Folate Receptor-Targeted, ROS-Responsive Dextran-Block-Poly(propylene sulfide) Copolymer Nanoparticles: A Bioinformatics-Guided Approach for Prostate Cancer Therapy

Yixun Zhang[1], Zhenguo Liang[2], Zeheng Tan[3], Jundong Lin[4], Xinqing Jiang[1], Ruimeng Yang[1], Jian Zhang[1], Huijing Tan[1], Wenjie Xie[1], Yangjia Zhuo[1], Fen Zou[1], Jianheng Ye[1], Jianming Lu[1], Yingke Liang[1], Ronghua Yang[1], Weide Zhong[1], and Hui-Kang Yang[1]

[1]the Second Affiliated Hospital, School of Medicine, South China University of Technology
[2]the First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University
[3]Nanfang Hospital, Southern Medical University
[4]Affiliation not available

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Abstract

This study presents a novel approach in nanomedicine for targeted cancer therapy by leveraging folic acid-receptor (FR) interactions and reactive oxygen species (ROS)-responsive nanocarriers. Utilizing big data to advance nanomedicine, we developed an innovative amphiphilic conjugate, folic acid (FA) decorated dextran-block-poly copolymer (FA-Dex-b-PPS), designed to serve as ROS-responsive nanocarriers specifically for prostate cancer treatment. The chemical structure of FA-Dex-b-PPS was confirmed via Fourier transform infrared spectroscopy and proton nuclear magnetic resonance. The self-assembly into ROS-responsive nanoparticles and subsequent degradation were characterized through fluorescence spectroscopy, dynamic light scattering, and transmission electron microscopy. Therapeutic nanocarriers encapsulating doxorubicin (Dox) were prepared via dialysis, demonstrating efficient oxidant-triggered Dox release in vitro. Cytotoxicity assays revealed high biocompatibility, with cell survival rates exceeding 85% at 400 μg/mL. Confocal laser scanning microscopy confirmed the efficient internalization of FA-Dex-b-PPS-Dox nanoparticles by PC3 cells via FR-mediated endocytosis, surpassing non-targeted Dex-b-PPS-Dox nanoparticles. Furthermore, in vitro and in vivo xenograft mouse model analyses consistently demonstrated that FA-Dex-b-PPS-Dox nanoparticles exhibited superior anti-tumor efficacy against PC3 cells compared to non-targeted and free Dox counterparts. These findings underscore the potential of tumor-targeted, ROS-responsive nanocarriers in enhancing the efficacy of cancer therapy.

1.Bioinformatics analysis

Prostate cancer

Targeting folate receptor

2.Design targeted nanomedicines

Dox

ROS

ROS-responsive

FA   Dex   PPS   Self-assembly

Dis-assembly

3.Biological experiments

In vitro

In vivo

Record

Tumor volume

Tumor weight

Body weight

Immunohistochemistry

Dis-assembly

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Propylene sulfide

(1) SH DBU

(2) Br

Alkyne-PPS

Dextran

(3) HCl H₂N Cl
NaBH₃CN, 50°C, 7days

(4) NaN₃,70°C, 1day

Azido-Dextran

Alkyne-PPS
+
Azido-Dextran

CuSO₄.5H₂O, NaAcs
DMSO, 50°C, 3days

Dex-*b*-PPS

Folic acid
EDCI/HOBT

FA-Dex-*b*-PPS

Folic acid

A.

B.

C.

D.

E.

F.

A.

B.

C.

D.

A. Cell Viability (%) vs FA-Dex-*b*-PPS concentrations (µg/ml): 25, 50, 100, 200, 400

B. Cell Viability (%) vs Dox concentrations (µg/ml): 0.625, 1.25, 2.5, 5, 10 — 24h and 48h. Legend: Free Dox, Dex-*b*-PPS-Dox, FA-Dex-*b*-PPS-Dox

C.
(a) Control
(b) Free Dox
(c) Dex-*b*-PPS-Dox
(d) FA-Dex-*b*-PPS-Dox

D. Control, Free Dox, Dex-*b*-PPS-Dox, FA-Dex-*b*-PPS-Dox; 0 h, 24 h

E. Invasion cells per field — Control, Free Dox, Dex-*b*-PPS-Dox, FA-Dex-*b*-PPS-Dox

F. Wound healing rate (%) — Control, Free Dox, Dex-*b*-PPS-Dox, FA-Dex-*b*-PPS-Dox

G. Dox, Hoechst, Merge — Control, Free Dox, Dex-*b*-PPS-Dox, FA-Dex-*b*-PPS-Dox

A.

NOX4

β-actin

Control Free Dox Dex-*b*-PPS-Dox FA-Dex-*b*-PPS-Dox

62 kDa

42 kDa

B.

Relative protein levels of NOX4/β-actin

C.

ROS fluorescence intensity (fold of control)

D.

(a) (b)

(c) (d)

(a) Control   **(b) Free Dox**
(c) Dex-*b*-PPS-Dox **(d) FA-Dex-*b*-PPS-Dox**

E.

GSH levels (fold of control)

F.

SOD levels (fold of control)

G.

Apoptosis Rate (%)

H.

Cleaved Caspase-3

β-actin

Control Free Dox Dex-*b*-PPS-Dox FA-Dex-*b*-PPS-Dox

17 kDa

42 kDa

I.

Relative protein levels of Cleaved Caspase-3/β-actin

6

A.

B.

C.

A.

B.

C.

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a, Department of Radiology, the Second Affiliated Hospital, School of Medicine, South China University of Technology, Guangzhou, Guangdong, China

b, Department of Burn and Plastic Surgery, the Second Affiliated Hospital, School of Medicine, South China University of Technology, Guangzhou, Guangdong, China

c, Department of Urology, the Second Affiliated Hospital, School of Medicine, South China University of Technology, Guangzhou, Guangdong, China

d, Guangdong Provincial Key Laboratory of Urology, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, 510230, Guangzhou, Guangdong, China.

e, Guangzhou Laboratory, No. 9 XingDaoHuanBei Road, Guangzhou International Bio Island, Guangzhou 510005, Guangdong, China

f, State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Taipa, 999078 Macau, China

g, Guangdong Provincial Institute of Nephrology, Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China

* To whom correspondences should be addressed: E-mail: 21720091@qq.com, zhongwd2009@live.cn; eyyanghk@scut.edu.cn.

[1] These authors contributed equally to this work.

ABSTRACT

Utilizing big data to advance nanomedicine, especially via folic acid-receptor (FR) interactions for targeted cancer treatment, underscores a novel approach in oncology. Reactive oxygen species (ROS)-responsive nanocarriers, in particular, can rapidly release drugs in response to ROS stimulation, making them highly effective for tumor therapy. In this study, we fabricated a novel amphiphilic conjugate composed of folic acid (FA) decorated dextran-block-poly copolymer (FA-Dex-*b*-PPS) to serve as ROS-responsive nanocarriers for the treatment of prostate cancer. The chemical structure of FA-Dex-*b*-PPS was confirmed using Fourier transform infrared spectroscopy and proton nuclear magnetic resonance. The self-assembling behavior of FA-Dex-*b*-PPS into ROS-responsive nanoparticles and their degradation was characterized using fluorescence spectroscopy, dynamic light scattering, and transmission electron microscopy. Therapeutic nanocarriers encapsulating doxorubicin (Dox) were formed via dialysis. The FA-Dex-*b*-PPS nanoparticles demonstrated excellent oxidant-triggered Dox release ability *in vitro*. The outcomes from the cytotoxicity assays indicate that the FA-Dex-*b*-PPS nanocarriers exhibit high biocompatibility, with cell survival rates exceeding 85 % even at concentrations of 400 μg/mL. The utilization of confocal laser scanning microscopy for analysis demonstrated that the FA-Dex-*b*-PPS-Dox nanoparticles were efficiently internalized by PC3 cells through FR, in contrast to the non-FA coated drug-containing nanoparticles (Dex-*b*-PPS-Dox). Furthermore, both in vitro and *in vivo* xenograft mouse model analyses consistently demonstrated that FA-Dex-*b*-PPS-Dox nanoparticles exhibited the best anti-tumor effect toward PC3 cells compared to non-targeted Dex-*b*-PPS-Dox nanoparticles and free Dox at the same Dox dosage. These combined data suggest that nanocarriers with tumor-targeting capabilities and ROS response have greater potential for application in tumor therapy.

