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Chemotherapy for gastric cancer by finely tailoring anti-Her2 anchored dual targeting immunomicelles

Wei Li [a,b,*,1], He Zhao [a,1], Weizhu Qian [a,b,1], Huafei Li [a], Li Zhang [a], Zengwei Ye [a], Ge Zhang [a], Mao Xia [a], Jinfeng Li [a], Jie Gao [a,b], Bohua Li [a,b,c], Geng Kou [a,b,c], Jianxin Dai [a,b,c], Hao Wang [a,b,c], Yajun Guo [a,b,c,*]

[a] International Joint Cancer Institute, The Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, PR China
[b] National Engineering Research Center for Antibody Medicine, State Key Laboratory of Antibody Medicine and Targeting Therapy and Shanghai Key Laboratory of Cell Engineering, 399 Libing Road, Shanghai 201203, PR China
[c] PLA General Hospital Cancer Center, PLA Graduate School of Medicine, Beijing 100853, PR China

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ABSTRACT

Micelles with high *in vivo* serum stability and intratumor accumulation post intravenous (*i.v.*) injection are highly desired for promoting chemotherapy. Herein, we finely synthesized and tailored well-defined anti-Her2 antibody Fab fragment conjugated immunomicelles (FCIMs), which showed interesting dual targeting function. The thermosensitive poly(N-isopropylacrylamide-*co*-N,N-dimethylacrylamide)$_{118}$ (PID_{118}) shell with volume phase transition temperature (VPTT: 39 °C) and the anchored anti-Her2 Fab moiety contributed to the passive and active targeting, respectively. The doxorubicin (DOX) loading capacity of such FCIMs was successfully increased about 2 times by physically enhanced hydrophobicity of inner reservoir without structural deformation. The cellular uptake and intracellular accumulation of DOX by temperature regulated passive and antibody navigated active targeting was 4 times of Doxil. The cytotoxicity assay against Her2 overexpression gastric cancer cells (N87s) showed that the IC50 of the FCIMs was ~9 times lower than that of Doxil under cooperatively targeting by Fab at $T >$ VPTT. FCIMs showed high serum stability by increasing the corona PID_{118} chain density (S_{corona}/N_{agg}). *In vivo* tissue distribution was evaluated in Balb/c nude mice bearing gastric cancer. As observed by the IVIS® imaging system, the intratumor accumulation of such finely tailored FCIMs system was obviously promoted 24 h post *i.v.* administration. Due to the high stability and super-targeting, the *in vivo* xenografted gastric tumor growth was significantly inhibited with relative tumor volume <2 which was much smaller than ~5 of the control. Consequently, such finely tailored FCIMs with anti-Her2 active and temperature regulated passive dual tumor-targeting function show high potent in chemotherapy.

1. Introduction

Block copolymer micelles with well-defined core-corona structure provide a unique and powerful nanoplatform for drug delivery in chemotherapy for overcoming the traditional formulation's drawbacks such as the side toxicity, *in vivo* instability and the fast clearance in the circulation [1–5]. The hydrophobic core can provide a natural hydrophobic environment that allows easy encapsulation of poorly soluble anticancer drugs via the similar-to-similar interaction. On the other hand, the densely packed hydrophilic corona-forming chain can protect micellar system from the reticuloendothelial system (RES) by reducing the interaction with serum proteins and renal filtration [6–8]. Additionally, the size of polymeric micelles, 10–100 nm, can be easily regulated by varying the block compositions of the amphiphilic copolymer. The unique physiochemical properties and tunable size increased micelles preferentially accumulate in solid tumor through the enhanced permeability and retention (EPR) effects [9,10].

Despite many advantages of block copolymer micelles for *in vitro*/*vivo* applications, several challenges still exist for translating the micellar drug delivery system to clinical application. For example, the small micellar size of 10–100 nm limits the amount of drug that can be incorporated inside the core and the premature release prior to the micelle reaching its intended site of action. Although chemical conjugation strategies increased compatibility of drug in the micelle core, the aggregation number (N_{agg}) of polymer chains inside one micelle can not be changed at

* Corresponding authors. International Joint Cancer Institute, The Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, PR China. Tel./fax: +86 21 81870801.
E-mail addresses: liwei.dds@gmail.com (W. Li), yjguo@smmu.edu.cn (Y. Guo).
[1] The authors contributed equally.

