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

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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 (PID118) 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 DOX by temperature regulated passive dual tumor-targeting function show high potent in chemotherapy.

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1. Introduction

Block copolymer micelles with well-defined core-corona structure provide a unique and powerful nanoplatinform 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 (Nagg) of polymer chains inside one micelle can not be changed at
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 polylactic-co-glycolic acid has been commonly used to enhance entrapment 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–drug complex in the tumor tissue is another big issue for efficient drug delivery. Specific targeting strategy can lower the cargo rate than those in normal tissues, which results in the larger dimerisation [22] polymeric micelles, that is, the immunomicelles, can improve drug delivery at target site can be achieved by functionalizing the micellar surface to form a Fab conjugated immunomicelles (FCMs). 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-PID118-OH, the compound 1 with phenyl end group as marked in the synthesis process) containing N-isopropylacrylamide (NIPAM) and N,N-dimethylacrylamide (DMAAm) was a gift from the Prof. Okano’s lab (Tokyo Women’s Medical University, Tokyo, Japan). α-Lactide (LA) (Aldrich) was recrystallized from ethyl acetate. Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), N,N-dimethylecetamide (DMac), xylene, diethyl ether, sodium thiosulfate (Na2S2O4), Na2HPO4, NaH2PO4, hydroxyl phosphoglucose (PTA), 4% paraformdehyde 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-hydroxyethylmethacrylate (Kanto Chemical, Tokyo), tin(II) 2-ethyl-hexanoate (Aldrich), 2-Ethanolamine (Kanto Chemical Co., Inc., Tokyo, Japan) were used as received. Maleimide derivatized PEG2000-DSPE (Male-PEG-DSPE) purchased from American Polarg 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® (Changhui 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-PID118-OH, 2 mol equivalents Na2S2O4 and 40 mol equivalents of maleimide (vs. terminal groups) were dissolved in 5 mL THF pre-degassed by N2 for 2 h. 2-Ethanolamine (20 mol equivalents vs. terminal dithiobenzoate groups) in 1 mL pre-deoxygenized THF was slowly dropped into the polymer solution under N2 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 10000, Spectra/Por 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 (Male-PID118-OH, compound 2 with maleimide end group) [32,33].

2.2.2. PID118-PLA7 diblock copolymers synthesis and characterization

The block copolymers with hydrophobic block length 71 was synthesized using male-PID118-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 weighed, the PID118 (0.75 g, 6 × 10⁻⁷ mol) and N-isopropylacrylamide (NIPAM) copolymer (PID118) shell was finely fabricated. The hydrophilic to hydrophobic transition as Tₘ > 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 micelle with poly (N-isopropylacrylamide) (PNIPAM) copolymer (PID118) comprising poly (N-isopropylacrylamide) (PNIPAM) copolymer (PID118) shell was finely fabricated. The hydrophilic to hydrophobic transition at Tₘ > VPTT of corona significantly enhanced the intracellular uptake [32].

2.3. Fabrication of antibody decorated immunomicelles

2.3.1. Formation of micellar complex

Fig. 1(c) shows the process of the micelle (Ms) complex formation. The micelles complex, drug loaded micelles and QS 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 PID118-b-PLA7 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/Por 6, Spectrum Medical
Industries, Los Angeles, CA) at 4 °C for about 3 days with regularly water changing. The final concentration of the reactive P(DAm-b-PLA71) micellar solutions was about 1.8 mg/mL by weighing method.

For optimizing the micellar size and structure, some other solvents including DMSO, THF, AC and AN were used in the dialysis method. In addition to the dialysis methods, we also used the rotation evaporation methods to prepare the micelles. A proportional amount of polymers (P(DAm-b-PLA71), PLGA and/or MAL-PEG-DSPC) were dissolved in 10 mL chloroform. Then the chloroform was evaporated under N2 to form a thin polymer film. The polymers were then eluted by Milli-Q water or PBS at temperature below/above the volume phase transition temperature (VPTT) of P(DAm-b-PLA71), namely, T_solution > VPTT or T_solution < VPTT.

2.3.2. The reactive anti-Her2 Fab fragment preparation

As shown in Fig. 1(d), the Fab fragment from anti-Her2 monoclonal antibody (mAb) was prepared by our group as reported previously [35]. Briefly, about 10 mg/mL mAb (0.1 M acetate buffer, pH 4.5) was incubated with 0.25 mg/mL of pepsin at temperature for about one day with regularly water exchanging resulting in the Fab fragment. Then this Fab was reduced with 20 mM dithiothreitol for about 1.5 h under argon at room temperature. The impurity was removed by Sephadex G-150 column (Pharmacia) which was pre-equilibrated by the buffer 1 (0.1 M NaCl, 0.1 M Tris, 0.05 M citrate and 2 mM EDTA, pH 5.5). Such Fab(2) solution was concentrated to 10 mg/mL and was further digested by the enzyme papain. The Fab fragment solution was purified by the same procedure as mentioned above resulting in the single Fab fragment. Then this Fab was reduced with 20 mM dithiothreitol for about 1.5 h under argon at room temperature. The impurity was removed by Sephadex G-150 column (Pharmacia) pre-equilibrated by the deoxygenated buffer 1. Then the solution was dialedysis against the 50 mM HEPES (pH 7.4) resulting in the final reactive targeting moiety Fab—SH which was stored at 4 °C and N2 environment for future use.

2.3.3. Fabrication of the anti-Her2 Fab conjugated immunomicelles

The anti-Her2 Fab conjugated immunomicelles (FCMIs) were then prepared by coupling the reactive Fab—SH fragment onto the micellar surface via the reaction between the —SH and Male group under room temperature and N2 for about 8 h. After the reaction, the un-conjugated Fabs were removed by dialysis method. Namely, the mixture was dialyzed against the Milli-Q water by the dialysis membrane (MWCO 100000, Spectrum/Pro 6, Spectrum Medical Industries, Los Angeles, CA) at room temperature for about one day with regularly water exchanging resulting in the Fab conjugated immunomicelles shown in Fig. 1(e).