1. Introduction

Cancer ranks as a predominant factor contributing to mortality worldwide[1] . Prostate cancer (PCa) is frequently diagnosed among men across various cancer types,

56 characterized by high recurrence, metastatic rates, and mortalities. Effective therapies

57 for PCa remain an unmet clinical need. Consequently, identifying targeted therapeutic

58 approaches has become one of the pressing challenges in current clinical medical

59 research. By deeply analyzing the pathogenesis and clinical characteristics of PCa,

60 researchers are striving to explore novel therapeutic strategies and methods to

61 enhance treatment efficacy and improve patient prognosis[2]. Concurrently, the

62 advancement of science and technology has introduced emerging treatment modalities

63 and techniques, offering new hope and possibilities for PCa therapy[3].

64   Conventional chemotherapy is one of the treatments for PCa; however, the

65 clinical application of chemotherapeutic agents is constrained by their limited water

66 solubility, absence of specificity, and elevated toxicity[4]. In order to surmount these

67 challenges, scientists have engineered advanced drug delivery platforms including

68 liposomes, hydrogels, and exosomes, with nanomedicine at the forefront of this

69 research area[5]. Extensive research has been conducted on polymeric nanoparticles

70 owing to their desirable attributes, which include biocompatibility, stability, and a

71 substantial drug-loading capacity[6]. Unfortunately, most polymeric drug carriers lack

72 the ability to actively target tumors and release drugs rapidly, which reduces the

73 effectiveness of chemotherapy. Consequently, there exists an urgent requirement to

74 engineer sophisticated polymeric nanoparticles that possess both tumor-targeting and

75 rapid drug release functionalities for the treatment of PCa.

76   With the rapid development of big data analysis technologies, integrating these

77 technologies to advance nanomedicine research has become a new trend in medical

78 research[7]. Specifically, the specific interactions between folic acid (FA) and its

79 receptors have recently become a focal point in academic research on targeted

80 delivery of nanomedicines. FOLH1, also known as FA, is a gene that encodes a type

81 II transmembrane glycoprotein of the M28 peptidase family. FA is a crucial molecule

82 involved in several biological processes, such as RNA and DNA preparation,

83 methylation, and modification. FA serves as the principal binding agent to the FR, a

84 protein that is excessively expressed in the majority of cancerous cells[8, 9] . The

85 specific binding of FA and the folic acid receptor has been utilized as the basis for

86 many targeted therapies to achieve precise drug delivery[10]. For example,

87 FA-modified poly(ε-caprolactone) block poly(2-methacryloxoethyl phosphorylcholine)

88 copolymers have shown higher cellular uptake compared to non-FA-modified

89 counterparts in some cancer cells[11]. Moreover, studies have documented that the

90 modification of FA-DABA-SMA copolymers is capable of decreasing the expression

91 of HES1 and NOTCH1 proteins, thereby enhancing the levels of FRα expression in

92 breast cancer cells[12]. In recent years, researchers have utilized FA conjugation to

93 nanoparticles to achieve targeted cancer therapy. For instance, mixed miRNAs-loaded

94 FR-conjugated PLGA nanoparticles have been designed to eliminate cancer cells from

95 spermatogonia stem cells[13]. Similarly, Dox-conjugated glycol-block copolymer

96 coated multiwall carbon nanotubes decorated with FR have been used to enrich Dox

97 accumulation in tumor tissue and achieve targeted therapy of breast cancer[14]. In

98 summary, the conjugation of FA has demonstrated potential in facilitating targeted

99 drug delivery for the treatment of cancer. The exploitation of the interaction between

100 FA and its receptor for targeted therapeutic approaches holds significant promise for

101 the advancement of future strategies in cancer treatment. However, current research

102 on the application of clinical data analysis in the bioinformatics study of FA in

103 pan-cancer or PCa is still inadequate. Moreover, there is a notable lack of

104 nanomedicine designed and constructed based on bioinformatics analysis results for

105 the targeted treatment of PCa.

106   In recent years, the domain of pharmaceuticals and biomedicine has witnessed a

107 growing trend in the development of smart drug delivery systems that respond to

108 various stimuli[15]. Researchers have designed and synthesized a variety of

109 stimuli-responsive polymeric nanoparticles to achieve targeted release of

110 chemotherapy drugs and improve the antitumor effect, based on the characteristics of

111 tumor tissue such as temperature, pH, ultrasound, reactive oxygen species (ROS), and

112 enzyme stimulation[16, 17]. It is well known that ROS, including H_2O_2, superoxide,

113 hydroxyl radical, and others, are highly expressed in cancer cells. ROS-responsive

polymeric nanoparticles, as drug delivery systems, have developed as an effective strategy for precise therapy, based on the higher ROS concentration in tumor tissues compared with normal tissues[18-20]. Drug delivery from ROS-responsive polymeric nanoparticles occurs mainly through two different pathways, namely, hydrophobic-hydrophilic transition and the degradation of polymeric chain segments[21, 22]. On one hand, ROS can oxidize hydrophobic polymers, which have chalcogen elements (such as S, Se, Te) in their backbone or side chain, into hydrophilic polymers. This hydrophobic-hydrophilic transition leads to dissociation of the polymer self-assembly and ultimately results in the rapid release of the drug. On the other hand, ROS-sensitive chemical structures, such as boronic acid esters, proline oligomers, thioketal, or diselenide groups, can be introduced into the polymer structure. The presence of ROS can then degrade the polymer, enabling rapid release of the drug. Therefore, ROS-responsive polymeric nanoparticles have great potential as drug delivery platforms in the future.

Polypropylene sulfide (PPS) is regarded as an ideal ROS-responsive polymer. The rationale for this lies in the facile synthesis of PPS through an anion-initiated ring-opening polymerization of propylene sulfide. Additionally, under the influence of ROS, the hydrophobic sulfur atoms within the PPS backbone can undergo oxidation, transitioning into hydrophilic sulfoxide or sulfone groups[23, 24]. This allows the polymer to undergo a hydrophobic-hydrophilic transition for rapid and controlled release of the drug[25]. Currently, polyethylene glycol-block-polypropylene sulfide (PEG-*b*-PPS) amphiphilic block copolymers are widely used as ROS-sensitive nanocarriers for the treatment of tumors, inflammation, and other diseases[26, 27]. This is mainly due to the fact that PEG is already approved by the FDA for improving the pharmacokinetics and bioavailability of drugs[28]. In addition, PEG-*b*-PPS can be easily synthesized by an anionic ring-opening polymerization reaction using PEG as a macromolecular initiator[29]. Nonetheless, it is important to acknowledge that polyethylene glycol (PEG) is a non-biodegradable biomaterial, and the existence of anti-PEG antibodies in patients treated with PEG-conjugated pharmaceuticals could

143 potentially diminish the therapeutic efficacy[30, 31]. Furthermore, the lack of

144 functional groups on the PEG segment makes it difficult to introduce targeting ligands

145 on PEG-*b*-PPS. Overall, PEG-*b*-PPS amphiphilic block copolymers remain a

146 promising platform for drug delivery. Nevertheless, further research is required to

147 address the limitations of PEG and optimize their performance for specific

148 applications.

149 Renewable and biodegradable natural polysaccharides, including starch, dextran,

150 hyaluronic acid, and cellulose, have attracted substantial interest due to their potential

151 utility in the biomedical field[32]. Dextran stands out as a polysaccharide of choice

152 for in-depth exploration, largely due to its desirable characteristics including aqueous

153 solubility, compatibility with biological systems, and a reduced potential to elicit an

154 immune response[33]. While dextran-based block polymers have not been extensively

155 studied, recent literature reports have focused on the preparation of

156 dextran-containing amphiphilic block polymers using click reactions[34, 35]. This

157 method offers meticulous regulation of the material's architecture and attributes,

158 enabling the integration of dextran's benefits with those of other substances. Moreover,

159 the hydroxyl groups on the dextran allow for further modifications by coupling

160 various targeting ligands, fluorescent molecules, and drugs[36-38]. Despite the

161 promising applications of dextran-based block polymers, there is currently no

162 research on dextran-block-polypropylene sulfide copolymers. This is an exciting area

163 of potential research, as polypropylene sulfide is another biodegradable material that

164 exhibits favorable properties for biomedical applications.

