Zinc Finger-Inspired Nanohydrogels with Glutathione/pH Triggered Degradation based on Coordination Substitution for Highly Efficient Delivery of Anti-Cancer Drugs

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ABSTRACT

Biodegradable materials used for drug delivery is of great demand due to their ability to degrade into low molecular weight species and further excrete from body by metabolism. Herein, we report a new kind of zinc(II) crosslinked poly(methacrylic acid) nanohydrogels (ZCLNs) inspired by zinc finger proteins with dual stimuli-triggered degradation (glutathione and pH) for the first time. Compared with the disulfide bond crosslinked nanohydrogels, this new kind of ZCLNs is benefit on the degradation in wide range of cells, including the normal cells. Ex vivo fluorescence images showed that the DOX-loaded folate-PEG conjugated zinc(II)-crosslinked polymeric nanohydrogels (FPZCLNs-15) preferentially accumulated in tumor tissue with minimal accumulation in normal tissues compared with DOX-loaded PZCLNs-15 (non-targeted nanohydrogels) and free DOX, the FPZCLNs-15 (targeting system) delivered DOX to the tumor site with approximate 3.6 and 1.6 fold higher than free DOX and PZCLNs-15, respectively. Meanwhile, the PZCLNs-15 and FPZCLNs-15 reduced the concentration of DOX in heart by 3.2 and 5.0 fold respectively, as compared to the free DOX. Moreover, a superior tumor growth inhibition and negligible damage to normal organs like heart and kidney, which is reported to be vulnerable to DOX-associated side effects, are further demonstrated.

Keywords: zinc(II)-crosslinked nanohydrogels, folate-PEG conjugation, glutathione/pH triggered degradation, coordination substitution, targeted drug delivery

1. Introduction

Drug delivery systems (DDS), based on particulate carriers (primarily composed of lipids or polymers), have demonstrated its superiority in preclinical tumor diagnosis and therapeutic treatment compared with conventional free drug in chemotherapy [1-10]. Especially, the so-called enhanced permeability and retention (EPR) effect, which means particles of certain size **tends** to penetrate interstitial tumor vasculature with higher retention time and accumulate more preferentially in tumor tissue than normal tissues, endows the targeted distribution of drug-loaded nanoparticles in vivo and enhances therapeutic efficiency of cancer and other inflammation for parenteral (primarily intravenous) applications [11-17]. However, to make drug carrier safe and potency, some requirements about the materials of the carrier should be met, for example, low biotoxicity [18-20], long circulation time in vivo [21, 22], biodegradability and metabolizability [23, 24]. Furthermore, drug carriers are expected to achieve a rapid and complete release of drug in tumor tissues while no premature drug leakage in blood circulation [25]. The dilemma between intracellular dissociation versus the extracellular stability still remains a big challenge.

Besides taking advantage of EPR effect as passive targeting, the active targeting can also be used in the particulate delivery system to further enhance their tumor cells recognition and internalization by targeting tumor cells and tissues, mostly via conjugating with a targeting moiety to offer preferential accumulation of the drug within selected tissues [26, 27]. The folate receptor, a glycosylphosphatidylinositol anchored on the cell surface, is overexpressed on the vast majority of cancer cells, while its expression is limited in healthy cells and tissues. This expression can be exploited to target imaging molecules and therapeutic compounds directly to cancerous tissues, thus avoid any possible side effect caused by nonspecific accumulation to non-tumor tissues [28-34]. However, to achieve this, the nanoparticles should have long blood circulation time without being eliminated by **reticuloendothelial system** (RES) and nonspecific attack of macrophages after intravenous injection [35]. PEGylation, the process of covalently attaching hydrophilic polyethylene glycol (PEG) polymer chains to nanocarriers, is an efficient and widely used method to mask the nanoparticles from the immune system and prolongs its circulatory time by reducing renal clearance and depressing nonspecific binding [36-39]. However, the PEG coating also screens the desired interactions between nanoparticles and tumor cells, leading to lower uptake [40]. It still remains a problem that the PEG polymer chains will cover the targeting ligands on the surface of nanoparticles to impede them from conjugation with the receptors in tumor cells, thus may negatively affect cellular uptake and intracellular distribution at the target site due to the steric hindrance.

Recently, stimuli-responsive nanohydrogels have already been widely used for the fabrication of targeted drug carriers, for they can respond to the environmental difference between the tumor and the normal cells, especially pH difference and concentration of glutathione (GSH) difference [41-44]. The loading and releasing of drug inside the nanohydrogels can be effectively controlled by environmental conditions. As in vivo circumstances, pH values are usually 5~6 in tumorous cells while 6~7 in normal cells, which can be used by smart materials to trigger drug release. Besides, the concentration of GSH outside tumor cells is reportedly as low as 2~20 μM whereas GSH levels within tumor cells range from 2~10 mM [45]. The significant concentration difference of GSH between the extracellular and intracellular compartments of tumor cells guide people to use material that sensitive to those stimuli as targeted drug delivery carriers. Disulfide bond, a widely existed structure in the human body, is extensively studied for its redox-responsive character that can be broken in reducing environments. Up till now, a tremendous amount of work have been reported

about the disulfide bonds structure materials in the drug delivery system to selectively deliver anti-cancer drugs to tumor cells under redox-stimulus response via a thiol-disulfide exchanges [46, 47]. Previously, our group has developed a nanohydrogel carrier system based on poly(methacrylic acid) crosslinked by *N,N*-bis(acryloyl) cystamine (BACy), which endowed the carrier with redox-stimuli property that can be degraded into low molecular weight species in the intracellular compartments of tumor cells and further excrete from body by metabolism [48]. However, as an ideal biodegradable material, the nanohydrogels are also required to be capable of decomposing not only in tumor cells, but normal cells as well, which needs the materials to be degraded responding to more than GSH stimulus. Unfortunately, disulfide bonds **crosslinked materials are** difficult to achieve that.