2.4. Characterization of the immunomicelles

2.4.1. Size distribution and morphology by LLS and TEM

The hydrodynamic diameter (D) and size distribution (PD) were determined by ZetaSizer (Nano-ZS, Malvern Instruments, Worcestershire, UK) equipped with a He–Ne laser (633 nm) at the scattering angle 173°. The stock micelle solution was diluted by MilliQ-H2O with concentration ∼0.6 mg/mL. Such diluted micellar solution was filtrated by a hydrophilic membrane 0.22-μm Millipore filters. To prepare stained specimens for the typical TEM (Hitachi, H-7000 Electron Microscope) experiments, about 5 μL micellar solution with a concentration 1.8 mg/mL was dropped on 200-mesh Formvar-free carbon-coated copper grids (Ted Pella Type-A; nominal carbon thickness 2–3 nm). After the water evaporating by exposing the sample to air at room temperature, the sample was inversely covered on a small drop of hydrated phosphotungstic acid (PTA) solution with a mass fraction of 2%. The conventional TEM images were obtained at 100 kV.

2.4.2. Surface chemistry

XPS experiments were carried out on an RBD upgraded PHI-5000 ESCA system (Perkin Elmer) with Mg Kα radiation (hν = 1253.6 eV). In general, the X-ray anode was run at 250 W and the high voltage was kept at 14.0 kV with a detection angle at 54°. The pass energy was fixed at 23.5, 46.95 or 93.90 eV to ensure sufficient resolution and sensitivity. The base pressure of the analyzer chamber was about 5 × 10^-9 Pa. About 10 mg IMs or FCMIs powder was directly pressed to a self-supported disk (10 × 10 mm) and mounted on a sample holder then transferred into the analyzer chamber. The whole spectra (0 – 1100/1200 eV) and the narrow spectra of all the elements with much high resolution were both recorded by using RBD 147 interface (RBD Enterprises, USA) through the AugerScan 3.21 software. Binding energies were calibrated by using the containment carbon (C1s = 284.6 eV). The data analysis was carried out by using the RBD AugerScan 3.21 software provided by RBD Enterprises or XPSPeak4.1 provided by Raymund W.M. Kwok (The Chinese University of Hongkong, China).

2.4.3. Temperature sensitive properties

UV (UV–VIS spectrometer, Cary-100, Varian) fitted with temperature and stirring controller was used to monitor the sample absorption profile at wavelength 300 nm. The incident light passes through the center of sample cell fitted with a thermometer. About 4 mL (C = 2 mg/mL) micelle solution tuned by PBS (pH 7.4) was added to a UV cell with a stirring bar and located on the cell holder. The VPTT of the micelle solution was defined as the absorption of the micellar solution sharply increased to 50%.
2.5. ADR loading and releasing profile

2.5.1. Preparation of DOX-loaded immunomicelles

About 12 mg PID118-b-PLA71 block copolymer was weighted and dissolved in DMAC with a concentration around 2 mg/mL. The DOX was also dissolved in DMAC with a concentration about 2 mg/mL. Then prepare a TEA solution with a 1.5 molar equivalents to the DOX in DMAC, and diluted it to 10 times by DMAC. The TEA solution was slowly dropped to the DOX solution and kept stirring for about 5 min. Then the DOX and polymer was mixed with a volume ratio 5/5 followed by 2 min stirring. The mixture was dialyzed against Milli-Q water in dark at room temperature. The dialysis was continuing for about 1 day with regularly water changing.

The FCIMs—DOX complex was collected and stocked for further use. Its concentration and loading content were measured by UV at 480 nm and calculated by the following function:

\[
\text{Loading content} \% = \left( \frac{W_{\text{DOX inside FCIMs}}}{W_{\text{FCIMs}}} \right) \times 100
\]

2.5.2. In vitro DOX releasing profile

A dialysis bag (Special dialysis membrane, MWCO 1000) containing about 3.0 mL FCIMs—DOX solution was put in a beaker with 500 mL Milli-Q water tunned by Dulbecco’s phosphate-buffered saline (PBS) at pH 7.4. The beaker was fixed in a water bath kept at 37 °C with continuous stirring. The initial DOX concentration in the micelle is about 0.5 mg/mL. The DOX—FCIMs mixture was continuing dialysis against with the PBS buffer solution. About 0.5 mL PBS solution outside the dialysis bag was sampled at the specific time intervals. Subsequently, 0.5 mL AN was added into the collected solution and analyzed by UV to determine the accumulated drug release profile.

2.6. Cell culture

Cell culture: the human stomach cancer cell N87 which overexpressed Her2 antigen, cultured in the medium: Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% fetal bovine serum (FBS), 50 unit/mL penicillin, and 50 µg/mL streptomycin at 37 °C with 5% CO2. Before all the cell line experiments, the cells were pre-cultured overnight until confluence was reached to 75%.

2.7. Cytotoxicity evaluation of the micelles

N87 cells were seeded into 96-well microplates (100 µL/well, 3.0 × 10^4 cells/mL) and cultured for 12 h. Then N87s were incubated with the IMs and FCIMs micelles with concentration 0.125, 0.25, 0.5 and 1.0 mg/mL in DMEM/FBS for 36 h at 37 °C and 4% O2. The N87s were gently rinsed with DMEM/FBS twice. The media were replaced with 100 µL of Cell Counting Kit-8, following by 2.5 h incubation. The absorbance was measured at 450 nm using a microplate spectrophotometer to check the cells survival profile.