165 In this study, we initially extracted the expression data of FA across 33 pan-cancer

166 types from the publicly available repository, The Cancer Genome Atlas (TCGA).

167 Thereafter, we analyzed the role of FA in tumor cell stemness. Progressing from this,

168 our study explored the relationship between FA and PCa in terms of TNM staging,

169 prognostic survival, and tumor infiltrating immune cells. Building on the critical

170 theoretical foundation of FA's high expression in PCa, we report the synthesis of FA

171 decorated dextran-block-polypropylene sulfide polymer using click chemistry and

EDCI/HOBT-catalyzed esterification reactions. The obtained amphiphilic polymer's chemical structure was characterized using Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). Furthermore, the self-assembly behavior of the amphiphilic block polymer was demonstrated using dynamic light scattering (DLS), transmission electron microscopy (TEM), and fluorescence spectroscopy. Additionally, Dox-loaded FA-Dex-*b*-PPS nanoparticles were found to exhibit ROS-responsive drug release behavior under high ROS environments (Scheme 1). *In vitro* and *in vivo* experiments were conducted to compare the anti-tumor effect of FA-Dex-*b*-PPS-Dox nanoparticles, non-targeted Dex-*b*-PPS-Dox nanoparticles, and Free Dox, all at the same Dox dosage. The results demonstrated that FA-Dex-*b*-PPS-Dox nanoparticles exhibited the best anti-tumor growth effect towards PC3 cells. Drawing from both in *vitro* and in *vivo* experimental outcomes, we observed that employing FA as a tumor-targeting ligand can augment the intracellular drug concentration and potentiate the antitumor efficacy of Dox-loaded nanoparticles.

2. RESULTS AND DISCUSSION

2.1 Pan-Cancer Analysis of FOLH1 Transcriptomic Expression and Its Correlation with Tumor Stemness and Prostate Cancer Pathology

To thoroughly investigate the transcriptomic expression pattern of FOLH1 across various cancers, we utilized pan-cancer data encompassing 34 common cancer types from the TCGA database. As depicted in Figure 1A, FOLH1 expression was found to be significantly altered in multiple cancer types, such as UCEC, LUAD, ESCA, STES, KIPAN, COAD, COADREAD, PCPG, PRAD, KIRC, LUSC, THCA, OV, PAAD, UCS, LAML, GBM, GBMLGG, LGG, BRCA, KIRP, STAD, LIHC, WT, SKCM, KICH, and CHOL. These findings underscore the pivotal role of FOLH1 in cancer pathogenesis (all P values <0.05). Then, to investigate the role of the FOLH1 gene in tumor stemness, we calculated the Spearman correlation between FOLH1 gene expression and tumor stemness scores within each cancer type. As depicted in Figure 1B, significant correlations were observed in 18 cancer types, with 4 of these demonstrating a significant positive correlation. These included

BRCA(N=1080)(R=0.148942256393485,P=8.80956467409179e-7),PRAD(N=491)(R =0.258044667531986,P=6.55838181611533e-9),LUSC(N=483)(R=0.2065064681831 96,P=0.0000047371602794732),ACC(N=76)(R=0.289905397385117,P=0.011077122 6466697). In contrast, a significant negative correlation between FOLH1 gene expression and tumor stemness scores was found in 14 other cancer types, such as LGG(N=507)(R=-0.123664750206764,   P=0.00529765414468569)   , CESC(N=301)(R=-0.19327883282091,   P=0.000748542829133704), COAD(N=281)(R=-0.314768065937981,   P=7.02241656987141e-8), COADREAD(N=369)(R=-0.280195437770192,   P=4.3960481873053e-8), SARC(N=253)(R=-0.153905971298841,   P=0.014460193903918)   , KIPAN(N=860)(R=-0.589272769954414,   P=1.55736718515943e-81)   , UCEC(N=177)(R=-0.265404997445562,   P=0.000356694282028656)   , KIRC(N=512)(R=-0.407254021627045,   P=7.08589079849258e-22), THYM(N=119)(R=-0.264175478120932, P=0.00369422930699814). These findings provide crucial insights for further exploration of the mechanism by which the FOLH1 gene influences tumor stemness. It is evident that FOLH1 is highly expressed in prostate cancer, rendering it a promising target for future investigative research.

In light of the above bioinformatics analysis of FOLH1 across pan-cancer datasets and drawing upon the foundation of our previous research efforts, we have chosen PCa as the tumor type for our subsequent studies. The relationship between FOLH1 and pathologic T stage, pathologic N stage, clinical M stage from TCGA-PRAD dataset were further analyzed. As illustrated in Figures 1C and 1D, PCa patients with high levels of FOLH1 expression are correlated with more severe pathological T stages (T3 and T4) and elevated pathological N stages (N1). However, there was no significant association between FOLH1 expression status in clinical M stage (Figure 1E). The area under the receiver operating characteristic (ROC) curve (AUC) for FOLH1 reached 0.828 (Figure 1F), signifying that FOLH1 expression possesses considerable diagnostic utility in the context of PCa.

The collective bioinformatics findings indicate that the strategic design of

nanomedicines targeting FOLH1 harbors significant potential for therapeutic advancement. FOLH1, also known as the folate receptor, has been the subject of numerous studies wherein folate-conjugated nanotherapeutics have been employed to target tumor cells expressing the folate receptor, thereby facilitating precision-targeted therapy.

2.2 Preparation and Characterization of FA-Dex-*b*-PPS

The number of polysaccharide-based block polymers is significantly lower when compared to polyethylene glycol-based block polymers. This is mainly due to the large number of reactive groups on the polysaccharides need to protect, the lower activity of the terminal reducing groups and difficult to find good solvent for both blocks that limit the preparation of polysaccharide-based block polymers[39]. Currently, chemical conjugation is widely used to synthesis polysaccharide-based block polymers. However, the reaction efficiency of coupling reactions between two large molecular blocks is usually low. Therefore, coupling reactions with high efficiency and selectivity are the basic requirements for the preparation of polysaccharide-based block polymers. Cu(I)-catalyzed "alkyl/azide" click reactions occurring under mild reaction conditions are highly selective, efficient, and reliable and play a crucial role in the preparation of polysaccharide block polymers[35]. In this study, dextran/poly(propylene sulphide) block polymer was prepared by using the Cu(I)-catalyzed "alkyl/azide" click reactions. To achieve this, α-azide-dextran was synthesized using the reductive amine reaction and α-alkyl-PPS was synthesized through anionic ring-opening polymerization. The dextran/poly(propylene sulphide) amphiphilic block polymer was then prepared by click reaction. Finally, folic acid molecule was grafted onto the dextran chain segment by esterification to enable the block polymer the ability to target the folic acid receptor, as shown in Scheme 2.

The chemical structure of the polymers was characterized by infrared spectroscopy, as illustrated in Figure 2A. For the α-azide Dex, the stretching vibration absorption peak of the hydroxyl group on the polysaccharide was located at 3500 cm^{-1}, and the characteristic peak of the azide group appeared at 2106 cm^{-1}. This indicated

that the azide group was successfully introduced to the reducing-end of the dextran via the reductive amine reaction. The C-H stretching vibration of PPS was observed in the region of 2850-3000 cm^{-1}. The spectrum for FA-Dex-*b*-PPS clearly showed that the azide group absorption peak at 2106 cm^{-1} had completely disappeared, indicating that the product was successfully prepared through the click reaction. Furthermore, the hydroxyl absorption peak on the dextran chain segment appeared at 3500 cm^{-1} and the absorption peak located at 1430 cm^{-1} was attributed to PPS block.

The molecular structure of α -alkyne-PPS was elucidated using NMR spectroscopy, with the resulting data depicted in Figure 2B. The chemical shift of the H-atom of the methyl group on the initiator n-butyl mercaptan was located at 0.90 ppm. The peak attributed to the methyl groups of the propylene sulphide repeated units appeared at 1.35 ppm, and the H-atom attributed to the methylene group was located at 2.52-2.65 ppm. The peak located at 2.85-3.00 ppm belonged to the hypomethyl group. By comparing the integrated area of the methyl group on the repeat unit of PPS with that of the initiator methyl group, we were able to calculate the polymerization degree of PPS to be approximately 78, and the molecular weight to be approximately 5911 g/mol, which was calculated based on the following equation:

$M_{\text{1-buthane thiol}}$ + $M_{\text{propylene sulfide}}$ × 78 + $M_{\text{Propargyl bromide}}$ - M_{HBr} = 90.19 g/mol + 74.14 g/mol×78 + 118.96 g/mol – 80.91 g/mol = 5911 g/mol (3), where M represents the molar mass.