Zinc is one of the essential trace elements in human body, which plays an important role in the physiological process of human growth, development, reproduction, immunity, endocrine and so on. Zn(II) ion can be chelated by two thiol groups to form relatively stable bonds, which is a very common structure in zinc finger proteins in human cells. Meanwhile, zinc ions are also capable of being replaced or removed by other ligands, **which display** considerable versatility in binding modes [49, 50]. Inspired by this nature system, we herein deliberately **designed** and prepared a new kind of folate-PEG conjugated zinc(II)-crosslinked polymeric nanohydrogels (FPZCLNs) for the first time. It had the capacity of glutathione/pH dual stimuli-triggered disassembly in normal cells and malignant cells by coordination substitution, thus significantly reduce the biotoxicity of the nanocarriers produced by the accumulation in human body. The biological evaluation of the FPZCLNs both in vitro and in vivo showed that the as-prepared nanohydrogels had great potentials as highly efficient drug delivery system for cancer treatment.

2. Materials and methods

2.1 Materials

Methacrylic acid (MAA) was purchased from Sigma Aldrich and distilled under reduced pressure prior to use. Zinc chloride, ethanol and sodium hydroxide were purchased from Shanghai Chemical Reagents Company and used without a further purification. *t*-Boc-NH-PEG-NH₂ (MW 1000) was purchased from Laysan Bio, Inc (Arab, AL, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), Glutathione (GSH), *N*-hydroxysuccinimide (NHS) and folic acid, were purchased from Shanghai Aladdin Chemistry Co. Ltd. 2, 2-azobisisobutyronitrile (AIBN) was obtained from Sinopharm Chemical Reagent Company and recrystallized from ethanol. Doxorubicin (DOX), in the form of a hydrochloride salt, was obtained from Beijing Huafeng United Technology Company. Acetonitrile (AN) and dimethyl sulfoxide (DMSO) were purchased from Shanghai Lingfeng Chemical Reagent Company, and dried with calcium hydride and purified by distillation before use. Other reagents were purchased from Chinese National Medicine Corporation and used as received.

2.2 Synthesis of zinc dimethacrylate as a crosslinker

Zinc dimethacrylate (ZDMA) was synthesized according to the published paper [51]. As a typical example, 1.6 g sodium hydroxide was dissolved in 10 mL deionized water and added 3.44 g methacrylic acid after sufficiently stirring to ensure they react completely, pH was adjusted to 7.0 before dropping ZnCl₂ solution. The solid precipitate was collected by vacuum filtration and washed it for several times. After drying under vacuum, the product was obtained (Yield: 87 wt%). The ¹H

NMR spectra of ZDMA in DMSO- d_6 were shown in Fig. S1. δ (ppm) 1.8 (a, 3H, $-\text{COCC}H_3-$), 5.3 (b, H, $-\text{CCH}_3=\text{CH}H$), 5.7(c, H, $-\text{CCH}_3=\text{CH}H$).

2.3 Synthesis of NH_2 -PEG-Folate

The synthesis of NH_2 -PEG-Folate was accomplished according to the literature [52]. First, folic acid (60 mg, 1.4 mmol) was dissolved in 20 mL of DMF and reacted with NHS for 12 h in the presence of EDC. Then the product was precipitated in a mixture of 30/70 acetone/diethyl ether and dried under vacuum. Folate-NHS was dissolved in 500 mL of DMSO after addition of *t*-Boc-NH-PEG- NH_2 (924 mg, 280mmol) and reacted for 12 h in the presence of *N,N*-diisopropylethylamine (DIEA). The product was precipitated in cool ether, reconstituted in an aqueous solution of NaOH and dialyzed for 48 h. The resultant *t*-Boc-NH-PEG-Folate was lyophilized and reconstituted in 700mL of TFA to remove the protecting group and get the final product.

2.4 Preparation of Zn-crosslinked PMAA nanohydrogels

Reflux-precipitation polymerization was used to fabricate the Zn-crosslinked PMAA nanohydrogels. A typical procedure is as following: MAA (500mg, 5.808 mmol) and AIBN (16.7 mg, 0.102 mmol) were dissolved in 40 mL of acetonitrile (AN) in a dried 100-mL triple-necked flask with the aid of ultrasonic for 10 min. Afterwards, the flask was submerged in a heating oil bath attached with a spherical condenser pipe. The reaction mixture was heated from room temperature to the boiling state within 30 min. After that, zinc dimethacrylate (125mg) was dissolved in 5 mL ethanol and added it dropwise to the flask within 20 min, the reaction was ended in 2 h. The obtained Zn-crosslinked PMAA nanohydrogels were separated and purified for three times by repeating centrifugation (12,000 rpm for 5min)/redispersion in deionized water with the aid of ultrasonic bathing.

2.5 Modification of Zn-crosslinked PMAA nanohydrogels with NH_2 -PEG-Folate hydrophilic chains

The NH_2 -PEG-Folate was conjugated to PMAA nanohydrogels using the carbodiimide coupling method. Typically, PMAA nanohydrogels (30 mg) was dispersed in aqueous solution (20 mL). After addition of EDC·HCl (48 mg, 0.25 mmol) and NHS (28.75 mg, 0.25 mmol), the mixture was magnetically stirred for 3 h. NH_2 -PEG-Folate (15mg, 0.015 mmol) was then added, and the reaction mixture was stirred at room temperature overnight. The obtained PMAA-PEG-FA nanohydrogels were separated and purified by repeating ultracentrifugation (12,000 rpm for 3 min)/decantation /resuspension in pure water with ultrasonic bathing for three times. The purified nanohydrogels were lyophilized to get the final product.

2.6 GSH/pH-triggered disassembly of Zn-crosslinked PMAA nanohydrogels

Monitored by dynamic light scattering (DLS) measurement, the turbidity change of the ZCLNs in response to GSH and pH can be regarded as an important index in the degree of disassembly. The relative turbidity is measured by the ratio of the scattering light intensity relative to the initial sample (no GSH). In the experiment, 5 mg ZCLBs was dispersed into 10 mL phosphate buffer (10 mM, pH 7.4) solution and then 10 mM GSH was added. The solution was placed in a shaking bed at 37°C with a rotation speed of 200 rpm. At predetermined intervals, samples were collected and their scattering light intensities were determined by using DLS. After complete degradation, the polymer

solution was filtered through 0.45 μm filter and measured by GPC to determine the molecular weight of the degraded polymers chains from nanohydrogels.

