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Hyperbranched polydiselenide as a self assembling broad spectrum anticancer agent

Jinyao Liu [a], Yan Pang [b,*], Jun Chen [c], Ping Huang [a], Wei Huang [a,*], Xinyuan Zhu [b], Deyue Yan [a,*]

[a] School of Chemistry and Chemical Technology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China
[b] State Key Laboratory of Metal Matrix Composites, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China
[c] Research Institute of Micro/Nano Science and Technology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

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ABSTRACT

This work presents a highly efficient, broad spectrum and self-delivery anticancer agent, which is the hyperbranched polydiselenide (HPSe) consisting of alternative hydrophobic diselenide groups and hydrophilic phosphate segments in the backbone framework. The data of systematic evaluations demonstrate that HPSe is very potent to inhibit the proliferation of many forms of cancer cell. The dose of HPSe required for growth inhibition of 50% (IC_{50}) in all of the tested cancer cell lines is within the concentration range between 1 and 2.5 μg mL^{-1} with the incubation time of 72 h. Furthermore, the amphiphilic HPSe can self-assembly into nanomicelles with an average diameter of 50 nm and spontaneously enter into tumor cells by the enhanced permeability and retention (EPR) effect. Besides, other hydrophobic anticancer drugs such as doxorubicin (DOX) can be encapsulated into HPSe micelles for combining therapy.

1. Introduction

Up to date, great efforts have been paid to the synthesis of potent anticancer drugs and their prodrugs, respectively [1–3]. Prodrugs made of hydrophobic anticancer drugs with a hydrophilic polymer (for instance, polyethylene glycol) delivered into tumor cells with more effective cancer treatment [4–8]. However, the polymer itself has no direct therapeutical effect, and its metabolites might lead to unexpected side effects, such as toxicity and inflammation [9]. It would be significant to search a highly efficient, broad spectrum and self-delivery drug for cancer therapy. This work synthesized a macromolecular anticancer agent consisting of alternative hydrophobic diselenide and hydrophilic phosphate groups in the dendritic backbone. Interestingly, this polydiselenide exhibits a very potent anticancer effect in a broad spectrum. Benefiting from its amphiphilicity, the obtained macromolecular anticancer agent can self-assemble into nanomicelles, and then self-deliver into tumor cells through the enhanced permeability and retention (EPR) effect [10].

During last decades, selenium compounds have been proved to be a category of good anticancer agents because of their remarkable ability to enhance the immune response and produce anticancer metabolites which can efficiently perturb cancer cell metabolism, inhibit angiogenesis and induce apoptosis of cancer cells [11–18]. A great number of selenium compounds including inorganoselenium and organoselenium compounds have been reported as anticancer agents [19–21]. However, previous work in this research area focused on the design and synthesis of small-molecular-weight selenium drugs, probably due to the limited solubility and poor stability of the macromolecular selenium compounds.

Macromolecular compounds, such as proteins, genes, antibodies, peptides, oligonucleotides and some special synthetic polymers, have emerged as efficient medicines for various types of human diseases [22–27], and this research field has grown exponentially in recent years [28–31]. Due to the existence of many binding entities per molecule, macromolecular drugs exhibit multivalent interactions which can be found throughout the biological systems and can lead to a synergistic enhancement of a certain activity compared to the corresponding monomeric interaction [32]. It has been demonstrated that macromolecular drugs possess highly efficient and special pharmaceutical properties not found in small-molecular-weight drugs. Furthermore, their high-molecular-weight characteristics make them systemically non-absorbed, providing many advantages over small-molecular-weight drugs, for example, the long-term safety profiles. Therefore, the synthesis and application of macromolecular selenium anticancer agents would provide a favorable platform to develop potent drugs for cancer therapy. Last year we reported the

* Corresponding authors. Tel.: +86 2154742665; fax: +86 2154741297.
E-mail addresses: py-1985@sjtu.edu.cn (Y. Pang), hw66@sjtu.edu.cn (W. Huang), dyyan@sjtu.edu.cn (D. Yan).

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preparation of the hyperbranched polydisulfide (HPS) through self-condensing ring opening polymerization, which is a smart redox-responsive drug carrier without any therapeutic activity for anti-cancer therapy [33]. In present work, we synthesized the hyperbranched polydiselenide (HPSe) through (A_2 + B_3) type polycondensation, which is an extensively efficient anticancer agent. The systematic evaluations of HPSe, i.e., the cytotoxicity effect, the cancer cell proliferation inhibition and the related apoptosis mechanism, were well performed. Meanwhile, the self-assembly of HPSe in water as well as the self-delivery and co-delivery of other drugs such as doxorubicin (DOX) for combining therapy were investigated carefully.