We employed NMR s spectroscopy to determine the detailed chemical structure of the dextran/poly(propylene sulphide) block polymer synthesized via the click reaction in DMSO. As exhibited in Figure 2C, all the H-atom peaks attributed to dextran and PPS blocks were identified. Moreover, the H-atom on the triazole ring which was produced after the click reaction was located at 8.10 ppm. The presence of the triazole ring provided compelling evidence for the successful preparation of dextran/poly(propylene sulphide). Using the ratio between the integrated area of the methyl H-atom signal of PPS and the methyl H-atom of dextran, we calculate that the molar ratio of the hydrophobic and hydrophilic repeating units in the polymer is about

288 2.22:1, which corresponded to a mass fraction of hydrophobic chain segments of

289 approximately 50 %. We cannot provide GPC data for dextran/poly(propylene

290 sulphide) mainly due to the block copolymer can only be dissolved in DMSO,

291 however, we do not have a GPC with DMSO as the mobile phase.

292   The folic acid molecule was grafted onto the dextran block via the

293 EDCI/HOBT-catalyzed esterification. Since folic acid contains unsaturated structures

294 such as benzene rings, the H on this type of unsaturated structure appears at high

295 chemical shifts. As shown in Figure 2D, the characteristic peaks of unsaturated cyclic

296 structures on folic acid were appeared at 6.50-8.50 ppm, confirming the successful

297 synthesis of target product. The degree of substitution of folic acid was calculated

298 from the NMR spectra as approximately 1.7 folic acid molecules per dextran block by

299 the ratio of the integrated area of the H-atom signal of the benzene ring on the folic

300 acid to the integrated area of the methyl H-atom signal on the n-butyl mercaptan.

301 **2.3 Preparation and characterization of ROS sensitive-nanoparticles**

302   The FA-Dex-*b*-PPS block polymer composed of hydrophilic dextran block and

303 hydrophobic PPS block, allowing it to self-assembly into nanoparticles in selective

304 solvents. In this study, FA-Dex-*b*-PPS based nanoparticles were obtained through a

305 nanoprecipitation process. It's well known that the size of nanoparticles plays an

306 important role in drug delivery, as it determines whether the drug can reach the tumor

307 site by passthrough the biological barriers and escaping clearance by the immune

308 system during *in vivo* circulation[40]. Figure 3A shows the DLS result of the blank

309 nanoparticles in aqueous solution. The size of the nanoparticles was approximately

310 101 nm when the nanoparticles were dispersed at a pH=7.4 aqueous solution

311 mimicked the human physiological environment and in the absence of H_2O_2. The

312 morphology of the nanoparticles was observed by transmission electron microscopy

313 (TEM). As shown in Figure 3C, the nanoparticles were spherical in structure.

314 Subsequent, we investigated the particle size and morphology of nanoparticles after

315 encapsulated with the hydrophobic antitumor drug Dox. From Figure 3B, we could

316 find that after loading with hydrophobic drug, the particle size of the nanoparticles

317 was increased from 101 nm to 130 nm. This is mainly due to the encapsulation of

318 hydrophobic drug in the hydrophobic core of the nanoparticles through hydrophobic

319 interaction[41]. As Figure 3D shows that the drug-loaded nanoparticles maintained

320 their spherical like morphology, and exhibited a larger size than the blank

321 nanoparticles.

322   The stability of nanoparticles is crucial in *in vivo* drug delivery since it prevents

323 drug premature release, extends circulation time of drug in the blood vessel, and

324 enhances drug accumulation at the tumor site[42]. Critical micelle concentration is a

325 widely used parameter to characterized the stability of nanoparticles. Pyrene can act

326 as fluorescent probe to measure the cmc value. Figure 4C shows the fluorescence

327 spectroscopy of pyrene at different polymer concentrations at pH 7.4 and 0 μM H_2O_2.

328 As the polymer concentration increases, a noticeable red-shift in the fluorescence

329 spectroscopy of pyrene was occurs. This phenomenon is due to the gradual formation

330 of nanoparticles with hydrophobic cores as the polymer concentration increases. The

331 hydrophobic pyrene is encapsulated within the hydrophobic cores of the nanoparticles

332 through physical interactions, causing a change in their chemical environment and

333 resulting in a red-shift in their fluorescence spectra[43]. The cmc value of

334 FA-Dex-*b*-PPS was approximately 0.0093 mg/mL, calculated from I_{337}/I_{335} versus the

335 logarithm of the concentration (Figure 4C). However, when the polymeric

336 nanoparticle in a solution with pH 6.5 and 100 μM H_2O_2, the amphiphilic polymer

337 was oxidized to a fully hydrophilic polymer. Consequently, even though the polymer

338 concentration increase, it is impossible to form nanoparticles. The fluorescence

339 spectra of pyrene at different polymer concentrations under these conditions (Figure

340 4B) indicate that the fluorescence intensity of pyrene is weak, and there is no red-shift

341 in the spectra. As a result, the cmc value cannot be obtained.

342 **2.4 Drug loading and ROS triggered *in vitro* release**

343   Doxorubicin is a broad-spectrum chemotherapeutic agent; however, its therapeutic

344 utility is compromised by its poor aqueous solubility and the occurrence of

345 toxicological effects on non-targeted organs. Amphiphilic block polymers are widely

used as drug nanocarriers to enhance the water solubility of antitumor drugs and minimize their side effects. In this study, Dox was encapsulated within the hydrophobic core of FA-Dex-*b*-PSS nanoparticles using a nano-precipitation method. The drug loading content was approximately 6.3 %, indicating the potential of FA-Dex-b-PSS as a promising nano-drug delivery system for tumor therapy.

Further, the release of the encapsulated drug from the drug-laden nanoparticles was investigated *in vitro*. As shown in Figure 4D, under normal physiological conditions (pH=7.4, 0.0 μM H_2O_2), Dox was slowly released from the nanoparticles, and the cumulative release was no more than 20 % over 72 h. At a weak acid environment (pH=6.5, without H_2O_2), the release rate of Dox increased slightly, and the cumulative release of approximately 30 % at the same period. A faster drug release rate was observed under a pH=4.5 acidic conditions (without H_2O_2), and almost 70 % of the Dox was released over 72 h. This phenomenon could be attributed to the improved solubility of Dox under acidic conditions[44]. Under simulate human physiological conditions (pH 7.4 contain with 100 μM H_2O_2), the cumulative 72 h release of Dox was less than 20 %. Maintaining the same pH of 7.4 and increase the H_2O_2 concentration to 500 μM did not significantly increase the release of Dox, with the cumulative drug release only approaching 28 %. This indicated that PPS could not be oxidized only in the presence of H_2O_2. However, when the pH value was lowered to 6.5 and H_2O_2 concentration was set at 100 μM, approximately 60 % of the Dox was released from the nanoparticles. As mentioned above, the PPS block could be oxidized under weak acidic conditions, containing 100 μM H_2O_2, resulting in a hydrophobic to hydrophilic transition. Hence, under this condition, a faster release of Dox phenomenon could be observed.

2.5 The antitumor effects of FA-Dex-*b*-PPS-Dox and cellular uptake on prostate cancer cells in vitro

It has also been reported that targeted therapy of prostate cancer can be achieved by targeting PC3 cells with folic acid decorated nanoparticles[45, 46]. These results suggest that we can achieve prostate cancer precise therapy by targeting folate

receptor of PC3 cells. Hence, PC3 cells were selected as our research cells in this study based on the above basis. Firstly, the biocompatibility of the synthesized FA-Dex-*b*-PPS was evaluated by CCK-8 assay in vitro. When the concentration of FA-Dex-*b*-PPS reached 400 μg/mL, the PC3 cells survival rate was still more than 80 %, indicating there was good biocompatibility of FA-Dex-*b*-PPS as nanocarriers (Figure 5A). Then, after FA-Dex-*b*-PPS encapsulating with Dox, we quantified it with Dox as the gradient concentration to evaluate the inhibition of cell proliferation by CCK-8 assay. After 24 h incubation, the cell viability of FA-Dex-*b*-PPS-Dox group was 41.46 ± 0.31 % compared with the control group, while cell viability dropped to only 17.61 ± 0.47 % after 48 h incubation. FA-Dex-*b*-PPS-Dox inhibited the growth of PC3 cells more obviously than Free Dox and Dex-*b*-PPS-Dox (Fig. 5B). Furthermore, the invasion and migration with different treatment on PC3 cells were also evaluated by transwell and wound healing assays. As shown in Figure 5C, 5D, 5E, and 5F, different treatments could inhibit the invasion and migration on PC3 cells. FA-Dex-*b*-PPS-Dox exhibited great stronger inhibition both invasion and migration among them.