2.7 Loading of DOX in Zn(II)-crosslinked PMAA nanohydrogels

DOX, in the form of a hydrochloride salt, was chosen to investigate the drug loading capability. Typically, DOX was dissolved in 20 mL of phosphate buffer solution (pH 7.4) with various concentrations, then 10 mg dry nanohydrogels were dispersed under stirring for 24 h at room temperature. The dispersion was then centrifuged to collect the DOX-loaded nanohydrogels and washed three times with distilled water to remove the surface adsorbed DOX. The sample was then freeze-dried and all the supernatant was collected. The concentration was measured by a UV-visible spectrophotometer at 480 nm according to DOX calibration curve. The amount of loaded DOX was calculated by subtracting the weight of DOX in the total supernatant from the DOX weight in the initial solution. The drug loading capacity (DLC) was calculated to be the ratio of loaded DOX in whole drug loading nanohydrogels.

2.8 In vitro release of drug from Zn-crosslinked PMAA nanohydrogels

Typically, the DOX-loaded nanohydrogels (10 mg) were dispersed separately in 10 mL phosphate buffer (pH 7.4) and acetate buffer (pH 5.0). Then the dispersion was divided into five equal fractions. Each 2 mL of the fraction sample was then transferred into a dialysis bag (molecular weight cut off 14,000) and dialyzed against 80 mL of the corresponding buffer (pH=7.4 or 5.0) with or without GSH and gently shaken (200 rpm) at 37 °C in shaking bed respectively. The drug release was believed to start as soon as the dialysis bags were set into the reservoir. At predetermined periods, 3 mL of the solution was obtained periodically from the reservoir as the sample to be tested and 3 mL of fresh buffer medium was added back to the reservoir to keep a constant volume. The amounts of released DOX were analyzed by UV-vis at 480 nm. All the data of DOX drug release were averaged over three measurements

2.9 In vitro cell assays

The cytotoxicity was performed by cell counting kit-8 (CCK-8) assay. The HeLa cells and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin at 37 °C, 5% CO_2 . The media were changed every three days, and the cells were passaged by trypsinization before confluence. Cells were seeded onto 96-well plates (10, 000 cells per well) and incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were washed with PBS, and the medium was replaced with a fresh medium containing the pure nanohydrogels, free drugs, or the drug-loaded nanohydrogels of known concentrations. After 24 h, the cells were washed with PBS and incubated in 100 mL DMEM containing 10mL CCK-8 solution for another 1 h. The absorbance of each well was measured by a micro-plate reader (model FL 311, Bio-Tek Instruments, Winooski, VT, USA) at the wavelength of 450 nm. The results were expressed as mean values of four measurements. Then the cells were washed with PBS and incubated in fresh medium for an additional 24 h and measured again. Since the absorbance is proportional to the number of viable cells in the medium, the cell viability was determined by using a previously prepared calibration curve (Dojindo Co.).

2.10 Confocal laser scanning microscopy (CLSM) observation

The cellular uptake of the drug-loaded FPZCLNs and free DOX was confirmed by confocal laser scanning microscopy (CLSM) observation. Typically, the HeLa cells were seeded in 6-well culture plates (a clean cover slip was put in each well) and grown overnight, then the cells were incubated with drug-loaded FPZCLNs and free DOX at 37 °C for 24 h. Afterwards, the cells were rinsed with PBS for three times, fixed with 2.5 % formaldehyde (1 mL per well) at 37 °C for 10 min, and then rinsed with PBS three times again. To perform nucleus labeling, the nuclei was stained with Hoechst33324 solution (20 mg·mL⁻¹ in PBS, 1 mL per well) for 10 min and then rinsed with PBS three times. The cover slips were placed on a glass microscope slide, and the samples were visualized using CLSM (Olympus, FV10-ASW).

2.11 Flow cytometry experiment

HeLa cells were seeded in 6-well plates with a density of 5×10^5 cells per well in 2 mL of DMEM and incubated for 24 h, and then the original medium was replaced with free DOX, PZCLNs and FPZCLNs (at a final DOX concentration of 0.5 mg L⁻¹) containing DMEM. The cells were incubated for 24h at 37 °C, and then washed for three times with phosphate buffered saline (PBS). The harvested cells were suspended in PBS and centrifuged at 1000 rpm for 5 min at 4 °C. The supernatants were discarded and the cells were washed with PBS to remove the background fluorescence in the medium. After two cycles of washing and centrifugation, the cells were resuspended with 500 mL PBS, and flow cytometry was done using a BD FACS Calibur flow cytometer from BD Biosciences.

2.12 Quantification of DOX in tissues and blood

H22 tumors reaching approximately 100 mm³ were selected for our study. Selected mice were randomized into 3 groups of at least 4 mice per group for the following i.v. treatments: free DOX (6.0 mg /kg), PZCLNs (6.0 mg/kg DOX equivalent), FPZCLNs (6.0 mg /kg DOX equivalent) or no treatment (control group). At 2, 8, 24 h after injection, the mice were sacrificed, and the tumors as well as normal tissue were collected, dipped in the lysis buffer and homogenized. After determination of the amount of homogenate tissue extracts, it was mixed with methanol by the ratio 1:1 and centrifuged at 3000×g for 15 min. Blood sample was collected and centrifuged at 3000×g for 20 min to obtain plasma. To determine the concentration of DOX, the supernatant and plasma were quantified fluorometrically (excitation 488 nm and emission 545 nm). To correct for nonspecific background fluorescence, the samples were analyzed using a standard curve made on the basis of tissue extracts derived from drug-free mice. The data are expressed as nanogram of DOX per mg of tissue extracts or as μ gram per mL of plasma [60].

2.13 Ex vivo DOX fluorescence imaging and biodistribution

Female Balb/c mice, aging 4-5 weeks and weighing 20-25 g, were supplied by Department of Experimental Animals, Fudan University (Shanghai, China), and maintained under standard housing conditions. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University. H22 tumor-bearing mice model was established by subcutaneous injection of 1×10^6 cells suspended in PBS into the flank region, and in vivo imaging was carried out 3 weeks after the inoculation. The DOX-loaded FPZCLNs, DOX-loaded PZCLNs, free DOX and physiological saline were injected into mice bearing H22 tumor via lateral tail vein (5 mg·kg⁻¹ on DOX basis). The mice were sacrificed 2, 8 and 24 h

post-injection. The tumor and major organs (heart, liver, spleen, lung and kidney) were excised, followed by washing with physiological saline three times for ex vivo imaging of DOX fluorescence using the In Vivo Xtreme (Bruker, USA). The average signals were also quantitatively analyzed using In Vivo Xtreme software.