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a given block copolymer composition which still limited the maximum of drug loading content [11,12]. On the contrary, it is known that drug loading and entrapment efficiency depend on drug solubility in the core-forming matrix material including the core-forming polymer molecular weight and the matrix composition [13,14]. The noncovalent encapsulation strategy makes it feasible to entrap drugs without structural deformation by regulating the core compositions. The biodegradable polymers such as poly(D,L-lactic-co-glycolic acid) has been commonly used to enhance entrapping of therapeutic compounds [15,16]. This alternative ideal method for improving drug loading is attributed to the increase of similar-to-similar interaction between the drug and hydrophobic core-forming polymers or the hydrophobic enhancers [17].

In addition to the high drug loading capacity, the specific *in vivo* accumulation of the micelle–dug complex in the tumor tissue is another big issue for efficient drug delivery. Specific targeting strategy can lower the cargo *in vivo* circulation time which limits the leakage and RES [18]. Such specific delivery at target site can be realized by surface decorating with ligands such as antibodies or peptides that specifically bind to biomarkers overexpressed on cancer cells [19]. Among all specific ligands, antibodies provide the broadest opportunities in terms of diversity of targets and high antibody–antigen affinity. Covalently anchored an antibody onto polymeric micelles, that is, the immunomicelles, can improve drug at desired site [20,21]. Her2 is a member of tyrosine specific protein kinase family consists of four EGF receptors i.e. EGFR (ErbB1), ErbB2 (Her2), ErbB3, and ErbB4 which contain a cytoplasmic tyrosine kinase domain, a single transmembrane domain, and an extracellular domain that is involved in ligand binding and receptor dimerisation [22–24]. The overexpression of Her2 at stomach cancer cells (N87s) surface may lead to potentiate dysregulated growth, angiogenesis, metastasis and resistance against apoptosis-inducing therapeutic agents [25].

Even if large amount of cargos safely arrive at the tumor site, successful therapy requires transferring the systems into the cells for realizing the therapeutic effect. It is known that the endothelial cells of the tumor blood vessels proliferate at a 30–40-fold higher rate than those in normal tissues, which results in the larger endothelial cells gaps (200–700 nm, or sometimes even larger, up to 1.2 μm) than 7 nm in the normal tissue. Additionally, the high metabolism of tumor cells requires much more oxygen, nutrients, gas exchange, and waste removal. But the heterogeneous structure and distribution of the tumor blood vessels as well as the blood capillaries slows down the energy exchanging between intra- and extra-tumor. All these result in unique characteristics of tumor, that is, the unmoral tumor blood vessels with gap in 200–700 nm [26], the relative high temperature of tumor ($T > 37$ °C) [27] and the relative low pH (5 ~ 6) [28]. Such unique characteristics of solid tumors offered new challenge and opportunities for fabricating micelles with properties such as pH, temperature and reductive conditions [3,29–31]. In our previous study, a thermosensitive block copolymer micelle with poly (N-isopropylacrylomide) (PNIPAM) copolymer (PID_{118}) shell was finely fabricated. The hydrophilic to hydrophobic transition as $T >$ VPTT of corona significantly enhanced the intracellular uptake [32].

In order to promote the *in vivo* tumor inhibition, ideal polymeric micellar delivery system should have a simple composition with high drug loading capacity, long circulation in blood, specific tumor cellular affinity. In this study, a thermosensitive block copolymer PID_{118}-*b*-PLA_{71} was finely synthesized with tunable VPTT ~ 39 °C. The micellar formation was finely regulated and tailored by the selfassembly process with the aid of laser light scattering (LLS) and TEM. The hydrophobic biodegradable PLGA was physically condensed into the micellar core as the drug loading reservoir. Then the anti-Her2 Fabs were conjugated onto the micellar surface to form a Fab conjugated immunomicelles (FCIMs). It is expected that the both the Fab and temperature could be used to regulate intracellular uptake and intratumor accumulation.