Next, to visualize distribution of the nanoparticles, PC3 cells were incubated with 5 μg/mL Dox of nanoparticles for predetermined time durations. Then specificity and cellular accumulation of Free Dox or nanoparticles by PC3 cell lines were confirmed by confocal laser scanning microscopy (CLSM) through intracellular Dox accumulations (red-fluorescence) (Figure 5G). The cell nuclei were labeled with Hoechst dye, exhibiting a blue fluorescence. Notably, following incubation, the free form of Dox preferentially accumulated within the cell nuclei, having been transported into the cells via osmosis. This is consistent with previous literature reports that the uptake of Dox was mainly dependent on a diffusion mechanism[47]. Compared with Free Dox, it is of more accumulation of Dex-*b*-PPS-Dox nanoparticles in cytoplasm. While FA-Dex-*b*-PPS-Dox distributed diffusely both cell nuclei and cytoplasm of cells, which was speculated to the uptake of Dox through the folic acid receptor-mediated endocytosis process, leading to its uniform distribution in

404   the whole cell[48]. All the above results demonstrates that FA-Dex-*b*-PPS-Dox has a

405   good antitumor effect *in vitro* and is a kind of nanoparticles with great potential value.

406   **2.6 FA-Dex-*b*-PPS-Dox induced apoptosis of PC3 cells through ROS production**

407   As research in nanotechnology for cancer treatment intensifies and its

408   applications broaden, the design of ROS-responsive nanodrug delivery system has

409   become an important platform for cancer diagnosis and therapy[49]. NADPH oxidase

410   4 (NOX4) is a member of the NADPH oxidase, which can catalyze the reduction of

411   molecular oxygen to various ROS[50]. The ROS produced by NOX4 are involved in

412   various biological functions such as cell apoptosis, differentiation and tumor

413   growth[51]. Dox, a kind of the most common cancer chemotherapeutic drugs, is

414   widely used in multiple clinical cancer therapy. It was reported that Dox can induce

415   the increase H_2O_2 levels, breaking the balance of oxidative stress in cells by

416   regulating the NOX4 expression levels[52]. These indicated that Dox could be used as

417   a potential drug to enhance H_2O_2 levels of cells. In this study, FA-Dex-*b*-PPS

418   nanocarriers were used to load traditional chemotherapy drug Dox to form a novel

419   nanomedicine (FA-Dex-*b*-PPS-Dox). On the one hand, Dox exerted traditional

420   chemotherapy effects. On the other hand, Dox increased the production of

421   intracellular H_2O_2 through NOX4 pathway, which further oxidized the PPS block,

422   thereby inducing a hydrophobic-hydrophilic transition, leading to the dissociation of

423   the polymer self-assembly and ultimately results in the rapid release of the drug. As

424   shown in Figure 6A and 6B, both Dex-*b*-PPS-Dox and FA-Dex-*b*-PPS-Dox could

425   increase the expression levels of NOX4. Further, flow cytometry (Figure 6C) and

426   CLSM (Figure 6D) were used to evaluate qualitatively and quantitatively the

427   production of intracellular ROS, respectively. FA-Dex-*b*-PPS-Dox significantly

428   increases intracellular ROS levels, demonstrating the targeted enhancement of

429   Dex-*b*-PPS-Dox with FA modification. In addition, glutathione (GSH) and superoxide

430   dismutase (SOD) levels reflect the antioxidant abilities of intracellular cells. As shown

431   in Figure 6E and 6F, compared with control group, Free Dox could increase both

432   intracellular GSH and SOD levels. FA-Dex-*b*-PPS-Dox significantly reduced GSH

and SOD levels, while there were no significant SOD levels differences after Dex-*b*-PPS-Dox treatment. These results illustrated cell antioxidant status of different treatments. Due to a series of intracellular redox chemical reactions after FA-Dex-*b*-PPS-Dox treatment, all those reduced the antioxidant capacity of cancer cells, resulting the reduction of GSH and SOD levels. In general, FA-Dex-*b*-PPS-Dox elevated intracellular ROS levels, while depleted GSH and SOD levels.

Next, we further evaluated cell apoptosis using flow cytometry and western blot assays, including the expression levels of Cleaved Caspase-3, a key factor of apoptosis. The results, as shown in Figure 6G, 6H, and 6I, revealed that FA-Dex-*b*-PPS-Dox treatment significantly induced apoptosis of PC3 cells when compared with the control group. Additionally, the expression levels of Cleaved Caspase-3 were significantly increased after Free Dox and FA-Dex-*b*-PPS-Dox treatment, while Dex-*b*-PPS-Dox showed no significant difference. These findings demonstrate that FA-Dex-*b*-PPS-Dox induces apoptosis of PC3 cells through ROS production. Significantly, the findings of the present investigation imply that FA-Dex-*b*-PPS-Dox may represent a potential therapeutic strategy for targeting PC3 tumor cells.

2.7 **The antitumor efficacy of FA-Dex-*b*-PPS-Dox *in vivo***

To assess the efficacy of various treatments *in vivo*, male nude mice harboring PC3 tumors were employed as the experimental animal model. The animal tumor models were established by subcutaneously injecting 5×10^6/mL PC3 cells. When the tumor volume reached approximately 50-100 mm^3, all mice were randomly divided into 4 groups, namely Control, Free Dox, Dex-*b*-PPS-Dox, and FA-Dex-*b*-PPS-Dox, and were injected through the tail vein with a dosage of 5 mg/kg Dox. Figure 7A and 7B display the tumor volume and weight after different treatments. The tumor volume in mice treated with FA-Dex-*b*-PPS-Dox decreased notably compared to the control groups, but there was no difference observed in the tumor volume of FA-Dex-*b*-PPS-Dox compared with Dex-*b*-PPS-Dox. However, there was a statistically significant difference between the tumor weight of FA-Dex-*b*-PPS-Dox

462 and Dex-*b*-PPS-Dox, indicating that FA-Dex-*b*-PPS-Dox effectively inhibited tumor

463 growth. This phenomenon could be explained by the specific absorption of

464 FA-Dex-*b*-PPS-Dox by the folic acid receptor on tumor cells.

465 The antitumor efficacy of FA-Dex-*b*-PPS-Dox was further validated through

466 hematoxylin-eosin and immunohistochemical staining of the tumor tissues, as

467 demonstrated in Figure 7C. Representative images showed a greater number of

468 obviously necrotic regions in tumor tissue after FA-Dex-*b*-PPS-Dox treatment using

469 hematoxylin-eosin staining. In addition, compared to the other groups, the ratio of

470 positive cells of Cleaved Caspase-3 was elevated, and the ratio of negative cells of

471 proliferating cell nuclear antigen (PCNA) was reduced after FA-Dex-*b*-PPS-Dox

472 treatment using immunohistochemistry staining. These findings indicate that

473 FA-Dex-*b*-PPS-Dox is capable of triggering apoptosis and inhibiting the proliferation

474 of cancer cells. Moreover, there were no significant changes in the body weight of the

475 four groups, as demonstrated in Figure 8A. Notably, the survival quality of mice after

476 Free Dox treatment was very poor, and the mortality rate of conventional

477 chemotherapy drugs Free Dox treatment was also high, with obvious hepatorenal

478 toxicity, as shown in Figure 8B and 8C.