2.14 In vivo antitumor activity evaluation

To evaluate the antitumor activity of DOX-loaded FPZCLNs, DOX-loaded PZCLNs and free DOX, H22 tumor-bearing mice were randomly divided into different groups (six mice each group), and treated with DOX-loaded FPZCLNs (4.5 mg kg^{-1} on DOX basis), DOX-loaded PZCLNs (4.5 mg kg^{-1} on DOX basis), free DOX (4.5 mg kg^{-1}) and physiological saline via tail vein injection respectively on days 0, 4, and 8. The tumor size was measured using vernier calipers, and the tumor volume (mm^3) was calculated using $V = a \cdot b^2 / 2$, where a and b were the longest and shortest diameter axes of the tumors. The body weight of the mice was measured simultaneously as an indicator of systemic toxicity.

2.15 Characterization

The nanohydrogels were characterized for their morphology on a JEOL1230 transmission electron microscope (TEM) at an accelerating voltage of 75 kV. Both the FTIR spectra and UV-vis of the nanohydrogels were obtained on a PerkinElmer Lambda 35 spectrophotometer and a NEXUS-470 Fourier transform infrared spectrophotometer, respectively. Hydrodynamic diameter and zeta potential of the nanohydrogels were measured by dynamic light scattering (DLS) using an autosizer 4700 (Malvern) at scattering angle of 90° and room temperature. The average molecular weight and polydispersity index (M_w/M_n) were estimated by Gel Permeation Chromatography (GPC). For the measurement of low-molecular-weight PMAA linear chains, GPC was performed on an Agilent 1100 equipped with a G1310A pump, a G1362A refractive index detector, and a G1315A diode-array detector, poly(ethylene oxide) (PEO) standard samples were used for calibration, the measurement condition is 0.1M NaNO_3 aqueous solution at 40°C with an elution rate of 0.5 mL/min . Inductively coupled plasma atomic emission spectrometry (ICP-AES) was performed to determine the Zinc contents in the ZCLNs by a P-4010 spectrometer (Hitachi, Tokyo, Japan).

2.16 Statistical analysis

All the experiments in this work were done independently at least three times and the data was expressed as the mean average $\pm$ standard deviation (s.d.).

3. Results and discussion

3.1. Preparation and characterization of zinc(II) crosslinked nanohydrogels (ZCLNs)

The strategy to prepare ZCLNs and FPZCLNs is depicted in **Scheme 1**. Briefly, the ZCLNs were fabricated by using a concise one-pot reflux-precipitation polymerization approach [53]. All the reagents of methacrylic acid (MAA) as monomer, zinc dimethacrylate (ZDMA) as crosslinker, azodiisobutyronitrile (AIBN) as initiator and acetonitrile as solvent were added together in the reaction flask before heating to reflux. During this process, the polymers with critical chain length formed suspended particles by precipitation and aggregation from the poor solvent. After removing the acetonitrile by centrifugation, ZCLNs could be redispersed in water. It was of note that a small amount of zinc ions **was good enough** to stabilize the nanoparticles in aqueous environment.

The proportion of **carboxyl group** in the nanohydrogel network could be rationally tuned for physicochemical properties of the nanohydrogels by adjusting the feed ratio of MAA and ZDMA, leading to the control of size, stimuli response, degradation rate as well as optimizing the conjugation of therapeutic compounds. In this way, we prepared P(MAA-co-ZDMA) with different crosslinking density from 15% (ZCLNs-15) to 30% (ZCLNs-30). The typical **formulations** were shown in **Table 1**. The nanohydrogels were characterized with respect to their morphology, size distribution, composition, and surface charge property. The TEM photographs showed that all groups of nanohydrogels were mono-dispersed with uniform shape. What's more, with an increase in the feeding amounts of ZDMA from 15 wt% to 30 wt% (ZCLNs-15 to ZCLNs-30), the average diameter of the as-prepared ZCLNs (TEM) increased from 80 nm to 146 nm (**Fig. 1**), which was also confirmed by DLS measurements data (**Table 1, Fig. S2**). This trend was attributed to the high reaction activity of the ZDMA monomer, which increased the number of the primary nuclei in the early polymerization stage before aggregating into larger polymer particles to lower their surface tension. The trend of the hydrodynamic diameter (D_h) of the nanohydrogels, as determined by DLS measurements, were consistent with the variation trends of the TEM observations, while the comparatively bigger size from DLS measurements should be due to the swollen state after being immersed in water, as the TEM samples were prepared in the desiccation state. Zeta potential analysis supported the successful copolymerization of MAA and ZDMA monomer, for the surface charge of the nanohydrogels decreased from -30.1 mV to -16.5 mV with the increase of ZDMA amount from 15 wt% to 30 wt%, which was due to the consumption of the **carboxyl groups** by Zn ion. However, as the amount of ZDMA was increased over 30 wt%, the narrow dispersed nanohydrogels couldn't be obtained, which might contribute to the destabilization and precipitation of the nanohydrogels. Quantitative amounts of Zn element in ZCLNs could be determined by inductively coupled plasma-atomic emission spectroscopy analysis (ICP-AES), showing the amount of Zn increased from 6.42 % to 11.66 %, in accord with the trend of the theoretical amount of Zn from 3.60 wt% to 6.38 wt% (**Table 1**), which further confirmed that the reaction activity of ZDMA was higher than MAA monomer.

The FTIR spectra were also employed to evaluate the existence of the MAA monomer and ZDMA crosslinker (**Fig. 2A**). The typical metal carboxylate bands could be clearly observed at 1540 and 1427 cm^{-1} and the C=O stretching vibration of MAA at 1706 cm^{-1} . Besides, it could also be found that the intensity of the metal carboxylate bands became stronger as the crosslinker concentration increased from 15, 20, 25 to 30wt%, indicating the growing amount of coordination crosslinking bonds between Zn and carboxyl group.