2.8. Cellular uptake and internalization by flow cytometer and fluorescent microscopy

N87 cells were seeded into 24-well microplate (0.5 mL/well, 2 × 10^5 cells/mL) and cultured for about overnight till the cells reached 70% confluence. Then N87s were incubated with Doxil, IMs—DOX FCIMs and FCIMs—DOX complex solutions for different times at 37 °C and 4% O2 for about 6 h. Then the N87s were gently rinsed with DMEM/FBS twice. The media were piped out and use trypsin to digest the cell for about 3 min. The N87s were rinsed by PBS and centrifuged twice with 800 rpm and 5 min. Finally the flow cytometry was used to check the fluorescence intensity of IMs—DOX and FCIMs—DOX. The FITC labeled anti anti-Her2 antibody (FITC-Ig) was used to evaluate cellular uptake of FCIMs. This FITC-Ig specifically attached to the Fab on the cell surface. After the FITC-Ig labeling, the FCIMs sample was also viewed by inverse fluorescent microscopy and checked by the flow cytometer.

The time dependence of cellular internalization of FCIMs was studied using FITC and QD as indicator. About 2 mg PID118-b-PLA71 block copolymer was dissolved in DMAC with a concentration around 2 mg/mL. Then about 100 µL of the mixture was mixed with 100 µL of cell suspension. The mixture was added on Millicell-Q water at room temperature under dark conditions for about 12 h resulting QD loaded micellar solutions. For the FCIMs case, the mixture was dried by lyophilization. The micellar solutions with gently stirring for about 8 h. Both the IMs and FCIMs solutions were loaded with 4% paraformaldehyde. The samples were visualized by TCS SP confocal laser scanning microscopy (Leica, Germany). For the blank FCIMs sample, an FITC labeled anti anti-Her2 antibody (FITC-Ig) was attached to the Fab on the cell surface. After the FITC-Ig labeling, the FCIMs can be viewed by CLSM.

2.9. In vitro cytotoxicity assay

For cytotoxicity measurement, N87 cells were cultivated in 96-well transparent plates (5000 cells/well) and incubated for overnight at 37 °C. The old medium was removed and the cells were incubated with Doxil in DMEM, IMs—DOX and FCIMs—DOX with different polymer concentration at the equivalent drug concentration. Kit-8 assay was used to measure the cell viability at the given time. The absorption of the samples in each well was measured by the microplate reader with wavelength 490 nm. The percentage of surviving cells was calculated according to the following equation [26]:

\[
\text{Surviving cells} = \left( \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right) \times 100
\]

where the A_sample, A_blank and A_control are UV absorption at 485 nm from cell incubated with immunomicelles, the culture medium and the cell without micelles.

2.10. Confocal laser scanning microscopy (CLSM)

The IMs—DOX, FCIMs and FCIMs—DOX were prepared by the dialysis method as mentioned above. N87 cells were seeded into special dish for CLSM (0.5 mL/well, 2 × 10^5 cells/mL) and cultured for about overnight till the cells reached 70% confluence. Then N87s were incubated with IMs—DOX, FCIMs and FCIMs—DOX for about 6 h at 37 °C and 4% O2. Then the N87s were gently rinsed with DMEM/FBS twice and fixed with 4% paraformaldehyde. Samples were visualized by TCS SP confocal laser scanning microscopy (Leica, Germany). For the blank FCIMs sample, a FITC labeled anti anti-Her2 antibody (FITC-Ig) was attached to the Fab on the cell surface. After the FITC-Ig labeling, the FCIMs can be viewed by CLSM.

2.11. Serum stability evaluation by LLS

For evaluating effect of the hydrophilic corona chain PID118 content on the FCIMs stability, we prepared two FCIMs solution with PID118-b-PLA71 content of 2.4 mg and 1.4 mg. The FCIMs micellar solution was prepared as mentioned above. An Alumina from bovine serum (BAS, minimum 96%) aqueous solution was prepared in PBS with a concentration about 50 mg/mL. Then the FCIMs solution was mixed with BSA solution at the same volume resulting in a FCIMs and BSA mixture. The LLS was used to measure the size (diameter) and size distribution profile of BSA, FCIMs and their mixture respectively.

2.12. Animal studies

The experimental animals (Balb/c nude mice, female, 4 – 6 weeks, ~20 g) were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, P. R. China). Balb/c nude mice were maintained in a pathogen-free environment and allowed to acclimate for at least one week before tumor implantation. All studies were performed in accordance to the guideline of the Committee on Animals of the Second Military Medical University, Shanghai, PR China.

2.13. Tumor implantation

The Balb/c nude mice were inoculated subcutaneously on the right back with 5 × 10^6 N87 cells (in 100 µL culture medium) to develop xenografts tumor. After about 2 weeks, the volume of tumors reached about 50 mm^3. The successful tumor xenograft model was demonstrated by tumor luminescent images. Briefly, mice were given an intraperitoneal injection of luciferin (Promega) at a dose of 150 mg/kg. The tumor xenograft model was viewed by IVIS® Lumina II Imaging System (Xenogen), which was taken to capture the visible light photograph and luminescent image (Supplementary Fig. 1). As shown in Supplementary Fig. 1, immunofluorescence revealed a high EGFR expression in MDA-MB-231 tumor tissues, indicating that the tumor was successfully xenografted in vivo.


In the study of in vivo distribution, mice bearing N87 tumors were randomly assigned to 2 groups with 3 mice/group. Targeting and non-targeting micelles (FCIMs, IMs) loading fluorescein isothiocyanate (FITC) with equivalent to 5 mg/kg dosage were administered via tail vein. 24 h later, the mice were anaesthetized by 1.5% isoflurane in 1:2 O2/N2. Noted here, the mice with FITC administration at 0 h were used as control. The in vivo images were observed with IVIS® imaging system (excitation 500 nm) and recorded by a built-in CCD camera. Mice were sacrificed after 24 h and the excised heart, liver, kidney, tumor and spleen. These organs were also imaged with the same excitation wavelength. Then the organs were collected and immediately were fixed in formalin 1 h. The organs were frozen in tissue-Tek-OCT and cryosections. Frozen sections were cut at 10 µm sections and fixed with acetone at ~20 °C. After washing with PBS, sections were counterstained with 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, Fluka Chemie, Buchs, Israel) and visualized by the inverse fluorescent microscopy.

