced to solvent signals. The number-average molecular weight (M_n) and molecular weight distribution (PDI = M_w/M_n) of polymers were determined at RT using a Waters GPC liquid chromatograph equipped with four TSK HXL series of polystyrene divinylbenzene gel columns (300×7.8 mm). Calibration was established with polystyrene standards from Polymer Laboratories. THF having a flow rate of 1 mL min^{-1} was used as solvent. SEM images were obtained on an S-4800 scanning electron microscope (Hitachi) with a working voltage of 5 kV. A drop of the micelle aqueous solution (0.05 mg mL^{-1}) was deposited onto a silicon slice and allowed to dry at RT before measurement. The mean size of the micelles was determined by DLS using a Malvern Nano S instrument (Malvern, UK).

3 Synthesis of 3-bromopropyl 3-azido-propanol (1)

To a solution of 3-bromo-propan-1-ol (1.02 g, 7.3 mmol) in water (10 mL) was added sodium azide (0.95 g, 14.6 mmol) and the solution was heated at 80°C for 18 h. The aqueous solution was extracted with EtOAc (4×20 mL). The organic layers were washed with brine (30 mL), dried over $MgSO_4$, subject to filtration, and concentrated under reduced pressure to give pure 3-azido-propanol as colourless oil (0.57 g, yield = 76.5%).

^1H NMR (400 MHz, $CDCl_3$): δ=3.73 (t, 2H, $OHCH_2$), 3.44 (t, 2H, $CH_2CH_2N_3$), 2.51 (s, 1H, OH), 1.80-1.86 (m, 2H, $CH_2CH_2CH_2$);

^{13}C NMR (100 MHz, $CDCl_3$): δ =59.65, 48.41, 31.43.

4 Synthesis of 3-azidopropyl 2,4-dinitrobenzenesulfonate (Dns-N_3)

A solution of 3-azido-propanol (0.57 g, 5.6 mmol) and Et_3N (1.56 mL, 11.2 mmol) in dry CH_2Cl_2 (10 mL) was cooled to

0°C. With the addition of 2, 4-dinitrobenzene-1-sulfonyl chloride (1.7 g, 6.7 mmol) in dry dichloromethane (10 mL), the solution was stirred at RT for 18 h. After quenching of the reaction with water (40 mL), the organic layer was separated, dried over $MgSO_4$, subject to filtration, and concentrated under reduced pressure. The as-obtained crude oil was purified by column chromatography (eluent: ethyl acetate/pet ether = 1/5, v/v) to yield 3-bromopropyl 2,4-dinitrobenzenesulfonate as colourless oil (1.33 g, yield = 71.1%).

^{1}H NMR (400 MHz, $CDCl_3$): δ= 9.00 (s, 1H, ArH) , 8.67 (d, 1H, ArH), 8.45 (d, 1H, ArH), 4.03-4.25(m, 2H, $CH_2CH_2CH_2$), 3.32 (t, 2H, OCH_2CH_2), 1.86(t, 2H, $CH_2CH_2N_3$);

^{13}C NMR (100 MHz, $CDCl_3$): δ =152.19, 145.22, 142.19, 123.88, 123.47, 115.71, 62.00, 42.49, 24.34.

5 Synthesis of 2,2-Bis (hydroxyl methyl) propionate (2)

In a 250 mL round-bottom flask, 2,2-bis(hydroxyl methyl)-propionic acid (2.24 g, 16.72 mmol), KOH (1.01 g, 18.04 mmol), and DMF (100 mL) were added. The mixture was stirred at 100°C for 2 h, and then propargyl bromide (2.13 g, 18.04 mmol) was added dropwise over a 30-min period. After 72 h of reaction, the reaction mixture was subject to filtration, and the solvent was evaporated under reduced pressure. The residues were dissolved in 50 mL of DCM and extracted three times with saturated salt water (20 mL×3). The organic phase was concentrated to yield the crude product, which was purified by column chromatography (eluent: ethyl acetate/petroleum ether = 1/5, v/v). Yield: 1.3 g (45.1%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.76 (d, 2H, $CHCCH_2CO$), 3.93 (d, 2H, CH_2OH), 3.73 (d, 2H, CH_2OH), 2.87 (s, 2OH), 2.51 (t, 1H, $CHCCH_2CO$), 1.11 (s, 3H, CH_3CC);

^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.01, 75.20, 67.82, 60.37, 52.43, 49.29, 16.95.