3.2. Folate-PEG conjugation

Crosslinking of the hydrophilic polymer chains may result in decreased hydrophilicity, thus compromising the stealth effect as well as reducing the circulation time of nanoparticles in the bloodstream. The decoration of hydrophilic PEG corona on the surface of nanoparticles is one of the universal strategies to prolong the in vivo circulation time. However, it may negatively affect the tumor cellular uptake at the target site by steric hindrance. To solve this problem, we introduced FA-PEG-NH₂ on ZCLNs-15 through carbodiimide reaction, which was a widely used coupling reaction between the **carboxyl groups** and amino groups. UV-vis spectroscopy was used to study the conjugation of FA-PEG-NH₂ on the nanohydrogels (**Fig. 2C**). The folate molecule had strong absorbance at 283 nm, while the pure nanohydrogels did not. Compared with the UV-vis spectra

before and after reaction with FA-PEG-NH₂, we could confirm the successful modification of FA-PEG-NH₂ on the surface of the nanohydrogels. FTIR spectra (**Fig. 2B**) further confirmed the successful conjugation of FA-PEG-NH₂. The PEG had the absorbing peak at 1100, which could be found after conjugation. However, compared with the PMAA nanohydrogels (Sample ZCLNs-15), the TEM image of FA-PEG modified nanohydrogels (Sample FPZCLNs-15) did not show much change in size and morphology (**Fig. S3**), while the hydrodynamic diameter measured by DLS (**Fig. 2D**) rise from 191 nm to 255 nm, due to the stretch of hydrophilic PEG chains in aqueous environment.

3.3. The stability of FPZCLNs in aqueous solution.

As a drug nanocarrier, one central issue is their stability under physiological conditions, which need to avoid dynamic dissociation in the circulation system in vivo before reaching their destination (the tumor tissues). To investigate the stability of FPZCLNs in aqueous solution, we dispersed the samples from FPZCLNs-15 to FPZCLNs-30 into the phosphate buffered saline (PBS) (0.01M, PH=7.4) over a storage period of 1 week at room temperature (25°C) and monitored the diameter and relative scattering intensity change with time. In **Fig. 3A** and **B**, we could see the size and relative scattering intensity of the nanohydrogels in PBS tracking by DLS did not show remarkable changes even after one week of storage period. This result suggested that the nanohydrogels were stable enough as a hydrophilic nanomaterial capable of being the drug carrier.

3.4. The dual stimuli-triggered disassembly behavior of PMAA nanohydrogels

Apart from excellent extracellular stability, ideal drug carriers should also be able to specifically and rapidly release the drugs in targeted places in response to internal or external triggers, thus minimizing the probability of drug resistance and systemic side effect.

Using the ZCLNs-15 as an example, upon the addition of water-soluble GSH or citric acid to the nanohydrogels aqueous solution and set the temperature to 37 °C, the state of the milky emulsion would gradually became transparent and colorless, as the nanohydrogels were quickly degraded into individual short polymeric chains which were easily soluble in water. To quantitatively evaluate the disassembly efficiency of ZCLNs triggered by GSH and pH, we employed the turbidity measurement and designed several batches of parallel experiments by varying the concentration of GSH as well as pH under the same condition and observed the relative turbidity (the real-time turbidity compared with the original data) change with time. As is shown in **Fig. 3C** and **D**, an increase in the GSH concentration (from 0 to 10 mM) could increase the disassembly rate of nanohydrogels, while a decrease in pH value (from 7.4 to 5.0) could also lead to an increase in disassembly. Meanwhile, the relative turbidity was almost unchanged in the absence of GSH or citric acid.

The significant difference may be attributed to both the removal of the Zn(II) ions from the crosslinked nanohydrogels framework by thiol groups in GSH molecules via coordination substitution mechanism and acid cleavable metal chelation bond caused by carboxyl protonation, which simultaneously lead to the cleavage of chain crosslinking bonds, degradation of polymer networks, and complete destruction of polymer and drug interaction to release of DOX. This result demonstrated the superior dual stimuli-responsive degradation ability of the Zn-crosslinked PMAA nanohydrogels, which would further be used to the degradation of nanohydrogels in normal cells (acid environment in lysosome) and malignant tissues and cells (relatively acid environment organelles like endosomes, lysosomes and high GSH concentration in cytosol). The possible

mechanism was depicted in Scheme 2.

After degradation into transparent solution, we use GPC to detect the molecular weight of ZCLNs-15 and FPZCLNs-15 degraded polymer chains (**Fig. 4**). The GPC trace of degraded ZCLNs was unimodal distribution, revealing the number average molecule weight (M_n) of 1536, and polydispersity index (PDI, M_w/M_n) of 1.08. After decorating the ZCLNs-15 nanohydrogels with PEG-FA linear chain, the molecular weight distribution became wider with a shoulder peak shifting to the higher M_n region, which from another perspective proved the successful conjugation of PEG-FA on to the PMAA framework. The GPC result showed that our nanohydrogels could be degraded into low molecular weight and narrow molecular weight distribution polymer chains with the aid of pH/GSH, which was favorable for metabolizing in vivo, avoiding their accumulation in the body to produce unwanted toxicity, thus would be potential to apply them as excellent biodegradable drug nanocarriers.

3.5. DOX loading and in vitro dual stimuli-triggered release studies

The negative charges of nanohydrogels played a crucial role in its electrostatic interactions with oppositely charged ingredients. Because of the densely carboxyl groups at the frame network of nanohydrogels, DOX, an amine-carrying structure anti-carcinogen, could be successfully incorporated into the FPZCLNs through electrostatic attraction, which was later administered intravenously as the carboxylate salt to ensure them in the active form for maximum protection of the drug activity. GSH/pH dual stimulus could trigger the FPZCLNs to disassembly and release the DOX outside while mildly acidic environment tended to dissociate the electrostatic interaction by protonation and increase the hydrophilicity of DOX to be released from nanohydrogels.

To test the DOX loading properties, we used the nanohydrogels with different crosslinking degree and loaded the DOX within different drug concentration solution. DOX was loaded by simply mixing with FPZCLNs in aqueous solution. The drug loading capacity as a function of the DOX concentration in solution was determined as in **Fig. 5A**. The curve rises steeply in its initial part, which suggests that DOX was absorbed with high affinity at low DOX concentrations. Then it nearly reached a plateau after the DOX concentration was above 0.25~0.35mg/mL. The plateau value of the curve reflects the maximal DOX loading capacity of the nanohydrogels, which increased with decreasing the crosslink degree, i.e. with increasing the negative charge density in the network of the nanohydrogels to increase the interaction sites between DOX and the nanohydrogels. Based on the DOX loading experiment, we selected the 15% crosslinked group (FPZCLNs-15) with 0.35 mg/ml concentration of DOX aqueous solution (DLC is 34.7 wt% and DLE is 87.8%) as an optimal nanohydrogels and condition for drug loading.

