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# TPGS-functionalized and ortho ester-crosslinked dextran nanogels for enhanced cytotoxicity on multidrug resistant tumor cells

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ABSTRACT

Herein pH-sensitive nanogels (NG1) and P-glycoprotein-repressive nanogels (NG2) were prepared by copolymerization between an ortho ester crosslinker (OEAM) and tocopheryl polyethylene glycol succinate (TPGS)-free or conjugated dextran. Nanogels with or without TPGS possessed a uniform diameter (~180 nm) and excellent stability in various physiological environments. Doxorubicin (DOX) was successfully loaded into NG1 and NG2 to give NG1/DOX and NG2/DOX, both of them showed appropriate drug release profiles under mildly acidic conditions (pH 5.0). NG2/DOX possessed higher drug enrichment and lethality than NG1/DOX did on MCF-7/ADR cells. Analysis of corresponding index of efflux activity showed that NG2 could induce depolarization of mitochondrial membrane and interfere with ATP metabolism. NG2/DOX also displayed increased penetration and growth inhibition on MCF-7/ADR multicellular spheroids. These results demonstrated that pH-sensitive TPGS-functionalized nanogels (NG2) as drug carriers had great potential to suppress drug efflux in MCF-7/ADR cells and even overcome MDR on cancer cells.

## 1. Introduction

Multidrug resistance (MDR) has been observed on a variety of mechanistically and structurally unrelated anticancer drugs, and always compromises the chemotherapeutic effect in clinical trials (Dubey et al., 2016; Johnson & Chen, 2017; Krishna & Mayer, 2000). Recently, a large number of research has shown that MDR is associated directly with the overexpression of P-glycoprotein (P-gp), a transporter of exogenous substrates (anticancer agents) on cell membrane related to drug efflux (Braunová et al., 2017; Callies et al., 2016; Holohan, Van Schaeybroeck, Longley, & Johnston, 2013; Sun et al., 2004). Drug delivery systems based on inhibition of efflux have been widely utilized trying to overcome MDR, but no significant progress has been achieved due to undesirable side effects and inadequate curative performance (Bao et al., 2014; Bernabeu et al., 2014; Kapse-Mistry, Govender, Srivastava, & Yergeri, 2014; Li et al., 2016; Muthu, Kutty, Luo, Xie, & Feng, 2015). Therefore, it is urgent to develop a particular drug delivery system to enhance drug enrichment in MDR cells and inhibit drug efflux with minimal side toxicity (Koziolová et al., 2016).

As a commercially available polysaccharide, dextran (DEX) has been widely used to prepare drug delivery carriers because of its excellent biocompatibility, biodegradability, non-toxicity as well as easiness for modification (Du, Weng, Yuan, & Hu, 2010; Kaewprapan, Inprakhon, Marie, & Durand, 2012; Sun et al., 2010). Besides, DEX possesses excellent aqueous solubility, non-fouling and non-ionic properties, consequently generating a super stability in blood circulation (Bai et al., 2018; Kreuter, 1994, ch. 5; Su, Jia, & Shan, 2016). Fréchet et al. had successfully prepared a novel type of nanoparticles based on acetalated-dextran, and results indicated that their delivery system possessed a super-biocompatibility similar to the FDA approved material, poly (lactic-*co*-glycolic acid) (Bachelder, Beaudette, Broaders, Dashe, & Fréchet, 2008). Sagnella et al. proved that dextran-based nanocarrier possessed a strongly penetrating ability into 3D tumor spheroids (Sagnella et al., 2013). In addition, biodegradable dextran-based nanogels had been fabricated by Thienen et al., which showed excellent stability and wouldn't aggregate after incubation with human serum *in vitro* (Van Thienen, Raemdonck, Demeester, & De Smedt, 2007). Although these DEX-based carriers have such excellent physiology compatible properties, formulations from simply modified-dextran have failed to effectively inhibit the efflux of MDR cells, and it is necessary to incorporate a reasonable and appropriate functionalization into dextran to combat with MDR.