6 Synthesis of 5-Methyl-5-propargylxycarbonyl-1,3-dioxane-2-one (MPC)

Compound **2** (1.18 g, 6.88 mmol) was mixed with ethyl chloroformate (1.48 g, 13.76 mmol) and THF (20 mL) in a sealed vessel that was purged with nitrogen and cooled in an ice bath. After stirring for an hour, triethylamine (1.39 g, 13.76 mmol) was added dropwise over a 30-min period under nitrogen atmosphere. The reaction was conducted at 0°C with stirring for 3 h, then at 25°C under stirring overnight. The solution was then subject to filtration, evaporated to dryness, and the as-obtained product was precipitated in a mixture of ethyl acetate and diethyl ether (1:1) as white crystals. Yield: 1.24 g (91.1%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.82 (d, 2H, $CHCCH_2CO$), 4.75 (d, 2H, CH_2OCO), 4.27 (d, 2H, CH_2OCO), 2.57 (t, 1H, $CHCCH_2CO$), 1.39 (s, 3H, CH_3CC);

^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.31, 147.21, 76.35, 75.94, 72.70, 53.47, 40.17, 17.39.

7 Synthesis of PEG-*b*-poly(MPC)

The ROP of MPC was carried out under an inert atmosphere of nitrogen using the standard Schlenk-line technique. In a typical experiment, MPC (0.594 g, 3 mmol), PEG_{5k}(0.601 g, 0.12 mmol), TU (0.055 g, 0.15 mmol), DBU (0.005 g, 0.03 mmol) and dried DCM (10 mL) were placed in a dried Schlenk tube fitted with a rubber septum. The solution was further degassed through three freeze–pump–thaw cycles. The resulting mixture was stirred at RT for 7 h, followed by precipitation in ice-cold diethyl ether and centrifugation. The resulting product was collected by filtration and dried under vacuum to yield a white powder. Yield: 1.1 g (92.0%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.73 (d, OCH_2CCH), 4.28-4.32 (m, $OC(O)OCH_2$), 3.65 (s, OCH_2CH_2O), 3.38 (s, CH_3O), 2.55 (s, CH_2CCH), 2.19 (s, OH), 1.29 (s, CH_3).

GPC (THF, RI): M_n(PDI) =7263 g mol^{-1} (1.04).

8 Synthesis of PMPC-Dns via ''click'' chemistry

In a Schlenk tube, PEG-*b*-poly(MPC) (200mg, propargyl group, 0.023 mmol), Dns-N_3 (76 mg, 0.23 mmol), sodium ascorbate (4.5 mg, 0.023 mmol), and DMF (4 mL) were introduced. The tube was fitted with a rubber septum. The solution was further degassed through three freeze-pump-thaw cycles. A DMF solution of copper sulfate (2.8 mg, 0.012 mmol) was then added to the Schlenk tube. The solution was stirred at RT for 24 h. The crude material was purified by dialysis (dialysis tubing 3500 MWCO) against deionised (DI) water that was renewed regularly. After 3 days, the final product, PMPC-Dns, was obtained by lyophilization. Yield: 190.9 mg (81.3%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.07 (s, ArH), 8.75 (d, ArH), 8.49 (s, ArH), 7.72 (s, N_3CHC), 4.73 (s, $C(O)OCH_2$), 4.31 (s, $C(O)OCH_2$), 4.28-4.24 (m, $OCH_2CH_2CH_2N$), 3.64 (s, OCH_2CH_2O), 3.47 (t，CH_2CH_2O), 3.38 (s, CH_3O), 2.17 (s, NCH_2CH_2), 1.29 (s, CH_3).

GPC (THF, RI): M_n (PDI) =11353 g mol^{-1} (1.07).

9 Preparation of micelles

Micelles of PEG-*b*-poly(MPC) and PMPC-Dns were prepared by a dialysis method. First 25.0 mg copolymer was dissolved in DMF (1 mL), and then DI water (10 mL) was slowly added with vigorous stirring. After vigorous stirring for another 2 h at RT, the micelles were obtained and subject to further dialysis against DI water for 24 h to remove DMF (MWCO 1000 Da). The final polymer concentration was adjusted by adding DI water to 0.5 mg mL^{-1}.

10 Measurement of the critical micelle concentration (CMC)

The CMC of PEG-*b*-poly(MPC) and PMPC-Dns amphiphiles were determined by a dye solubilization method using NR as probe molecule. NR in THF (0.1 mg mL^{-1}, 30 μL) was added to a glass vial using a microsyringe. After the evaporation of THF, a portion of the micelle solution (2 mL) was added. The concentration of micelle solution was varied from 0.1 to 5×10^{-4} mg mL^{-1}. Then, the solution was stirred for 24 h. Fluorescence measurements were taken at an excitation wavelength of 550 nm and the emission was monitored from 570 to 750 nm.