479 To investigate the related toxicity of these nanomedicines *in vivo*, the major

480 metabolic organs, including the heart, liver, spleen, lung, and kidney, were evaluated

481 using hematoxylin-eosin staining. There was significant inflammatory cell infiltration

482 in the liver and kidney after Free Dox or Dex-*b*-PPS-Dox treatments, while

483 FA-Dex-*b*-PPS-Dox showed no obvious abnormalities in these major metabolic

484 organs. This indicates that the modification of FA could enhance the tumor targeting

485 of drugs and reduce accumulation in metabolic organs through the folic acid

486 receptor-mediated endocytosis process.

487 **3. Conclusion**

488 We report on the development of ROS-responsive Dex-*b*-PPS copolymers for

489 targeted oncotherapy. The Dex-*b*-PPS copolymers exhibit strong ROS responsiveness

490 and high loading capacity for Dox, and after modification with FA, they are capable

of targeted oncotherapy. Our studies have demonstrated that FA-Dex-*b*-PPS micelles release Dox faster in response to 100 μM H_2O_2. *In vitro* assays such as the CCK-8 assay, transwell assay, and wound healing assay, along with *in vivo* experiments on xenograft mouse models, demonstrate that FA-Dex-*b*-PPS-Dox exhibits greater antitumor effects compared to non-targeted Dex-*b*-PPS-Dox. These results suggest that folic acid decorated Dex-*b*-PPS copolymers are a promising class of therapeutic block copolymer nanoparticles that are ROS-responsive and have great potential in the field of oncotherapy.

4. EXPERIMENTAL SECTION

Materials: 1-buthane thiol, 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) and propylene sulfide (PS) (>96 %) were purchased from Tokyo Chemical Industry (TCI, Japan). Propargyl bromide (99 %) was supplied by Alfa Aesar and used as received. Dextran (Mn = 6600 g/mol) and Sodium azide (NaN_3, 99 %) were obtained from Sigma-Aldrich. Sodium cyanoborohydride ($NaBH_3CN$, 98 %) and 3-chloropropylamine hydrochloride (98 %) were acquired from Aladdin Chemical Company (Shanghai, China). Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 99 %) and sodium ascorbate (NaAsc, 99 %) were acquired from Macklin Chemical Company (Shanghai, China). Doxorubicin hydrochloride (Dox•HCl) was purchased from Meilunbio (Dalian, China). Tetrahydrofuran (THF, chromatographic grade, >99.9 %) and dichloromethane (DCM, Chromatographic grade, >99.9 %) were obtained from J&K Chemical Reagent Inc (Beijing, China) and used as received without further purification. All additional chemicals were utilized in their as-received state without undergoing any additional purification steps.

Methods—Bioinformatics analysis: The Cancer Genome Atlas (TCGA) pan-cancer dataset, which encompasses RNA-sequencing data and curated clinical phenotypes for 34 prevalent cancer types, was obtained from the Pan-Cancer Atlas Hub within the University of California Santa Cruz (UCSC) Xena dataset (https://xenabrowser.net/). Specifically, transcriptome data for the FOLH1 gene were extracted from 34 tumor types, including glioblastoma (GBM, t=153, n=1157),

glioblastoma multiforme (GBMLGG, t=662, n=1157), lower grade glioma (LGG, t=509, n=1157), uterine corpus endometrioid carcinoma (UCEC, t=180, n=23), breast invasive carcinoma (BRCA, t=1092, n=292), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, t=304, n=13), lung adenocarcinoma (LUAD, t=513, n=397), esophageal squamous cell carcinoma (ESCA, t=181, n=668), stomach and intestinal stromal tumor (STES, t=595, n=879), kidney renal papillary cell carcinoma (KIRP, t=288, n=168), kidney and pancreas cancer (KIPAN, t=884, n=168), colorectal adenocarcinoma (COAD, t=288, n=349), colorectal and rectal adenocarcinoma (COADREAD, t=380, n=159), prostate adenocarcinoma (PRAD, t=495, n=152), stomach adenocarcinoma (STAD, t=414, n=211), head and neck squamous cell carcinoma (HNSC, t=518, n=44), kidney renal clear cell carcinoma (KIRC, t=530, n=168), lung squamous cell carcinoma (LUSC, t=498, n=397), liver hepatocellular carcinoma (LIHC, t=369, n=160), Wilms tumor (WT, t=120, n=168), skin cutaneous melanoma (SKCM, t=102, n=558), bladder urothelial carcinoma (BLCA, t=407, n=28), thyroid carcinoma (THCA, t=504, n=338), rectal adenocarcinoma (READ, t=92, n=10), ovarian serous cystadenocarcinoma (OV, t=419, n=88), pancreatic adenocarcinoma (PAAD, t=178, n=171), testicular germ cell tumor (TGCT, t=148, n=165), uterine corpus sarcoma (UCS, t=57, n=78), acute lymphoblastic leukemia (ALL, t=132, n=337), acute myeloid leukemia (LAML, t=173, n=337), pheochromocytoma and paraganglioma (PCPG, t=177, n=3), adenocarcinoma of the breast (ACC, t=77, n=128), kidney chromophobe (KICH, t=66, n=168), cholangiocarcinoma (CHOL, t=36, n=9), as well as relevant paracancerous tissues.

The dataset for this study was sourced from the Uniform Standardized Pan-Cancer dataset provided by the University of California, Santa Cruz (UCSC) database (https://xenabrowser.net/) – the TCGA Pan-Cancer (PANCAN), which encompasses 10,535 samples and 60,499 genes. Building upon this foundation, we extracted the expression data for the ENSG00000086205 (FOLH1) gene across various samples. Subsequently, we further refined our selection to include samples derived from

549 primary blood-derived cancers (Peripheral Blood) and primary tumors. Following the

550 methodology outlined in the previous study (Malta, T. M. et al, 2018), we computed

551 the RNAss tumor stemness scores corresponding to the mRNA characteristics of each

552 tumor. Thereafter, we integrated the stemness indices with gene expression data and

553 applied a $log2(x+1)$ transformation to each expression value. After excluding cancer

554 types with fewer than three samples, we ultimately obtained expression data for 37

555 cancer types.

556 *Methods—Methods and Instrumentation:* The infrared absorption spectra of the

557 samples were measured using a Perkin-Elmer Paragon 1000 infrared spectrometer,

558 USA. The chemical structure of the polymers was analyzed using a Bruker NMR

559 instrument (Bruker AV-500). All deuterated solvents were purchased from Sigma and

560 tetramethylsilane (TMS) was used as an internal standard. Particle size and particle

561 distribution index (PDI) were determined by using a Malvern particle sizer (Malvern

562 Nano-ZS/ZEN-3600 Zetasizer). The dry-state morphology of the nanoparticles was

563 examined utilizing a transmission electron microscope (TEM, JEM-2100F, JEOL,

564 Tokyo, Japan). A minute quantity of the nanoparticle suspension was deposited onto a

565 copper grid, and following a one-minute interval, the excess solution was

566 meticulously blotted away using filter paper. Subsequently, the specimens were

567 subjected to negative staining with a 1 wt% phosphotungstic acid solution. A small

568 phosphotungstic acid solution droplest was cover the sample, after 20 seconds the

569 excess phosphotungstic acid solution was carefully blotted off with filter paper. Prior

570 to TEM observation, samples are stored in a desiccator to remove excess moisture.

571 The critical micelle concentration (cmc) for the block copolymer was ascertained

572 through the application of the widely employed pyrene fluorescence probe technique.

573 Briefly, 50 μL of acetone solution containing pyrene 6×10^{-5} mol/L was added to a 10

574 mL EP tube, and the acetone was removed via evaporation. Various concentrations of

575 the block polymer's aqueous solutions were introduced into EP tubes, which were

576 subsequently agitated in the absence of light for 24 h at 37 °C on a

577 temperature-controlled shaker before conducting the tests. The tests were carried out

using a Shimadzu RF-5301PC fluorescence spectrophotometer. The excitation spectrum was scanned in the wavelength range of 300-350 nm, with an emission wavelength of 373 nm and a slit width of 5 nm. The fluorescence spectra of pyrene in the nanoparticle solution were recorded separately under two different conditions, that is, PBS (50 mM, pH 7.4) without any H_2O_2 or PBS (50 mM, pH 6.5) with 100 μM H_2O_2. The intensity ratio of fluorescence intensity at 337 nm and 335 nm was used as the vertical coordinate and the logarithm of micelle concentration was used as the horizontal coordinate. The concentration value corresponding to the intersection of the data points is the measured cmc. The concentration of Dox in the aqueous solution was measured using a Shimadzu UV-3150 UV-Vis spectrometer and the absorbance intensity at 480 nm was recorded. A working curve was established by measuring the UV absorption at 480 nm for a series of known concentrations of aqueous Dox solutions.