Tocopheryl polyethylene glycol succinate (TPGS) is a non-ionic water-soluble compound formed by vitamin E and polyethylene glycol (PEG), as such it inherits advantages form these two materials, such as being amphiphilic as well as retaining benefits of PEGylation (Zhang,

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Tan, & Feng, 2012). TPGS has been used as emulsifier, solubilizer, absorption enhancer, and permeation enhancer based on the above excellent properties (Sadoqi, Lau-Cam, & Wu, 2009). More importantly, TPGS has also been extensively applied to combat MDR cancer because it can effectively interfere with cell membrane fluidity, and then, drug efflux is inhibited by interfering with energy metabolism and P-gp transport function (Collnot et al., 2010; Gottesman, Fojo, & Bates, 2002; Guo, Luo, Tan, Otieno, & Zhang, 2013; Silva et al., 2015). For instance, Zhu et al. prepared a series of PLGA nanoparticles encapsulated with various content of TPGS, and results indicated that TPGS-associated nanocarriers could prolong drug retention time and increase drug concentration by synergistically targeting mitochondria to decrease the mitochondrial membrane potential (MMP) (Zhu et al., 2014). Nevertheless, these P-gp inhibitors-contained carriers often caused undesirable off target toxicity and failed to quickly reach the cytotoxic threshold of anticancer drugs (Bernabeu et al., 2014; Ferry, Traunecker, & Kerr, 1996; Yu et al., 2015). Consequently, an accurate spatial-temporal drug release performance is highly required for TPGS-contained carriers to effectively induce MDR cells apoptosis.

Acid-triggered degradation is widely applied in controlled drug release, owing to the distinctive pH gradient among blood vessels (pH 7.4), extracellular (pH 6.5–7.2), and intracellular (pH 5.0–6.0) space in solid tumors (Lee, Gao, & Bae, 2008; Luo, Sun, Sun, & He, 2014). Hence, combining P-gp resistant molecules and pH-sensitive components is an effective method to combat MDR in cancer (Wang et al., 2011). Guo et al. fabricated pH-sensitive and TPGS-grafted chitosan nanoparticles to overcome the P-gp-induced MDR, and corresponding results showed that intracellular drug level significantly improved after treatment by nanoparticles with different TPGS grafting degree (Guo, Chu et al., 2013). Unfortunately, lack of acid-sensitivity of the above TPGS-contained delivery system limited its anti-cancer effect, only about 25% of DOX was released from nanoparticles after incubation at pH 5.5 even for 7 d. In the past several decades, ortho ester has been widely reported as an acid-sensitive linkage with great potential applications (Fu et al., 2017). Compared with other acid-labile bonds such as ketal and acetal, the hydrolysis rate of ortho ester could be increased by 1 to 4 orders of magnitude under acidic conditions (Yan et al., 2017). Recently, we reported an ortho ester-based compound (OEAM) as a cross-linker to prepare pH-sensitive nanogels. These carriers were highly stable under physiological condition while specifically showed swelling and dissociation in mildly acidic environments (such as pH 5.5) (Yang et al., 2017; Zha et al., 2017). Subsequently, encapsulated drugs were controllably enriched in cancer cells and quickly reached their effective cytotoxic threshold for enhanced cytotoxicity.

We hypothesized a desirable drug carrier for MDR cancer therapy, which could be realized by integrating TPGS and ortho ester-bonds into dextran nanogels to achieve an ideal delivery process: (i) remaining stable and long-circulating in blood vessels; (ii) triggering encapsulated drug release at tumoral intracellular pH; (iii) inhibiting cell efflux to prolong the retention time of anticancer agent and quickly reaching an effective cytotoxic threshold in MDR cells, and then to obtain enhanced cytotoxicity.

In this work, we prepared the proposed TPGS-grafted dextran nanogels crosslinked by OEAM. The structures of modified-dextran and physicochemical properties of these nanogels were investigated in details. The MDR-related analysis such as the change of ATP metabolism and mitochondrial transmembrane potential, were performed *in vitro* by using a MCF-7/ADR monolayer cell model and three-dimensional multicellular spheroids.