The controlled stimuli-responsive drug release of DOX-loaded FPZCLNs-15 in vitro mimicking the physiological environment and tumor intracellular environment was investigated at different pH levels to assess the drug release behavior upon different conditions. DOX was first loaded on FPZCLNs-15 under the optimum loading conditions, then they were dispersed in two different buffer solutions (phosphate buffer/pH=7.4; acetate buffer/pH=5.0). The percentage of DOX release from the nanohydrogels was measured as a function of time, as is shown in **Fig. 5B**. At physiological pH, there was approximately about 23.68 % release for the ZCLNs-15 after a 48 h incubation period, while only about 8.57 % release for the FPZCLNs-15, which was nearly **one third of** ZCLNs-15 release. Considering the premature free DOX release would lead to the unfavorable drug leakage in the blood circulation, the FPZCLNs with PEG corona displayed its great potential to encapsulate

DOX in the drug delivery carriers, which would **accumulate** tumor sites through enhanced penetration effect and increase the antitumor efficacy by continuous release in the tumor tissues.

In contrast, after adding 10 mM GSH, accelerated DOX release was observed for FPZCLNs-15, wherein approximately 50.11% of drugs were released after 48h, which might be attributed to the GSH triggered disassembly of FPZCLNs skeleton to set the drug free. Furthermore, when exposed to acid environment with pH 5.0, the drug released was much faster than that in the physiological solution and the cumulative release increased to 62.01% after 48h, which is due to the protonation of carboxylate groups and disassembly of nanohydrogels to induce the drug release. When **the DOX-loaded FPZCLNs-15 was exposed to pH 5 and 10 mM GSH environment**, the DOX release amount significantly accelerated to 50 % after 3 h, and reached 97.92 % after 48h. Obviously, the pH/GSH dual triggered drug release would offer a valid approach for drug delivery system and tumor therapy.

3.6. In vitro cell assays

The careful investigation on the cytotoxicity of FPZCLNs-15 had been investigated to check the proliferation inhibition capability for tumor therapeutic treatment. Time- and dose-dependent studies were undertaken to determine the effect of DOX delivery. Specifically, we analyzed the biocompatibility studies using HEK293T normal cells and in vitro cytotoxicity against HeLa cancer cells.

For HEK293T normal cells (**Fig. 6A**), FPZCLNs-15 had a negligible impairment in the cell viability, even after increasing the concentration of FPZCLNs-15 dosage up to the highest testing concentration of 200 $\mu\text{g/mL}$, which demonstrated its good biocompatibility and cytocompatibility with normal cells in a wide range of nanocarrier concentration (10~200 $\mu\text{g/mL}$). This result showed that FPZCLNs-15 were suitable as drug carriers in tumor therapy.

For HeLa cancer cells, in order to detect the targeted effect of FA, we prepared the PEG-conjugating Zn-crosslinked PMAA nanohydrogels (PZCLNs) product without FA modification as a control group. As is shown in **Fig. 6** (B and C), the cytotoxicity of free DOX, DOX loaded PZCLNs-15 and DOX loaded FPZCLNs-15 was dose-dependent, the DOX-loaded FPZCLNs-15 showed a higher cancer cell killing potency compared with ZCLNs-15, which was similar to pure DOX as the DOX concentration increased from 0.1-2.5 $\mu\text{g/mL}$. It was probably attribute to the highly efficient folate-receptor assisted endocytosis of DOX loaded FPZCLNs-15. In fact, for in vivo therapy applications, it was unlikely that such a high concentration of free DOX would be present for such a long treatment time. On the other hand, DOX-loaded nanohydrogels, especially the PEGylation formulation with long circulating ability might facilitate its passive accumulation at tumor tissue via EPR effect and endocytosis by tumor cells via receptor ligand effect. These data demonstrated that the DOX-loaded FPZCLNs-15 displayed high cytotoxicity with enough efficiency to kill the cancer cells. Compared with **free DOX that** would impair normal cells before reaching cancer cells, the drug-loaded FPZCLNs-15 could have a lower cytotoxicity to normal cells as the DOX hardly leaked from the FPZCLNs-15 in physiological environment, which significantly enhanced the therapeutic efficiency of DOX to tumor cells.

3.7. Intracellular drug delivery

To further investigate the cellular internalization and intracellular DOX release, we incubated the DOX-loaded PZCLNs-15, FPZCLNs-15 and free DOX with HeLa cells for 24 h at 37°C, and then

observed by confocal laser scanning microscopy. The nuclei of HeLa cells were stained with Hoechst 33342 (blue). As shown in **Fig. 7A**, red DOX fluorescence could be observed in both the cytoplasm and the cell nucleus following 24h incubation with DOX-loaded PZCLNs-15, FPZCLNs-15 and free DOX, demonstrating clear visual evidence of cellular internalization of DOX-loaded FPZCLNs-15 and free DOX. Since the nuclear pores were too small to allow the nanohydrogels in [53], it suggested that DOX molecules were released from drug-loaded nanohydrogels in the cytoplasm before entering into the cell nucleus subsequently. The DOX fluorescence intensity of PZCLNs-15 was weaker than that of FPZCLNs-15, demonstrating the efficient intracellular DOX delivery of FPZCLNs-15 via folate ligands-mediated endocytosis. In addition, fluorescence-activated flow cytometry was used to quantitative study the cellular internalization and intracellular release of DOX. The experimental results showed that the DOX fluorescence intensity in FPZCLNs-15 was 2.4 fold stronger than that of PZCLNs-15, which is consistent with the previous results.

It was worth noting that stronger DOX fluorescence could be observed in HeLa cells incubated with free DOX for 24h compared with the PZCLNs-15 and FPZCLNs-15, which in fact could be attributed to different cellular internalization mechanisms between the DOX loaded nanohydrogels (endocytosis) and free DOX (diffusion) [56]. Additionally, it was reported that free DOX possess stronger fluorescence intensity compared with the DOX loaded in the nanohydrogels at the same concentration due to the self-quenching effect of DOX [57, 58, 59]. Hence, the weaker DOX fluorescence of PZCLNs-15 and FPZCLNs-15 compared with free DOX might be explained by the slower cellular uptake of DOX-loaded nanohydrogels and the self-quenching effect of DOX in PZCLNs-15 and FPZCLNs-15.