2. Material and methods

2.1. Materials

The randomly copolymer macromolecular RAFT agent (*phenyl-PID_{118}-OH*, the compound 1 with phenyl end group as marked in the synthesis process) containing N-isopropylacrylomide (*NIPAM*) and N,N′-dimethylacrylamide (*DMAAm*) was a gift from the Prof. Okano's lab (Tokyo Women's Medical University, Tokyo, Japan). D,L-Lactide (*LA*) (Aldrich) was recrystallized from ethyl acetate. Dimethyl sulfoxide (*DMSO*), tetrahydrofuran (*THF*), N,N-dimethylacetamide (*DMAc*), xylene, diethyl ether, sodium thiosulfate ($Na_2S_2O_4$), Na_2HPO_4, NaH_2PO_4, hydrodated phosphotungstate (*PTA*), 4% paraformaldehyde and highly purified 1,4-dioxane were obtained from Wako Pure Chemicals and were used without further purification. PLGA (26,000 g/mol), Maleimide (*Mal*) (Aldrich), 2-hydroxyethylamine (Kanto Chemical, Tokyo), tin(II) 2-eth-ylhexanoate (Aldrich), 2-Ethanolamine (Kanto Chemical Co. Inc., Tokyo, Japan) were used as received. Maleimide derivatized PEG2000-DSPE (*Mal-PEG-DSPE*) purchased from Avanti Polar Lipids was used without any purification. Dulbecco's phosphate-buffered saline (*PBS*) and Albumin from bovine serum (*BAS*, minimum 96%, Sigma), the DOX and commercial DOX formulation Doxil® (Changhai Hospital, Shanghai) were used as received. Water used in terms of resistivity ~18.2 MΩ cm in this study was purified by a Milli-Q Synthesis A10 system (Millipore, Billerica, MA) unless otherwise mentioned.

2.2. Block copolymer synthesis and characterization

2.2.1. Aminolysis and conversion of polymer termini

Fig. 1(a) shows the end group conversion and synthesis of block copolymers. The first step is conversion of dithiobenzoate end group to the hydrophilic amine group. 0.015 mmol phenyl-PID_{118}-OH, 2 mol equivalents $Na_2S_2O_4$ and 40 mol equivalents of maleimide (*vs.* terminal groups) were dissolved in 5 mL THF predeoxidized by N_2 for 2 h 2-Ethanolamine (20 mol equivalents *vs.* terminal dithiobenzoate groups) in 1 mL pre-deoxidized THF was slowly dropped into the polymer solution under N_2 bubbling following by 23 h reaction at room temperature and dark. After reaction, the solution dialysis against Milli-Q water (resistivity of 18.2 MΩ, Millipore, CA) by membrane (MWCO 1000, Spectra/Pro 6, Spectrum Medical Industries, Los Angeles, CA) with water exchanging until removal most of the unreacted chemicals and the organic solvent THF. Then the final white product was recovered by freeze-drying (Mal-PID_{118}-OH, compound 2 with maleimide end group) [32,33].

2.2.2. PID_{118}-PLA_{71} diblock copolymers synthesis and characterization

The block copolymers with hydrophobic block length 71 was synthesized using mal-PID_{118}-OH as the macro-CTA via the conventional ring open polymerization (*ROP*) as shown in the second of Fig. 1(a). In the ROP process, proportional calculated amount of monomers were weighted, the PID_{118} (0.75 g, 6×10^{-5} mol), D,L-lactide (0.75 g, 15 mM) were mixed in a 25 mL nash-flask connected with a condenser. The mixed powder was dried at 25 °C under vacuum for about 3 h. Then 5.0 mL pre-degassed xylene was injected into each flask under N_2. The imitator tin(II) 2-eth-ylhexanoate (40 mg) was pre-dissolved in about 1.0 mL pre-degassed xylene and was injected in to the monomer solution under N_2. The polymerization was carried out at 130 °C for about 22 h. After polymerization, the solution was distillated at 50 °C under vacuum for removing the xylene. Then about 5 mL THF was added to the flask to form a solution with a little black precipitate, which was removed by centrifugation with 800 rpm and 10 min. The up clear solution was collected and precipitated in excess diethyl ether two times. The pale powder (compound 3) was obtained under vacuum drying [32]. The block copolymer composition was calculated from the characteristic signal in ^1H NMR (400 MHz, Varian Inc.) using chloroform-D ($CDCl_3$) as solvent as shown in Fig. 1(b).

2.3. Fabrication of antibody decorated immunomicelles

2.3.1. Formation of micellar complex

Fig. 1(c) shows the process of the micelle (IMs) complex formation. The micelles complex, drug loaded micelles and QD loaded micelles were prepared by dialysis method as mentioned in our previous publications [32,34]. The formation of micelles with reactive group on their surface was used as a sample: about 10 mg PID_{118}-b-PLA_{71} diblock copolymer and about 10% (weight percentage to the polymer) of MAL-PEG-DSPE were mixed together. This mixture was dissolved in 1.5 mL DMAc for about 2 h. Then the DMAC solution was dialyzed against Milli-Q water using the dialysis membrane (MWCO 1000, Spectra/Pro 6, Spectrum Medical