11Preparation of DOX-Loaded PMPC-Dns micelles and calculation of drug loading content (DLC)

In brief, PMPC-Dns (15.0 mg) and DOX (1.0 mg) were simultaneously added into dimethyl sulfoxide (DMSO, 5 mL) under vigorous stirring until complete dissolution. Then, the mixture was transferred into a dialysis bag (MWCO 3500 Da) and subject to dialysis in aqueous solution for 3 days for the formation of the DOX-loaded PMPC-Dns micelles. During the 3-day period, the DI water was renewed regularly. To determine the DLC and DLE, the DOX-loaded PMPC-Dns micelles were incubated in Sec solution for 24 h, then lyophilized, and dissolved in DMSO again. The drug concentration was determined by measuring the fluorescence intensity of DOX (excited at 490 nm).The DLC and DLE were calculated according to the following equations:

$DLC\ (\%) = W_{loaded}/(W_{polymer} + W_{loaded}) \times 100\%$

$DLE\ (\%) = W_{loaded}/W_{total} \times 100\%$

Where W_{total}, $W_{loaded}$and $W_{polymer}$ are the weight of total DOX used, theloaded DOX, and PMPC-Dnsmicelles, respectively.

12Cytotoxicity assay

Using Hela cells as model, the cellular cytotoxicity of the probe was evaluated by standard cell viability MTT assay.[27] Hela cells were seeded into a 96-well plate at a concentration of 5×10^3 cells $well^{-1}$ in 100 μL of MEM with 10% FBS. The plates were maintained at 37°C in a 5% CO_2 95% air incubator for 24 h. Then, the medium was removed and replaced with 200 μL polymer micelles. The aggregate concentrations of each formulation were prepared by serial dilution with DMEM. The cells incubated with the culture medium only were used as controls. The cells were washed with PBS solution for three times, and then 100 μL MTT solution (0.5 mg mL^{-1} in PBS solution) was added to each well. After the addition of DMSO (150 μL $well^{-1}$), the assay plate was shaken at RT for 10 min. Experiments were done in triplicate. The cell viability was calculated based on measurement of UV-Vis absorption at 570 nm using the following equation, where OD_{570} represents the optical density.[28, 29]

Cell viability = $[OD_{570(sample)} - OD_{570(blank)}] / [OD_{570(control)} - OD_{570(blank)}]$

13Cell Incubation and Imaging

The Hela cells were from the Biomedical Engineering Centre of Hunan University (Changsha, China). The cells were cultured using high-glucose DMEM (GIBCO) with 1% penicillin-streptomycin (10,000 U mL^{-1}, 10,000 μg mL^{-1}, Invitrogen) and 10% FBS(GIBCO) in an atmosphere of 5% CO_2 and 95% air at 37°C. For the detection of exogenous produced Sec, the cells were co-incubated with Sec (5 μM) for 2 to 12 h. After washing three times with PBS solution (pH = 7.4) to remove the remaining Sec, the cells were further incubated with PMPC-Dns micelles (0.05 μg mL^{-1}) for 60 min at 37°C. Then the incubated cells were washed with 3×1 mL of PBS solution (pH = 7.4), and fresh medium was added before imaging. To induce the endogenous Sec, the Hela cells were exposed to sodium selenite (5 μM) for 2 to 12 h, and then the cells were further incubated with the PMPC-Dns micelles (0.05 μg mL^{-1}) for 30 min at 37°C. After washing the cells three times with PBS solution (pH =7.4), fresh medium was added. All the fluorescence images were acquired using an Olympus FV1000 laser confocal microscope and analyzed by Image Pro Plus software.

14Preparation and staining of Hela cancer tissue slices

Tissue slices were prepared from Hela cancer neoplasm. A total of 2×10^6 Hela cancer diluted in 100 mL of serum-free DMEM was injected subcutaneously into the right flank of 6 to 8-week-old BALB/c nude mice for tumor inoculation. After 15-20 days, the mice were sacrificed, and the tumors were transferred and embedded with O.C.T (Sakura Finetek, USA, Torrance, CA) for frozen sections. The tissues were cut into 50 μm thick slices using a vibrating-blade microtome. For detection of exogenous produced Sec, the slices were co-incubated with Sec (20 μM) for 12 h. After washing three times with PBS solution (pH = 7.4) to remove the remaining Sec, the slices were further incubated with PMPC-Dns micelles (0.2 μg mL^{-1}) for another 12 h at 4°C. Afterward, the incubation slices were washed with 3×3 mL of PBS solution (pH = 7.4) before imaging. To induce the endogenous Sec, the tissues were exposed to sodium selenite (20 μM) for 12 h, and then the slices were further incubated with the PMPC-Dns micelles (0.2 μg mL^{-1}) for 12 h at 4°C. After washing three times with PBS solution (pH = 7.4), the slices were mounted with 10% glycerol and sealed with nail varnish on a glass substrate. The fluorescence images of Hela cancer tissue were carried out on an Olympus FV1000 laser confocal microscope and analysed by Image Pro Plus software.

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