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2.15. Antitumor activity assay

Bald/c nude mice were inoculated subcutaneously with N87 cells (5 × 10⁶ cells in 100 μl of PBS). Tumors were allowed to grow for 2 weeks to reach proliferative phase (~50 mm³). Then the mice were randomly assigned to 4 groups: 5 mice/group (for the control, the DOX, the targeting and non-targeting, respectively). Subsequently, 100 μl saline, free DOX in PBS, FCIMs—DOX and IMs—DOX complex solution (5 mg/kg/mouse) were injected into the tail vein 3 times at 3-day intervals. Tumor size was recorded every 2 days by a digital vernier caliper. The tumor volume (V) was calculated according to the following equation [36]:

\[ V_{tumor} = L W^2 / 2 \]  

(3)

where L and W are the longest and shortest diameters. Tumor progression was evaluated in terms of relative tumor volume (to day 0) over a period of 21 days.

2.16. Data analysis

Data were accumulated and expressed as the means with 95% confidence intervals. The statistical analysis of the samples was undertaken using a Student’s t-test, and p-values of 0.05 were considered statistically significant.

3. Results and discussion

3.1. Polymer synthesis and fabrication of FCIMs

To obtain amphiphilic block copolymer with tunable block lengths, the reversible addition fragmentation chain transfer polymerization (RAFT) which is the well-known living radical polymerization for control of molecular weight and applicable to a wide range of monomers under various experimental, was used in this study [34]. In our previous work, the amphiphilic poly(N-isopropylacrylamide-co-NN-dimethylacrylamide)-b-poly(L-lactic acid)₅₉ (PID₁₁₈-b-PLA₇₁) block copolymer was synthesized. And the drug loading content by the corresponding micelle was about 4%, which was similar to that as reported by others. But this loading content is still not so high. The drug loading content is affected by the hydrophobic block length. In order to increase the drug loading capacity, the block length of hydrophobic segment in our current study was increased to 71 by increasing the hydrophobic monomer ratio. The synthesis process of PID₁₁₈-b-PLA₇₁ was shown in Fig. 1(a). The end group of macromolecular RAFT agent phenyl-PID₁₁₈-OH (compound 1 present from Porf. Teruo Okano) was firstly converted to a reactive end group under dark condition for about one day resulting in the compound 2 Mal-PID₁₁₈-OH. Then the –OH end group was utilized to initiate the ring open polymerization (ROP) of lactic acid (LA) catalyzed by the salt Sn(Oct)₂. After reacted at the 130 °C for about 22 h, the resulting amphiphilic block copolymer PID₁₈₈-b-PLA₇₁ (compound 3) was obtained.

As shown in Fig. 1(b), the molecular weight (Mₘ) and composition of such thermosensitive biodegradable block copolymer PID₁₁₈-b-PLA₇₁ was characterized by the ¹H NMR (400 MHz, Varian Inc.) using chloroform-D (CDCl₃) as solvent. The characteristic signal at ~4.0 ppm was from –CH(CH₃)₂ of PNIPAM. Before end group modification, the signal of ~7.4 ppm, ~7.6 ppm and ~7.9 ppm confirmed the dithiobenzato group of phenyl-PID₁₁₈-OH. The signal of such phenyl group disappeared as it was converted to Mal group (the data was not shown here). Also shown in Fig. 1(b), we can find the characteristic peaks of PLA is around ~5.2 ppm form –CH₂CH₂–. The LA monomer unit was about 71 by the NMR signal of –CH(CH₃)₂ and –CH₂CH₂–. So, combining the living radical polymerization (RAFT) and ring open polymerization (ROP), the thermosensitive biodegradable amphiphilic block copolymer PID₁₁₈-b-PLA₇₁ was successfully prepared.

Fig. 1(c) shows the thermodynamic and dynamic conditions for spherical micelle formation in aqueous solution. It is known that the driving force of selfassembly is the direct solubility difference between hydrophilic and hydrophobic blocks in aqueous solvent [37]. The determining factor is the solubility parameter (β in the Flory interaction parameter as shown in following section) of hydrophilic and hydrophobic blocks. In addition, the difference of β between PID and PLA in water resulted in the volume difference of the hydrophilic head and the hydrophobic tail in water. This difference was described by the packing parameter β.

\[ \beta = V_H / L_C A_0 \]

(4)

where the Vₜ, L_C and A₀ are the volume occupied by the hydrophobic chain, the hydrophobic chain counter length and the surface area of hydrophilic chain, respectively [38]. Normally, the morphology of the polymeric aggregates changes as the β value such as spherical micelle (0 < β < 1/3), rod-like micelle (1/3 < β < 1/2), vesicle (1/2 < β < 1), and finally the planar bilayer (β > 1). The condition for spherical micelle formation is that the packing parameter β should be in the range of 0–1/3. For our previous case, the β of PID₁₁₈-b-PLA₅₉ was smaller than 0.1. In this case, the hydrophobic block length of PID₁₁₈-b-PLA₇₁ was a little longer that the 59 with little changes of β value, which indicated the core-corona spherical structural micelles [32,39]. So PID₁₁₈-b-PLA₇₁ can form a spherical structure micelle in aqueous solution from the point of thermodynamic and dynamic conditions.