2. Materials and experimental methods

2.1. Materials

Chloroform and methylene chloride were dried by refluxing with calcium hydride and distilled just before use. Triethylamine (TEA) was refluxed with phthalic anhydride, potassium hydroxide, and calcium hydride in turn and distilled just before use. Tetrahydrofuran (THF) was dried by refluxing with the fresh sodium–benzophenone complex under nitrogen and distilled just before use. Phosphorus oxychloride ($POCl_3$) was distilled just before use. Nile red, *p*-toluene sulfonylchloride (TsCl), glutathione (reduced) (GSH), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), Se powder, triethylene glycol (TEG), and sodium borohydride were purchased from Sigma and used as received. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Corporation and used as received. Alexa fluor® 488 annexin V/dead cell apoptosis assay kit was purchased from Invitrogen and used as received. Bradford protein assay kit and caspase-3 activity assay kit were purchased from Beyotime Instituted of Biotechnology and used as received. Clear polystyrene tissue-culture-treated 12- and 96-well plates were obtained from Corning Costar. All other reagents and solvents were purchased from the domestic suppliers and used as received.

2.2. Measurements

Nuclear magnetic resonance (NMR) analyses were recorded on a Varian Mercury Plus 400 MHz spectrometer with deuterated chloroform ($CDCl_3$) as solvent. The number-average molecular weight (M_n), weight-average molecular weight (M_w), and M_w/M_n were measured by gel permeation chromatography (GPC). GPC was performed on a Perkin–Elmer series 200 system (10 μm PL gel 300 × 7.5 mm mixed-B and mixed-C column, linear polystyrene calibration) equipped with a refractive index (RI) detector. DMF containing 0.01 mol L^{-1} lithium bromide was used as the mobile phase at a flow rate of 1 mL min^{-1} at 70 °C. Fourier transform infrared spectrometer (FTIR) spectra were recorded on a Paragon 1000 instrument by KBr sample holder method. Differential scanning calorimeter (DSC) was performed on a Perkin–Elmer Pyris 1 in nitrogen atmosphere. Both In and Zn standards were used for temperature and enthalpy calibrations. First, all the samples (about 5.0 mg in weight) were heated from room temperature to 150 °C, held at this temperature for 3 min to remove the thermal history and quenched to −80 °C. The samples were heated from −80 °C to 150 °C at 10 °C min^{-1} to determine the glass transition temperature (T_g). Dynamic light scattering (DLS) measurements were performed in aqueous solution using a Malvern Zetasizer Nano S apparatus equipped with a 4.0 mW laser operating at λ = 633 nm. The samples of 0.25 mg mL^{-1} were measured at 20 °C and at a scattering angle of 173°. Transmission electron microscopy (TEM) studies were performed with a JEOL 2010 instrument operated at 200 kV. The samples were prepared by directly dropping the solution of micelles onto carbon-coated copper grids. The samples were frozen by liquid nitrogen quickly and lyophilized by a freeze-dryer system (Martin Christ, α1-4) at −20 °C overnight before measurement.

2.3. Synthetic procedures

2.3.1. Synthesis of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate

In a typical procedure: TsCl (49.57 g, 0.26 mol) in 100 mL of methylene chloride was added dropwise over 2 h to a solution of TEG (195 g, 1.5 mol) and TEA (26.26 g, 0.26 mol) in 100 mL of methylene chloride at 0 °C. Then, the solution was stirred overnight at room temperature. The organic layer was washed with saturated $NaHCO_3$ solution and dried over anhydrous $MgSO_4$. After the evaporation of solvent, the crude product was purified by flash column chromatography (SiO_2, chloroform/methanol 98:2) to yield a colorless oil, yield 62%. ^{1}H NMR ($CDCl_3$, ppm): 7.80 (2H, ArH), 7.34 (2H, ArH), 4.17 (2H, OCH_2CH_2OTs), 3.71 (2H, OCH_2CH_2OTs), 3.61 (4H, OCH_2CH_2O), 3.57 (2H, OCH_2CH_2OH), 2.44 (3H, $ArCH_3$). The ^{1}H NMR spectrum of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate was shown in Fig. S1.

2.3.2. Synthesis of NaSeSeNa

The chemical equation is shown below:

$$2\ NaBH_4 + 2\ Se + 6\ H_2O \rightarrow NaSeSeNa + 2\ H_3BO_3 + 7\ H_2\uparrow$$

In a typical procedure: Sodium borohydride (4.921 g, 130 mmol) in 50 mL of water was added with magnetic stirring to selenium (5.132 g, 65 mmol) suspended in 50 mL of water at room temperature. After the initial vigorous, reaction had subsided (20 min), and additional equiv of selenium (5.132 g, 65 mmol) was added. The mixture was stirred for 10 min and then warmed briefly to dissolve selenium. The resulting brownish red aqueous solution of NaSeSeNa was then ready for further use.