3.8. Ex vivo DOX fluorescence imaging and in vivo biodistribution

To study the biodistribution, plasma pharmacokinetic and metabolism of free DOX, PZCLNs-15 and FPZCLNs-15 in vivo, the mice bearing H22 tumor were sacrificed at 2 h, 8 h and 24 h post tail vein intravenous (i.v.) injection. Various organs (heart, liver, spleen, lung and kidney) and tumors were collected and the DOX fluorescence intensity was analyzed using fluorescent imaging and fluorescent quantitative measurement, respectively. The results (Fig. 8A) show that at 2 h and 8 h after injection, heart, liver and kidney showed strong DOX fluorescence for free DOX group, suggesting that as foreign bodies in vivo, DOX molecules were mainly captured by the reticuloendothelial system (RES), uptaken by liver and metabolized by kidney [54, 55]. Nevertheless, the relatively weaker fluorescence in liver and kidney was observed for the injection of DOX-loaded PZCLNs-15 and DOX-loaded FPZCLNs-15, which could be attributed to the bulky hydrophilic outer PEG shell that allowed the drug to evade specific recognition by the RES system and preferentially accumulated in tumor tissue by EPR effect. In addition, a favorable biodistribution with increased accumulation in tumor and the decreased accumulation in normal tissues (especially for liver and kidney), could be observed for the injection of FPZCLNs-15, which proved the enhanced folate mediated tumor accumulation.

With the time went on, the fluorescence of the main organs in free DOX group became weaker and nearly faded off (24h), which indicated the drug molecules were gradually cleared from the mice body. However, higher intensities of fluorescence in the DOX-loaded PZCLNs-15 group and DOX-loaded FPZCLNs-15 group were found as compared to free DOX at 24 h, implicating that DOX-loaded nanohydrogels could significantly improve the pharmacokinetics of the DOX circulating time in vivo. The DOX-loaded FPZCLNs-15 group had a lower accumulation of DOX in

normal organs than the other groups (free DOX and PZCLNs-15), which confirmed that Zn(II) crosslinked nanohydrogels based drug delivery system, specifically nanohydrogels encapsulated with folate, were capable of limiting the distribution of the drug in normal organs. Furthermore, the accumulation of DOX in the tumors of the FPZCLNs-15 group was higher than that of the PZCLNs-15 group, suggesting that the folate conjugated nanohydrogels has the ability to keep therapeutic drug level at the tumor site for extended periods of time. These results indicated the Zn(II)-crosslinked nanohydrogels were able to alter the biodistribution of the DOX and provide significant benefits to enhance the tumor accumulation and reduce the drug's systemic toxicity. Notably, compared with the free DOX, the quantitative analysis results revealed that the DOX distribution in heart was reduced by 3.2 and 5.0 folds, while the DOX distribution in tumor was increased by 2.3 and 3.6 folds when delivered with PZCLNs-15 and FPZCLNs-15 respectively (Figure 8B). Furthermore, the pharmacokinetic data of DOX were obtained as presented in Figure 8C. The disappearance of DOX loaded in PZCLNs-15 and FPZCLNs-15 as well as free DOX from blood circulation occurred in an exponential manner, while plasma DOX concentration of PZCLNs-15 and FPZCLNs-15 system decreased slowly, which could keep DOX concentration at a relatively high level than free DOX, indicating an obvious retardation in clearance from the blood. The blood circulation time of PZCLNs-15 and FPZCLNs-15 was significantly extended compared with free DOX, which may promote accumulation in tumor through the EPR effect. The decreased clearance speed of DOX in plasma for PZCLNs-15 and FPZCLNs-15 compared with free DOX might be explained by the low premature drug release (shown in Fig. 2C) and enhanced circulation life in vivo of the nanohydrogels.

3.9. In vivo antitumor effect

With prolonged blood circulation, good tumor localization and stimuli-responsive biodegradability ability, the DOX-loaded FPZCLNs-15 might contribute to superior antitumor efficacy with minimal unexpected side effects. To further prove the in vivo antitumor potential of FPZCLNs-15 and PZCLNs-15, the antitumor efficacy was investigated on Balb/c mice bearing H22 tumor. The treatments were done by intravenously injecting physiological saline, free DOX, DOX-loaded PZCLNs-15 and FPZCLNs-15 respectively, into tumor-bearing mice.

The tumor volume after the treatments as a function of time is shown in **Fig. 9A**. Compared to the control group (treatment with physiological saline), free DOX group treatment showed limited tumor growth delay, while the tumor growth was effectively inhibited in both the groups treated with DOX-loaded PZCLNs-15 and FPZCLNs-15. Furthermore, FPZCLNs-15 group treatment showed more efficiency in inhibiting tumor growth compared with PZCLNs-15 group, which resulted in a significantly higher rate of tumor growth delay at the treatment dose within 16 days (**Fig. 9C**).

All the mice were alive during the experimental period. Moreover, there is no significant drop of body weight of the mice treated by PZCLNs-15 and FPZCLNs-15 groups within 30 days (**Fig. 9B**). However, an obvious weight loss (15.28%) was noted in the free DOX treatment groups at day 10, indicating that free DOX treatment has side effect which could become less toxic after loading to PZCLNs-15 and FPZCLNs-15.

Altogether, all the results and data strongly support the in vivo anticancer efficacy of DOX-loaded PZCLNs-15 and FPZCLNs-15 as well as the lower undesirable systemic toxicities to normal tissues.