It is known that the molecular weight of micelle is around 1.8 × 10⁵, which is very large comparing with that of block copolymer. On the other hand, the targeting function moiety mainly located on the Fab zone of antibody. In order to avoid the structure deformation generated from the antibody decorating, Fab (Mₚ ~ 45,000 g/mol) was prepared and functionalized as shown in Fig. 1(d). By the conventional pepsin and papain enzyme digestion, an anti-Her2 antibody Fab was easily obtained in our group. Shortly, the enzyme papain was used to cleave an immunoglobulin monomer into two Fab fragments (F(ab)₂) and an Fc fragment. The enzyme pepsin was further used to cleaves below hinge region of F(ab)₂ fragment resulting in a Fab fragment. As mentioned in the experimental section, this thiol group was then modified onto the Fab resulting in reactive Fab segment Fab–SH. The –SH group was easily coupled with maleimide group on the micellar surface at a mild condition. The un-conjugated Fab fragment was successfully separated from the FCIMs system by dialysis. By such systemically decorating, an anti-Her2 Fab conjugated immunoemacle can be prepared as shown in Fig. 1(e). The targeting moiety anchored on the micellar surface for the Her2 overexpressed stomach cancer cells N87. The hydrophilic thermosensitive PID shell can stabilize the FCIMs in water, suppress the RES system capturing and enhance the intracellular uptake. The hydrophobic core swollen by the PLGA can entrap hydrophobic anticancer drug DOX and some fluorescence probe like QD. Such multifunctional FCIMs are expected to improve the anticancer therapy.

3.2. Micellar structure and morphology tailoring

It is known that the size and size distribution of micelles strongly affect the micellar in vitro/vivo performance. In order to optimize the micellar structure for the drug loading, releasing and stability, we further systemically investigate the effects of solvent quality and selfassembly conditions on the micellar formation. Fig. 2(a) shows the effects of solvent quality on the micellar size. In this case, the polymers were dissolved in organic solvent following by the dialysis against water. It was found that the size of the micelles decrease with the trend as: DMF > DMSO > DMAC > AN > DMSO > AC > THF. As we known that the driving force of the selfassembly is the solubility difference between the hydrophilic and hydrophobic blocks [40]. At a given block copolymer composition, the size thus depended on the solvent.
quality which indicated that the organic solvent’s polarity has some effects on the micellar size [41]. This is reasonable because the higher organic solvent polarity indicates its higher solubility in water. The parameter of normalized solvent polarity ($E^N_T$) of H$_2$O is 1.0. $E^N_T$ of DMSO is about 0.444, but for THF it is 0.207 [42]. Low $E^N_T$ means the interaction between the hydrophobic block and organic solvent is strong. From the point of dynamic for assembly, it will take some more time for the solvent diffusion out for the low $E^N_T$ solvent. The time for the in-suit organic/aqueous solvent mixing for high $E^N_T$ solvent was shorter than that of low $E^N_T$ solvent. The polymer chain has enough time to selfassembly. The large size of micelles was similar to some large aggregates formed in the kinetically frozen process [43].

In addition to the dialysis method, we also used the rotation evaporation method to prepare the IMs as shown in Fig. 2(b). Due to the shell-forming polymer PID118 is a thermosensitive polymer, we further investigate the effect of elution temperature on the micellar formation. It was found that the size of IMs eluted at $T > VPTT$ was relatively larger than that eluted at $T < VPTT$. The size increase trend appeared both in water and PBS solution. It is helpful to know that PID chain will change from hydrophilic to hydrophobic as $T > VPTT$. This hydrophilic/hydrophobic transition enhanced the hydrophobicity of the block copolymer. Such hydrophobicity increase led to the aggregation number increase or some inter-micellar aggregation resulting in the large size [44]. The size plays an important role in the drug delivery in vitro/vivo. From the above experiments, we can easily tailor the micellar size as needed by regulating the solvent quality or the micellar formation temperature.

Fig. 2(c) shows the size and size distribution of the IMs, IMs-PLGA and FCIMs obtained by the rotation evaporation method. It was found that the size of empty IMs was about 100 nm. As the PLGA was entrapped into the hydrophobic core, the micellar size has little change. However, after the antibody decorating, the size becomes smaller than that of IMs. This hydrophilic Fab segment surrounding the micellar surface increased the hydrophilicity of the FCIMs, which resulted in the size decrease. Fig. 2(d) shows the morphology of the IMs and FCIMs. Both the spherical morphology from TEM and the similar size obtained from LLS clearly confirmed that the FCIMs were successfully prepared in our experiments. The micellar size and well-defined core-shell structure can be easily tailored from the above mentioned methods.

3.3. Properties of the FCIMs

The FCIMs with targeting function and temperature sensitive properties were then investigated as shown in Fig. 3. The antibody successfully anchored on the micellar surface was confirmed by XPS Fig. 3(a) and FITC-Ig labeling Fig. 3(b). Both IMs and FCIMs has similar signal in the range of binding energy from 150 eV to
In the range from 130 eV to 150 eV, which is special binding energy range for P(2p3), the signal appeared in FCIMs as shown in the insert Figure. The P signal came from the nucleic acid of Fab. Additionally, we used the FCIMs to incubate with N87 cells, and then used the FITC-Ig antibody to label the system after rinse the cell. The green color generated from FITC surrounding the cells was attributed to the FITC-Ig binding on the Fab shown in Fig. 3(b). Both the characteristic XPS peak of Fab and the confocal laser scanning confirmed that the Fab was successfully conjugated on the FCIMs.

PNIPAm was a well-known thermosensitive polymer with an LCST around 32 °C [45]. Its LCST can be regulated by hydrophilic or hydrophobic modification. It should be pointed out that the UV was always used to detect the transmittance of some PNIPAM or its copolymer solution with relative high concentration, in such case, the temperature at the phase transition should be named as volume phase transition temperature (VPTT). VPTT just reflected the aggregation phenomena before or after the LCST [46]. The PIND18 was copolymerized from PNIPAM and DMAAm. The DMAAm was a hydrophilic monomer. So the VPTT was shifted to about 45 °C which was larger than the LCST of PNIPAm homopolymer (data was not shown here). But when the hydrophobic PLGA was entrapped into the core leading to the hydrophobicity of the micellar system increase, the VPTT was thus decreased to about 38 °C. However, as the hydrophilic Fab was anchored onto the micellar surface, the stability of the FCIMs increase. The VPTT was thus increased a little to about 39 °C. Noted here, the phase transition temperature of such FCIMs can easily be tuned to some special temperature by regulating the micellar composition.