2.3.3. Synthesis of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl diselenide

In a typical procedure: NaSeSeNa (65 mmol) solution was added with magnetic stirring to 300 mL of THF solution of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (39.562 g, 130 mmol) with nitrogen protection. The mixture was stirred for 48 h at 50 °C. Then, the solvent was removed, and the crude product was redissolved in methylene chloride. The organic layer was washed twice with water and then dried over anhydrous $MgSO_4$. The product was purified by silica gel column chromatography eluting with 1:1 hexane/ethyl acetate. The evaporation of solvent gave a brown oil product, yield 65%. ^{1}H NMR ($CDCl_3$, ppm): 3.77 (4H, $SeCH_2CH_2O$), 3.72 (4H, OCH_2CH_2OH), 3.67 (4H, $OCH_2CH_2OCH_2CH_2OH$), 3.65 (4H, $OCH_2CH_2OCH_2CH_2OH$), 3.59 (2H, OCH_2CH_2OH), 3.12 (4H, $SeCH_2CH_2O$). ^{77}Se NMR ($CDCl_3$, ppm): 289.20. The (a) ^{1}H NMR and (b) ^{77}Se NMR spectra of 2-(2-(2-hydroxyethoxy)ethoxy)ethyl diselenide were shown in Fig. S2.

2.3.4. Synthesis of hyperbranched polydiselenide

In a typical procedure: 20 mL of chloroform solution of 2-(2-(2-hydroxyethoxy) ethoxy)ethyl diselenide (2.12 g, 5.00 mmol) and TEA (0.841 g, 8.33 mmol) was added to a 50 mL round-bottomed flask with nitrogen protection. A mixture of $POCl_3$ (0.426 g, 2.78 mmol) and 5 mL of chloroform was slowly added to the above flask at 0 °C using an ice bath and then kept at room temperature for 24 h. The by-product of triethylamine hydrochloride salt was removed by filtration. The obtained filtrate was twice extracted by HCl (1 M), $NaHCO_3$ (10%) and NaCl (saturated) aqueous solution, respectively. After drying over anhydrous $MgSO_4$, the solvent was removed under vacuum, obtaining a brown solid product, named HPSe, 72% yield. ^{1}H NMR ($CDCl_3$, ppm): 4.19 (2H, $POCH_2CH_2O$), 3.76 (4H, OCH_2CH_2Se), 3.72 (2H, OCH_2CH_2OH), 3.65 (12H, $POCH_2CH_2O$, $OCH_2CH_2OCH_2CH_2OH$), 3.10 (4H, $SeCH_2CH_2O$). ^{31}P NMR ($CDCl_3$, ppm): 1.27 (P in dendritic units), 0.16 (P in linear units), −11.72 (P in terminal units) [34]. ^{77}Se NMR ($CDCl_3$, ppm): 290.44. The FTIR spectrum and DSC curve of HPSe were shown in Fig. S3.

2.4. Cytotoxicity measurement

2.4.1. Cell culture

L929 cell (a murine fibroblasts cell line), NIH-3T3 cell (a mouse embryonic fibroblast cell line), HeLa cell (a human cervical carcinoma cell line), Cal-27 cell (a human head and neck squamous carcinoma cell line), HN-6 cell (a human primary squamous carcinoma of tongue cell line), MCF-7 cell (a human breast adenocarcinoma cell line), and Bel-7402 cell (a human hepatocellular carcinoma cell line) were cultured in DMEM. MGC-803 cell (a human gastric carcinoma cell line) and 95-D cell (a highly metastatic lung carcinoma cell line) were cultivated in RPMI-1640. LoVo cell (a human colon carcinoma cell line) was cultured in F-12K. MDA-MB-231 cell (a human breast adenocarcinoma cell line) was cultured in L-15. The culture mediums contain 10% FBS (fetal bovine serum) and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin). MDA-MB-231 cells were incubated at 37 °C under a non–CO_2–equilibrated humidified atmosphere. The other cells were incubated at 37 °C under a humidified atmosphere containing 5% CO_2.

2.4.2. MTT assay

The cells were seeded into 96-well plates at 8 × 10^3 cells per well in 200 μL of culture medium. After 12 h of incubation, the medium was removed and replaced with another 200 μL of culture medium containing serial dilutions of HPSe or HPS from 0.25 to 25 μg mL^{-1}. HeLa cells without the treatment were used as control. The cells were grown further for different incubation times from 6 to 72 h. Then, 20 μL of 5 mg mL^{-1} MTT assay stock solution in PBS was added to each well. After the cells were incubated for 4 h, the medium containing unreacted MTT was carefully removed. Then, the obtained blue formazan crystals were dissolved in 200 μL $well^{-1}$ DMSO, and the absorbance was measured in a BioTek Synergy H4 hybrid reader at a wavelength of 490 nm. The blank was subtracted to the measured optical density (OD) values, and the cell viability was expressed as % of the values obtained for the untreated control cells.

2.4.3. Apoptosis assay

HeLa cells were seeded in six-well plates at 5 × 10^5 cells per well in 1 mL of complete DMEM and cultured for 12 h, followed by removing culture medium and adding 1 mL HPSe solution of DMEM at various concentrations from 0.25 to 25 μg mL^{-1}. HeLa cells without the incubation of HPSe were used as control. After 12 h incubation, cells were rinsed by PBS twice and treated with trypsin. Then, 2 mL