4. Conclusions

In summary, we have fabricated a novel **kind of biodegradable nanohydrogels with pH/GSH dual stimuli-responsive character** inspired by zinc finger proteins via a facile method of reflux-precipitation polymerization, with ZDMA as biodegradable crosslinker and MAA as monomer. These nanohydrogels simultaneously showed excellent colloidal stability and folate-targeted ability after modification with FA. The as-prepared biodegradable nanohydrogels (FPZCLNs) could be degraded into low molecular polymer chains when exposed to slightly acidic and GSH rich environment, **which exhibited** its good biodegradability in wide range of cells, including in the reductive cytoplasm environment in tumor cells as well as acidic lysosome environment in normal cells. DOX could be easily loaded in the nanohydrogels via electrostatic attraction, which was relatively stable in physiological environment with minimal drug leakage. Under weak acidic pH and relatively high GSH concentration environment, the encapsulated DOX could be quickly released over 90% within 48h. Cell viability assays proved that DOX-loaded FPZCLNs could be internalized by HeLa cells and the released DOX could efficiently inhibit the growth of tumor cells, while FPZCLNs themselves showed nearly no toxic to HEK293T normal cells. Ex vivo DOX fluorescence images showed the DOX-loaded FPZCLNs preferentially accumulated in tumor tissue with minimal accumulation in normal tissues compared with non-targeted nanohydrogels and free DOX, **the DOX concentration delivered by FPZCLNs-15 in the tumor site was approximate 3.6 and 1.6 fold higher than free DOX and PZCLNs-15, respectively. Meanwhile, the PZCLNs-15 and FPZCLNs-15 could reduce the concentration of DOX in heart by 3.2 and 5.0 fold respectively, as compared to the free DOX group.** Moreover, the in vivo animal model experiments showed that the DOX-loaded FPZCLNs had a superior chemotherapeutic efficacy and tumor growth inhibition to tumor-bearing mice with no observable side effects on the animal behavior.

Acknowledgements

This work was supported by National Science Foundation of China (Grant No. 21474017), National Science and Technology Key Project of China (Grant No. 2012AA020204) and Science and Technology Commission of Shanghai (Grant No 13520720200).

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Schemes

Scheme 1. Schematic illustration of (a) preparation of DOX-loaded folate-PEG conjugated zinc(II)-crosslinked polymeric nanohydrogels (FPZCLNs) and (b) EPR effect and folate-mediate active-targeting of DOX-loaded FPZCLNs and stimuli-responsive DOX release profile.

Scheme 2. Illustration of the possible reaction mechanism for the nanohydrogels disassembly with stimuli-responses to GSH and weak acid.

Figures

Fig. 1. Representative TEM images of ZCLNs with different crosslinking density: (a) ZCLNs-15; (b) ZCLNs-20; (c) ZCLNs-25; (d) ZCLNs-30. All scale bars are 100nm.

Fig. 2. FTIR spectra of (A) ZCLNs with different crosslinking degrees from 0wt%, 15wt%, 20wt%, 25wt% to 30wt% (ZCLNs-0 to ZCLNs-30) and (B) ZCLNs-15 and FPZCLNs-15; (C) UV-vis spectra of Folate, ZCLNs-15 and FPZCLNs-15; (D) Dynamic light scattering of ZCLNs-15 and FPZCLNs-15

Fig. 3. Dynamic light scattering measurement of (A) the hydrodynamic diameter and (B) the relative scattering light intensity change of FPZCLNs-15, FPZCLNs-20, FPZCLNs-25 and FPZCLNs-30; Relative turbidity change of FPZCLNs-15 dispersions with (C) different GSH concentrations and (D) different pHs.

Fig. 4. GPC traces recorded for degradation of ZCLNs-15 and FPZCLNs-15.

Fig. 5. (A) Concentration-dependent of DOX loading capacity profile of FPZCLNs-15, FPZCLNs-20, FPZCLNs-25 and FPZCLNs-30; (B) DOX release profiles of ZCLNs-15 in physiological pH (7.4) condition, FPZCLNs-15 in physiological pH (7.4) condition, FPZCLNs-15 in 10 mM GSH and physiological pH (7.4) condition, FPZCLNs-15 in acidic pH (5.0) condition, FPZCLNs-15 in 10 mM GSH and acidic pH (5.0) condition.

Fig. 6. In vitro cytotoxicity of (A) FPZCLNs-15 to HEK293T cells; In vitro cytotoxicity of free DOX, DOX loaded ZCLNs-15 and DOX loaded FPZCLNs-15 to HeLa cells in (B) 24 h and (C) 48 h.

Fig. 7. Cellular uptake of free DOX, PZCLNs-15 and FPZCLNs-15 after incubation with HeLa cells for 24 h, observed by (A) confocal laser scanning microscopy and (B) flow cytometric analyses.

Fig. 8. (A) Ex vivo DOX fluorescence images and biodistribution of DOX in major organs (heart, liver, spleen, lung and kidney) and tumors of Balb/c mice bearing H22 tumor after treatment of free DOX, **PZCLNs-15** and **FPZCLNs-15** at different time points; (B) Quantitative analysis of DOX distribution in tumors and major organs examined at 2, 8 and 24 h post i.v. injection (**, $P < 0.001$; *, $P < 0.05$); (C) Plasma concentration of DOX versus time scales in mice after intravenous administration of free DOX, **PZCLNs-15** and **FPZCLNs-15** at a DOX dose of 5.0 mg kg^{-1} .

Fig. 9. (A) The relative tumor volume and (B) body weight of Balb/c mice bearing H22 tumor in (a) physiological saline, (b) free DOX (4.5 mg kg^{-1}), (c) DOX loaded **PZCLNs-15** (4.5 mg kg^{-1} DOX eq.), (d) DOX loaded **FPZCLNs-15** (4.5 mg kg^{-1} DOX eq.) groups versus the treatment time. The red arrows represent the day on which the intravenous tail vein injection was performed. (**, $P < 0.001$; *, $P < 0.05$); (C) Representative photographs of tumor tissues excised from tumor-bearing mice

Tables

Table 1 Formulations and characterization data of the ZCLNs with different crosslinking degrees (the solvent amount is 40 mL, AIBN is 15 mg).

Sample code	MAA (mg)	ZDMA (mg)	Zn amount (wt% _{Theor.})	D _{TEM} (nm)	D _{DLS} (nm)	PDI	Zeta (mV)	Zn amount (wt% _{Exper.})
ZCLNs-15	500	75	3.60	80	191	0.04	-30.1	6.42
ZCLNs-20	500	100	4.61	93	235	0.06	-26.4	8.03
ZCLNs -25	500	125	5.53	130	297	0.04	-19.1	10.35
ZCLNs -30	500	150	6.38	146	334	0.12	-16.5	11.66

Graphical abstract

A new kind of zinc(II) crosslinked poly(methacrylic acid) nanohydrogels(ZCLNs) inspired by zinc finger proteins with dual stimuli-triggered degradation has been prepared for high efficiency delivery of the anticancer drugs.