The non-cytotoxicity of drug carrier system is a necessary condition for the in vitro/vivo applications. For our thermosensitive Fab decorated micellar system, the incubation conditions will be at 37 °C and 40 °C. So the cytotoxicity of the IMs and FCIMs was checked by incubating with Her2 overexpressed stomach cancer cell N87.

![Figure 3](image-url)
concentration of 1 mg/mL. This cell viability profile indicated that the IMs and FCIMs are relative non-cytotoxicity.

3.4. Anticancer drug loading and releasing regulating

In our previous study and some others works, the anticancer drug DOX loading content (LC) by micelles was about 5% which limited the chemotherapeutic effect. So it is very important to enhance the drug accumulation in tumors. However, to our best knowledge, the low DOX loading content of micellar drug delivery system is still a pending issue because of the limited drug entrapping capacity by the hydrophobic core. Chemical binding drug on the core-forming segment was a solution for that issue [11]. On the other hand, to enhance the drug loading content by a physically increasing the hydrophobicity of core without any micellar size and structural deformation is an important alternative method for practical application. The driving force for the drug loading into the hydrophobic micellar core is similar-to-similar interaction as expressed by the modified Flory—Huggins parameter \( \chi_{\text{polymer–drug}} \) as [47]:

\[
N \chi_{\text{polymer–drug}} = \left( N \left( \delta_{\text{polymer}} - \delta_{\text{drug}} \right) V_{\text{drug}} / k_B T \right) + 0.34
\]

where \( N, \delta_p \) and \( \delta_d \) are the segment number of core-forming polymer, the solubility parameter of polymer and solvent, respectively, \( V_p \) is the molar volume of solvent, \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature and a value of 0.34 is entropic contribution. The Hildebrand—Scatchard solubility parameter (\( \delta \)), \( \delta = \sqrt{\Delta E_{\text{vap}} / V} \) was often used to describe the mechanism of solubility of solute, where \( \Delta E_{\text{vap}} \) is the energy of vaporization and \( V \) is the molar volume of the solvent. Such \( \chi_{\text{polymer–drug}} \) describes the interaction between hydrophobic segment and the drug. Noted here, the thermal translational energy per macromolecular is of the order of \( k_B T \), whereas the interaction energy per macromolecules is proportional to its composition (segment number \( N \)), namely, the product \( N \chi_{\text{polymer–drug}} \) [13]. So in our experiment, we firstly increased the hydrophobic block length to 71, but the drug loading content was still around 5%. PLGA homopolymer, which was hydrophobic polymer, was usually used to entrap hydrophobic drug such as docetaxel and DOX [41,48]. So the PLGA was physically entrapped into the core of FCIMs for enhancing the interaction strength with DOX. Fig. 4(a) shows the PLGA weight percentage (to the micellar weight) dependence of the drug loading improvement efficiency and FCIMs size. The DOX loading improvement efficiency (LCIE) was calculated as LCIE\% = \( \left( LC_{\text{FCIMs}} - LC_{\text{IMs}} \right) / LC_{\text{IMs}} \times 100\% \), where \( LC_{\text{FCIMs}} \) and \( LC_{\text{IMs}} \) are the loading content of IMs and FCIMs containing PLGA. As shown in Fig. 4(a), when the \( W_{\text{PLGA}} \% \) increased from 0 to 30%, the LCIE\% sharply increased about 2 times. On the contrary, the size of the PLGA entrapped FCIMs had little changed.

The effects of PLGA entrapment on the drug release profile from such FCIMs was also investigated. Fig. 4(b) shows the accumulated release profile of FCIMs with different \( W_{\text{PLGA}} \% \). The release rate decreased with the \( W_{\text{PLGA}} \% \) increased. The drug released out from the hydrophobic core was dominated by diffusion which was also affected by the interaction between drug and core-forming polymer [49]. For the FCIMs without PLGA, the interaction between the DOX and hydrophobic core was attributed to the PLA. On the other hand, for the FCIMs with PLGA inside core, the interaction strength was increased. At the same condition, the higher interaction between DOX and micellar core resulted in lower drug detaching from the core. Thus, the drug loading and releasing profile can be regulated by tailoring the FCIMs composition without any structural deformation. This is special important for the in vivo micellar drug delivery system.

3.5. Cellular uptake and intracellular internalization

As mentioned above, the FCIMs were responsive to temperature and bore the Fab moiety. In order to evaluating the effects of temperature and Fab on the intracellular uptake by stomach cancer cell N87. The flow cytometer (FC) and inverse fluorescent microscopy (FM) were used in this study with the QD (green color) and DOX (red color) as the fluorescent probe (Fig. 5). As shown in Fig. 5(a), as indicated by the DOX, the IMs and FCIMs significantly enhanced the intracellular uptake which was about 3 time that of free DOX commercial DOX formulation Doxil. In addition, the antibody guided FCIMs showed more enhancing than non-targeting IMs at the same incubation T. On the other hand, as the micelles were incubated at \( T > \) VPTT, the DOX intracellular uptake was further enhanced with about 4-fold increase of the fluorescence intensity. It was noted here that, first, the micelles showed obvious enhancement for the intracellular uptake of DOX which was similar to our previous work [32]. Second, the intracellular uptake could be further improved and regulated by either temperature or the antibody. These finding in the FC confirmed that the anti-Her2 antibody (Fab) conjugated immunocimelles were potent in anticanccer drug delivery. Moreover, combining the targeting moiety and temperature was much more promising effect than the separated one.