## 2. Experimental section

### 2.1. Materials

Dextran (20 KDa, α-(1–3) branch, 5%), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and poly (2-hydroxyenthyl methacrylate) (poly-HEMA) were purchased from Sigma-Aldrich (St Louis, MO, USA). *N*,*N*'-Carbonyldiimidazole (CDI) was gained by Tokyo Chemical Industry Co., Ltd (Shanghai, China). Methacrylic anhydride (MA), potassium peroxodisulfate (KPS, re-purified by distill waster) and TPGS were obtained from Shanghai Macklin Biochemical Co., Ltd. Dimethyl sulfoxide (DMSO) was dried over Na followed by reduced pressure distillation and dichloromethane (DCM) was dried by refluxing over $CaH_2$. N, *N*-((((Oxybis (methylene)) bis (1,3-dioxolane-4,2-diyl)) bis (oxy)) bis (ethane-2,1-diyl)) bis (2-methylacrylamide) (OEAM) was synthesized according to the previous work (Yang et al., 2017; Zha et al., 2017). Doxorubicin hydrochloride (DOX·HCl) and rhodamine-123(R-123) were provided by Meilun Biological Technology Co., Ltd. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Waltham, MA, USA). Human breast adenocarcinoma cells (MCF-7) and doxorubicin-resistant cells (MCF-7/ADR) were obtained from KeyGEN BioTECH (Nanjing, China). Annexin V-FITC/PI Apoptosis Detection and ATP assay Kit were purchased from beyotime @biotechnology Co., Ltd.

### 2.2. Synthesis of MA-DEX and TPGS-MA-DEX

Methacrylic anhydride-grafted dextran (MA-DEX) was synthesized according to previous reports (Qiao, Zhang, Du, Liang, & Li, 2011; Rao, Rao, Ramanjaneyulu, & Ha, 2015). Briefly, 1.0 g of dextran was dissolved in 100 mL of phosphate buffer (PBS, pH 8.0) and incubated in ice bath for 30 min. And then 1 mL of methacrylic anhydride (MA, 6.75 mmol) was carefully added under magnetic stirring for 24 h, and pH value of the reaction system was remained at 8.0 by adding NaOH solution (1.0 M). Subsequently, the mixture was purified by dialysis (MWCO 3500) against double-distilled water (DDW) for 3 d and then the final product was harvested by freeze-drying. Fourier transform infrared spectroscopy (FT-IR) and $^1$H NMR (400 MHz, $D_2O$ as solvent) were used to confirm the structure of MA-DEX. In addition, the degree of substitution (DS) of MA was calculated by the formula (Duong et al., 2012): $DS_{MA} = (S_b + S_c)/2S_a$, where $S_a$ represents the integral intensity of specific protons from dextran; $S_b$ and $S_c$ represent the integral intensity of olefinic protons of grafted MA.

The synthetic scheme of TPGS-modified MA-DEX (TPGS-MA-DEX) was shown in Fig. 1. Detailly, TPGS-MA-DEX was synthesized by a two-step process. Firstly, to achieve TPGS active ester (TPGS-CDI), TPGS and CDI (1:10 mol/mol) were dissolved in 20 mL of dry DCM and stirred constantly for 4 h at room temperature. DCM was discarded by a rotary evaporator, and then the crude product was dialyzed against DDW (MWCO 1000) for 2 d to get pure TPGS-CDI. The product was confirmed by $^1$H NMR (400 MHz, $CDCl_3$ as solvent) and FT-IR. In addition, the $^1$H NMR spectrum of TPGS-CDI was shown in Fig. S1 (Supporting information).

The second step was to carry out the reaction between MA-DEX and TPGS-CDI. Briefly, after MA-DEX (300 mg) was completely dissolved in 50 mL of dried DMSO, TPGS-CDI (200 mg) was added to the reaction system with constant stirring at room temperature for 6 h. The subsequent process of purification and verification was similar to that of MA-DEX. TPGS-MA-DEX was characterized by $^1$H NMR (400 MHz, DMSO as solvent) and FT-IR. Similarly, the DS of TPGS was calculated by analyzing the integral areas of typical protons of TPGS and dextran in $^1$H NMR spectra (Duong et al., 2012): $DS_{TPGS} = S_d/12(S_b + S_c) \times 2DS_{MA}$, where $S_d$ represents the integral area of methyl protons in TPGS.

### 2.3. Fabrication of nanogels

The free radical co-polymerization technique was used to fabricate pH-sensitive nanogels (NG1) and TPGS-grafted nanogels (NG2), which were built with ortho ester crosslinker (OEAM) (Wang, Zheng, Tian, & Yang, 2015). The preparation process of NG1 was taken as an example: first, MA-DEX (100 mg) and OEAM (100 mg) were homogenized in