Methods—Preparation of α-alkyne Poly(Propylene sulfide) (alkyne-PPS): α-alkyne poly(propylene sulfide) can be synthesized via a combination of anionic polymerization of propylene sulfide and termination with propargyl bromide. Prior to anionic polymerization, the monomer propylene sulfide is first purified by distillation. The anionic polymerization process of propylene sulfide is based on the method previously reported in the literature[23]. A 100 mL flame-dried round-bottom flask was filled with DBU (4 mmol, 0.60 mL) and 20 mL of ultra-dry THF, and then degassed with argon gas for 30 minutes. The flask was placed in a cold trap to keep the solution temperature below 0 °C. The pre-degassed THF solution containing 1-buthane thiol (1.0 mmol, 0.07 mL) was then slowly dripped into the flask via a dropping funnel, and the reaction was continued for 30 minutes to activate the initiator. The degassed propylene sulfide (80 mmol, 6.24 mL) monomer was then added to the round-bottom flask in one go using a syringe, and the reaction was stirred for 2 h at a temperature of no more than 0 °C. Propargyl bromide (2 mmol, 0.24 g) was added to the reaction system to terminate the anionic polymerization reaction and to introduce an alkyne group. After 12 h of reaction, the resulting salt was removed by filtration,

and most of the THF was distilled out under reduced pressure. The mixture was then dropped into cold anhydrous ether to give a white precipitate, which was collected, washed three times with ether, and dried under vacuum to yield a white viscous polymer. ^{1}H NMR (500 MHz; CDCl$_3$, δ): 0.9-0.95 (t, *CH$_3$*CH$_2$), 1.35-1.41 (s, CH$_3$*CH*), 1.45-1.52 (s, -CH$_2$*CH$_2$*CH$_2$), 1.53-1.60 (m, CH$_3$CH$_2$*CH$_2$*), 2.50-2.56 (t, *CH*≡C-), 2.56-2.70 (s, CH*CH$_2$*S), 2.83-2.98 (t, S*CH*CH$_2$).

Methods—Preparation of α-azide Dextran (azide-Dex): The azide functionality was incorporated into the reducing end of dextran by employing a previously documented procedure, albeit with slight modifications [53]. Initially, a mixture consisting of 2 g of dextran and 40 mL of deionized water was prepared in a 100 mL round-bottomed flask. After complete dissolution of the dextran, 15 g of 3-chloropropylamine hydrochloride and 2 g of NaBH$_3$CN were added to the flask. The reaction vessel was secured with a rubber stopper throughout the process. The reaction solution was agitated in an oil bath maintained at 50 °C for a duration of 7 days, during which time 3 g of NaBH$_3$CN was added daily. Upon completion of the reaction, the product was decanted into a dialysis membrane (1000 Da) and subjected to dialysis against deionized water for a period of three days to remove residual inorganic contaminants. It should be highlighted that throughout the dialysis procedure, the volume of the mixed solution in the dialysis tube increases significantly, and the collected dialysate must be concentrated before the next reaction. The aqueous fraction of the mixture was largely evaporated through vacuum distillation, resulting in a volume of approximately 30 mL of solution. Subsequently, 2 g of sodium azide were introduced into the solution, followed by stirring in an oil bath heated to 80 °C for a duration of 24 h. The product was then subjected to dialysis against deionized water over a three-day period to eliminate surplus inorganic salts. The process was concluded with the acquisition of a white powder, achieved by lyophilizing the dialysate.

Methods—Synthesis of dextran-block-poly(propylene sulfide) copolymer by Husigen Cycloaddition: Copper(I)-catalyzed azide/alkyne Husigen cycloaddition has

been widely utilized to prepare block polymers that incorporate polysaccharide chain segments[35]. In this work, a polysaccharide-based diblock copolymer was synthesized via the Husigen cycloaddition between alkyne-PPS and azide-Dex. In a representative procedure, 0.16 g of azide-modified dextran was transferred into a 50 mL round-bottomed flask, which already held 25 mL of DMSO. Viscous PPS is hard to dissolve directly in DMSO, so 0.1 g of alkyne-PPS was dissolved in 4 mL of THF and then slowly added dropwise to the DMSO solution. To prevent the oxidation of cuprous ions by oxygen dissolved in the DMSO, a high-purity argon gas was continuously passed through the DMSO to remove oxygen. $CuSO_4 \cdot 5H_2O$ and NaAsc were subsequently added, and the flask was further maintained under an argon atmosphere for 10 minutes. The flask was sealed with a rubber stopper, and the mixture was stirred in an oil bath at 50 °C for 3 days. After the reaction, the mixture was transferred to dialysis tubes (50 kDa) and dialyzed with deionized water containing 0.01 % EDTA-Na for 2 days, followed by dialysis with deionized water for another 2 days to remove excess inorganic salts and azide-Dex. The dialysate was lyophilized to obtain white powder.

Methods—Preparation of folic acid decorated dextran-block-poly(propylene sulfide) (FA-Dex-b-PSS) copolymer via esterification reaction: The hydroxyl groups present on dextran provides an opportunity for esterification with the carboxyl group of folic acid. In this study, folic acid decorated Dex-*b*-PSS was synthesized following a previously reported procedure[54]. Briefly, 0.02 g of FA was dissolved in 15 mL of anhydrous DMSO. After the folic acid had fully dissolved, to the flask, 0.1 g of Dex-*b*-PSS was introduced, after which EDCI (0.017 g, 0.09 mmol) and HOBT (0.012 g, 0.09 mmol) were sequentially added. Subsequently, the flask was sealed, wrapped in aluminum foil, and agitated at ambient temperature for a period of two days. After the reaction was complete, the reaction mixture was packed into dialysis bags (6 kDa) and dialyzed for three days to remove excess folic acid and DMSO. The dialysate was lyophilized, yielding a yellow powder. The degree of substitution of folic acid can be calculated using methods previously described in literature[54].

Methods— ROS sensitive-nanoparticles preparation and characterization: The FA-Dex-*b*-PSS amphiphilic copolymer is capable of undergoing self-assembly to produce nanoparticles in chosen solvents. Then, 10 mg of FA-Dex-*b*-PSS was dissolved in 1.5 mL of warm DMSO under continuous stirring. The combined solution was incrementally introduced into 5 mL of deionized water under continuous stirring for 1 h. Subsequently, the mixture was transferred to a dialysis sack with a 6 kDa molecular weight cutoff and subjected to dialysis against deionized water for a duration of 2 days to eliminate the organic solvents. Following this, the dialysate was filtered through a 0.22 μm pore-size syringe filter. The dimensions and dispersal of the synthesized nanoparticles were assessed utilizing a Malvern particle size analyzer. The dried state morphology of the nanoparticles was examined with a JEOL JEM-2100F transmission electron microscope. The critical micelle concentration of the block polymer was ascertained using the established pyrene fluorescence probe technique, employing a Shimadzu RF-5301PC fluorescence spectrophotometer.

Methods— Dox loaded and in vitro release: To incorporate Dox within the internal compartment of the nanoparticles, a solution containing 10 mg of FA-Dex-*b*-PSS and 1 mg of Dox•HCl was prepared by dissolving them in warm DMSO. Triethylamine was added in an equivalent amount to neutralize the hydrochloride, and the resulting mixture was dropped into deionized water with continuous stirring. Following this, the solution was decanted into a dialysis tube and subjected to dialysis against deionized water for a period of 24 h The resulting dialysate was then passed through a needle filter. The concentration of Dox was determined using an established standard curve, and the drug loading content (DLC) was calculated using the following formula:

$$DLC(\%) = w_1/w_2 \times 100 \qquad (1)$$

where w_1 is the weight of Dox loaded in the nanoparticles and w_2 represents the weight of FA-Dex-*b*-PSS.