The promoted intracellular uptake was also investigated using the QD as the fluorescent probe. Fig. 5(b) shows the inverse

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fluorescent microscopy image of intracellular uptake of QD-IMs and QD-FCIMs at 37 °C and 40 °C. When the incubation temperature $T > VPTT$, the QD transferred into the cell was much higher than that at $T < VPTT$ for both IMs and FCIMs. At the same incubation temperature $T < VPTT$ or $T > VPTT$, the intracellular uptake of QD by the FCIMs was much higher than that by IMs because of the Fab targeted improvement. Such intracellular uptake of QD by the IMs and FCIMs was similar to the trend of the intracellular uptake of DOX as shown in Fig. 5(a). Both the FC and FM successfully confirmed that the intracellular uptake of Her2 overexpressed stomach cancer N87 can be regulated by temperature or the Fab conjugation.

In addition, using the FITC-Ig as probe, we further investigate the internalization of the FCIMs at different conditions. It was interesting to find that the intracellular uptake of FCIMs was affected by the incubation time and micellar concentration shown in Fig. 5(c) and (d). At the low FCIMs concentration about 50 μg/mL, the difference intracellular uptake of the FCIMs at $T < VPTT$ and $T > VPTT$ was much larger than that at the high concentration around 100 μg/mL. This means that the antibody-antigen interaction can be saturated by the FCIM’s concentration. On the other hand, at the same concentration of FCIMs, the intracellular uptake was strongly affected by the incubation time. It was found from Fig. 5(c) and (d) that, at the same concentration of FCIMs, the internalization obtained at short incubation time 2 h was higher than that at the long incubation time 4 h. Moreover, higher FCIMs concentration resulted in lower intracellular uptaking. Comparing the intracellular uptake indicated by the FITC labeled Ig at different incubation time and concentration, it was helpful to find that there was a maximum concentration for intracellular uptake of the antibody conjugated FCIMs. This interesting finding was indicative and helpful for the in vitro/vivo cytotoxicity evaluation, which could facilitate us to optimize the FCIMs.

3.6. Intracellular DOX accumulation and cytotoxicity

Confocal laser scanning microscopy (CLSM) was used to evaluate the DOX accumulation profile inside the cell promoted by the IMs and FCIMs. As reported above, the effects of temperature and Fab on the DOX accumulation were systematically investigated as shown in
Fig. 6. Firstly, the incubation temperature was fixed at 37 °C, DOX accumulation was promoted by the anti-Her2 Fab conjugated FCIMs than that by IMs via antibody-antigen binding as shown in Fig. 6(a). The Her2 was an antibody with the function of promoting endocytosis. Secondly, the promoting effect of temperature on antibody-antigen interaction was checked by the FITC-Ig antibody marking in green color. As shown in Fig. 6(b), the green color surrounding cellular surface incubated at \( T < VPTT \) was weaker than that at \( T > VPTT \). This enhancement of antibody-antigen interaction was attributed to the hydrophilic to hydrophobic transition of thermo-sensitive PDL18 shell which increased the interaction between FCIMs and cell membrane [3,31,32]. Subsequently, the cooperative effects of temperature and Fab were further confirmed by indicating the fluorescence of DOX inside cells as shown in Fig. 6(c). The DOX loaded FCIMs were incubated with Her2 overexpressed stomach cancer cell N87 at temperature above and below the VPTT. In such case, both the temperature and Fab was evaluated at the same time. The intracellular DOX accumulation guided by FCIMs at \( T > VPTT \) was obviously higher than that by FCIMs at \( T < VPTT \). This observation was consistent with our previous finds in the intracellular uptake experiments, that is, the intracellular uptake of FCIMs and the DOX accumulation can be enhanced by the temperature and antibody cooperative effects. To the best of our knowledge, this is first report on DOX delivery by dual-functional FCIMs.

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Generally, the higher the DOC accumulation will result in the higher cytotoxicity. The cytotoxicity was then evaluated by the Doxil, the IMs-DOX and FCIMs-DOX as shown in Fig. 7. Fig. 7(a) shows the cell viability profile of Doxil, the IMs and FCIMs at \( T_{<VPTT} \) and \( T_{>VPTT} \). At the same temperature, the cell viability of IMs was lower than that of Doxil due to the intracellular DOX accumulation enhanced by IMs. While the cell viability of FCIMs was further decreased than IMs which was attributed to the antibody targeting effect. Similarly, for the FCIMs, the cell viability as incubated at \( T_{>VPTT} \) was lower than that at \( T_{<VPTT} \) because the temperature promoting intracellular uptaking \([3,31,32]\]. According to the DOX concentration dependence of the cell viability profile, the IC50, which indicated the half of the cell death, was used to evaluate the cytotoxicity profile of the drug delivery systems as shown in Fig. 7(b). The IC50 of FCIMs incubated with N87 at \( T_{>VPTT} \) was the lowest case, which was about 9 times lower than that of Doxil. These results also further confirmed that both the temperature and Fab successfully enhanced the cell cytotoxicity.

3.7. Serum stability evaluation

For clinical application, the in vivo stability of micellar system should be considered. Here, we use the Albumin from bovine serum (BSA) as a model serum protein to check the micellar serum stability profile. Fig. 8 shows the size and size distribution of FCIMs mixed with BSA with high (a) and low (b) hydrophilic PID118 chain density \( (S_{\text{corona}}/N_{\text{agg}}) \). The individual peak from BSA and FCIMs shown in Fig. 8 indicated that the narrow size distribution of each sample as separately measured in LLS. The well-separated peaks for BSA and FCIMs still appeared in their mixture when high PID 118 coated on the FCIMs (Fig. 8(a)). However, as the PID118 content decreased from 2.4 mg to 1.4 mg. The peak of BSA and FCIMs coupled together resulting a very broad distributed non-uniform peak as shown in Fig. 8(b). This coupling of individual size peaks of BSA and FCIMs at low PID118 content indicated the absorption of BSA onto the FCIMs. It is known that, the stability of the micellar system is attributed to the hydrophilic chain surrounding the micelle surface. The determining factor is the corona-forming chain density \( (r_{\text{corona}}) \) which defined as the ratio of micellar surface area \( (S_{\text{corona}}) \) to the aggregation number \( (N_{\text{agg}}) \), that is, \( r_{\text{corona}} = S_{\text{corona}}/N_{\text{agg}} \). High \( r_{\text{corona}} \) means the gap between the neighboring hydrophilic shell chains is small. Chain’s mobility is thus forbidden leading to the entropic loss of shell chain. This entropic loss increases the excluded volume of the micellar resulting in repulsion the proteins absorption which dominates the system in vitro/vivo stability as shown in Fig. 8(c) \([51]\).
3.8. In vivo distribution and intratumor accumulation

Although many micellar systems perform excellently in vitro, they showed unsatisfactory in vivo manners due to their instability, low intratumor accumulation. Based on the above discussion, it is helpful to know that the in vivo stability can be enhanced by micelles with high shell chain density. On the other hand, the intratumor accumulation can be promoted by active targeting through antibody. Balb/c nude mice bearing gastric cancer (N87 cells with Her2 overexpressed) was set-up for in vivo tissue distribution and tumor accumulation examination. The fluorescent indicator FITC loaded FCIMs and IMs micelles with high shell chain density was injected in to the mice bearing with human stomach tumor via tail vein and was observed by the IVIS® imaging system. Fig. 9A shows the in vivo tumor accumulation of targeted and non-targeted micelles. The living animal fluorescent images demonstrated that the FITC can be transferred to the tumors by micelles as marked by white arrows. Furthermore, comparing with the non-targeted micelles IMs, the FITC accumulation in tumors was obviously enhanced by dual targeting FCIMs. Such higher fluorescence intensity from the FCIMs clearly shows the antibody targeting can promote the intratumor accumulation. It should be noted here, the intensity on the rear end of the mice maybe also due to some staining from the mouse food or due to the background intensity (mouse body) [52,53].

In order to further investigate the tissue distribution profile of FITC loaded FCIMs and IMs micelles, the main organs such as liver, heart, spleen and kidney mice were harvested once the living animal fluorescent image experiments. The fluorescent intensity profile in different organs for targeting and non-targeting mice was shown in Supplementary Fig. 2. It was found that fluorescent molecules mainly distributed in tumors, especially for the dual targeting FCIMs. The FITC distributed in tumor and other organs was further visualized by inverse fluorescent microscopy showed in Fig. 9B. The cell nucleus was stained by DAPI shown in blue. The intensity of green color indicated the FITC molecular accumulation. This fluorescent image of different frozen sections further demonstrated the micellar accumulation in tumors. Moreover, the intratumor accumulation of FCIMs was much higher than that of IMs. The densely packed micellar corona chain increased the in vivo stability with long circulation time in blood. This is pretty important for tumor accumulation. As the micellar system arriving at the tumor site, both the EPR and the active targeting promoted the intratumor accumulation. Additionally, it was reported that the temperature of some solid tumor was 1.5–3 °C higher than that of normal tissue [27,32]. While the VPTT of the micelles was about 39 °C, which indicated that the micellar shell will transfer from hydrophilic to hydrophobic. This phase transition at lesion site could lead to the passive targeting effect [3,30,32]. On the other hand, such temperature regulated passive targeting could be very useful for the hyperthermia treatment. The high shell chain density, the passive targeting by temperature responsive shell chain and the active targeting via Her2 antibody accounted for the high intratumor accumulation.

3.9. In vivo antitumor activity

The therapeutic index of the dual targeting immunomicelles was subsequently examined by the Balb/c nude mice bearing gastric cancer. In the in vivo tumor inhibition experiments, as the tumor volume grown to about 50 mm³, mice with the Her2 overexpressed gastric tumor were randomly divided in four groups with 5 mice/group. Mice were then intravenously administrated 100 μL saline for control, free DOX in PBS, IMs-DOX and FCIMs-DOX solutions with equal drug dosage 5 mg/kg on day 1, 2 and 3. The tumor volume was recorded from day 1. The results of tumor inhibition as expressed by the relative tumor volume were shown in Fig. 10. The tumor volume of untreated (control) and the free
DOX group increased much faster than that of IMs and FCIMs group at the same time scale. This is due to the anticancer drug accumulation in tumor was enhanced by micelles via EPR, which became obvious around 6 days post i.v. injection. Most importantly, the relative tumor volume of group administrated with the dual targeting immunomicelles FCIMs was < 2, which was much smaller than that of control (~5), free DOX (~4) and IMs (~3). Such FCIMs showed the best therapeutic effect on the in vivo tumor inhibition. This further proved that the in vivo intratumor accumulation of FCIMs was much higher than IMs. Such in vivo tumor inhibition experiments clearly suggested that the antibody conjugated dual targeting immunomicelles (FCIMs) showed a considerable higher anticancer therapeutic potential than free DOX and non-targeted micelles.

4. Conclusions

In summary, an anti-Her2 antibody Fab conjugated temperature sensitive poly(N-isopropylacrylamide-c-เนื้อเยื่อน-dimethylacrylamide)-b-poly(0,1-Lactide) immunomicelle (FCIM) with dual targeting function and well-defined structure was finely assembled by composition regulation and solvent quality tuning. The micellar properties were clearly demonstrated. The effects of composition, temperature sensitive and Fab targeting on DOX loading, releasing, intracellular uptake and DOX accumulation was systematically investigated. Experimental data further showed that the cytotoxicity was significantly promoted by the cooperative effects of temperature and Fab moiety. Importantly, by the structural tailoring, such finely tailored immunomicelles obviously promoted in vivo stability, intratumor accumulation. Referring to the Balb/c nude mice bearing gastric cancer examination, the significant in vivo tumor inhibition by FCIMs indicated their high potent in Her2 overexpressed chemotherapy.

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Appendix A. Supplementary material


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