In vitro drug release was conducted under various conditions, including pH 7.4 without H_2O_2, pH 6.5 without H_2O_2, pH 4.5 without H_2O_2, pH 7.4 with 500 μM H_2O_2,

694   pH 7.4 with 100 μM H_2O_2, and pH 6.5 with 100 μM H_2O_2. To begin, 3 mL of

695   drug-loaded nanoparticle solution was placed into a dialysis bag and transferred to a

696   centrifuge tube containing 27 mL of PBS at different pH and H_2O_2 concentrations.

697   The tubes were then sealed and placed into a 37 °C water bath, where they were

698   agitated at 100 rpm. At predetermined time intervals, 3 mL of solution outside the

699   dialysis bag was withdrawn and replaced with 3 mL of fresh buffer solution. The drug

700   release experiment was repeated three times, and the Dox concentration was measured

701   at 480 nm using a UV-Vis spectrometer (UV-3150, Shimadzu, Japan). The

702   accumulated percentage of released Dox was calculated using the following equation:

703    $$E_r(\%) = \frac{V_e \sum_1^{n-1} C_i + V_0 C_n}{m_{DOX}} \times 100\% \qquad (2)$$

704   In which m_{DOX} denotes the mass of doxorubicin within the micelles, V_0 signifies

705   the total volume of the release medium (V_0 is 30 mL), V_e is the volume of the medium

706   exchanged ($V_e = 3$ mL), and C_i is the concentration of DOX in the ith aliquot.

707   *Methods—CCK-8 assay:* Human prostate cancer cell lines PC3 was maintained in

708   RPMI-1640 medium (Hyclone, United States) that contained 10 % fetal bovine serum

709   (FBS) and 1 % penicillin-streptomycin, within a 37℃ incubator under a 5% CO_2

710   environment. CCK-8 assay was conducted to evaluate the in vitro biocompatibility of

711   FA-Dex-*b*-PPS and the inhibition of cell proliferation of Free Dox, Dex-*b*-PPS-Dox,

712   and FA-Dex-*b*-PPS-Dox to PC3 cells. Briefly, cells were plated into 96-well plates at

713   a density of approximately 1×10^4 cells/well. After 24 h or 48 h incubation with

714   different treatments, 10 μL solution of different treatments concentrations were added

715   to the appropriate wells for appropriate time in a 5 % CO_2 incubator at 37 °C.

716   Subsequently, 10 μL of the CCK-8 reagent were added to each well, and the plate was

717   further incubated for a period of 2 to 4 h. The optical density of each well was then

718   quantified at a wavelength of 450 nm using a multimode plate reader.

719   *Methods—Transwell assay:* The upper surface of the lower membrane in the

720   transwell chamber was coated with a solution of Matrigel at a concentration of 50

721   mg/L, diluted 1:8. The cells were incubated in serum-free RPMI 1640 medium for a

722   duration of 12 h, and the 5×10^5 PC3 cells density of cell suspension was adjusted. A

200 μL volume of cell suspension was introduced into the upper compartment of the transwell insert, while 600 μL of medium supplemented with 10% FBS was placed in the lower chamber of the 24-well culture plate. The 24 well culture plates were placed in 5 % CO_2 incubator at 37 °C for 24 h. Afterward, the cells in the upper layer of the cell membrane were wiped with a cotton swab, and the rest of cells were fixed with 4 % paraformaldehyde for 20 minutes and dyed with crystal violet solution for 15 minutes. After the chambers were dried, pictures were taken under the microscope and conducted comparative analysis. Each sample is randomly counted for 10 visual fields, and the average value is taken.

Methods—Wound healing assay: PC3 cells were plated and grown to 90% confluence in a six-well plate overnight. Following this, wounds were created using a 200-mL sterile pipette tip, and any cellular debris was subsequently cleared away. The cells were incubated in the medium solution of different treatments: Control (PBS), Free Dox, Dex-*b*-PPS-Dox, and FA-Dex-*b*-PPS-Dox. The images of distance migrated by the cells were taken at 0 and 24 h after wound scratching through a microscope (Leica, Germany) in the same position of the plate. The experiments were conducted in triplicate independently.

Methods—Cellular uptake: PC3 cells were treated with PBS, Free Dox, Dex-*b*-PPS-Dox, and FA-Dex-*b*-PPS-Dox maintaining Dox content 5 μg/mL for 8 h with Hoechst for 10 min after washing with PBS. Then cellular uptake of different group by confocal laser scanning microscope.

Methods—Reactive oxygen species generation: The intracellular levels of ROS were quantified using a dedicated ROS assay kit (beyotime, China). The attached PC3 cells were treated with different treatments (Control, Free Dox, Dex-*b*-PPS-Dox, and FA-Dex-*b*-PPS-Dox) for 8 h. After the incubation, harvested cells were treated with 1 μL DHE (S0033, Beyotime Biotechnology, Shanghai, China) at 37 °C with CO_2 in the dark for 30 minutes. Subsequently, the cells underwent two PBS washes and were then disaggregated into a uniform single-cell suspension. The fluorescent intensity was analyzed by flow cytometry (BD Bioscience).

Methods— Apoptosis assay: The Annexin-V apoptosis detection kit was utilized to assess the apoptotic status of PC3 cells that had been subjected to Control (PBS), Free Dox, Dex-*b*-PPS-Dox, and FA-Dex-*b*-PPS-Dox. PC3 cells were seeded and incubated for 24 h to reach 80 % confluency in a six-well plate. Then, the cells were treated with different treatments for 24 h at 37 °C in a CO_2 incubator. Subsequently, the cells were harvested and rinsed with chilled PBS, and then redispersed in 1× binding buffer at a concentration of $1×10^6$ cells per milliliter. The cell suspension was then incubated with Annexin-V at ambient temperature in a light-shielded setting. Whereafter, before being analyzed by flow cytometry, the cells were washed, re-suspended, and PI was added. Using the FACS Calibur flow cytometer, the fluorescence intensity was analyzed and repeated three times. All analysis was carried out using FlowJo software.

Methods— Western blot analysis: PC3 cells subjected to various treatments underwent two PBS washes and were subsequently lysed using an ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer containing 1mM PMSF, from which total protein extracts were obtained. The concentration of total protein was quantified using the BCA protein assay kit. Separation of proteins was achieved through electrophoretic techniques utilizing 10% SDS-polyacrylamide gel matrices, followed by the complete transfer of the desired protein fractions onto polyvinylidene difluoride membranes. Subsequent to this, the membranes were subjected to a blocking step using 5% non-fat dry milk in TBST buffer at ambient temperature for a duration of 1 h. This was followed by an overnight incubation at 4°C with specific primary antibodies against NOX4, Cleaved Caspase-3, and β-actin. After thorough washing with TBST buffer on three separate occasions, the membranes were then probed with horseradish peroxidase-conjugated secondary antibodies for an hour at 37°C. β-actin served as the internal reference for normalization purposes. All experiments were carried out with three replicates. Blots were determined using chemiluminescence detection system membranes. Image J software (version 1.8.0, NIH, USA) was used to quantify the intensity of the immunoreactive bands.

Methods— In vivo antitumor study: Animal studies were conducted with BALB/*c* mice aged between 4 to 6 weeks. All experiments were handled based on the relevant principles of the Guide for the Care and Use of Laboratory Animals and approved by the ethics committee of the South China University of Technology (Approval no. S-2023-078-01). Experimental animals were allocated to various groups in a random fashion. For tumor induction, approximately 5×10^6 PC3 cells were harvested and administered subcutaneously to the right flanks of nude mice, and tumor growth was monitored until the volume reached 50-100 mm³. The test animals received a single intravenous dose of the therapeutic agent at 5 mg/kg body weight, administered every other day for a total of five injections. In contrast, the control group received an equivalent volume of PBS via the same route. Tumor dimensions and the body weight of the mice were recorded throughout the study. Following the completion of the treatment regimen, mice were euthanized via cervical dislocation. Subsequent to euthanasia, hematoxylin and eosin (H&E) staining was utilized to examine the histological features of tumor tissues as well as the primary organs, including the heart, liver, spleen, lungs, and kidneys.

810

Conflict of Interest

812   The authors report no conflict of interest in the present study.

813

Declaration of competing interest

815   The authors declare that they have no known competing financial interests or
816 personal relationships that could have appeared to influence the work reported in this
817 paper.

818

Data availability Statement

820   All the data associated with this study are presented in the paper or in the
821 Supporting Information.

822

Keywords

824   Folic acid, Copolymer nanoparticles, Prostate cancer.

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