In situ injection of phenylboronic acid based low molecular weight gels for efficient chemotherapy

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Abstract

Injectable low molecular weight gels (LMWGs) based on the derivatives of phenylboronic acid were prepared and used as substrates for efficient in situ chemotherapy. The gelators as well as LMWGs were characterized by ¹H NMR, UV-vis, FTIR, MS and SEM. Anticancer drug doxorubicin hydrochloride (DOX) was encapsulated in the gels. The rheological properties and rapid recovery capability of both blank and drug-loaded gels were tested. The LMWGs were non-toxic to both 3T3 fibroblasts and 4T1 breast cancer cells. The gels were formed rapidly after injected in vivo. The in vivo anticancer activities of DOX-loaded LMWGs were investigated in breast cancer bearing mice. The intratumoral injection of DOX loaded LMWGs with dose of 30 mg/kg revealed that the gels could coat around the tumor tissues to release DOX sustainingly and maintain effective DOX concentration for chemotherapy. The systemic toxicity of DOX was reduced significantly with the in situ administration of LMWGs formulations. The injectable LMWGs exhibited excellent therapeutic efficacy and low side effects in local chemotherapy.

Keyword: low molecular weight gels, drug delivery, intratumoral injection, in situ chemotherapy

Introduction

Chemotherapy is usually used in cancer treatment.[1] The rapid clearance in blood circulation and non-specificity of chemotherapeutics in usage resulted in high administration dose, which regenerated serious side effects due to the severe toxicity of chemotherapeutics.[2, 3] The development of nanomedicine provides a potential strategy to avoid the problems, however, the risk of vein injection administration and the comprehensive barriers in vivo are the challenges in clinical applications.[4] Local injection[5] administration directly delivers chemotherapeutics to tumor sites, which has attracted great interest to provide precise administration in tumors. In order to maintain the necessary concentration of chemotherapeutics for long effective therapy, local drug delivery systems are inspired to reserve the therapeutics in situ to prolong the sustaining release and reduce the systemic toxicity to normal organs and tissues.[6, 7] Injectable hydrogels

are used to localize the therapeutics around tumors. The injectable hydrogels with in vivo sol-gel transition were reported to trap therapeutics,[8, 9] the minimally invasive administration of injectable hydrogel caused smaller scar size and less pain to patients.[10]

To most polymeric injectable hydrogels, the gelation of the precursors was responsive to stimuli such as temperature, pH value, ionic concentration and so on.[11-14] The thermosensitive polymers including poly(N-isopropylacrylamide) and polyester-PEG block polymers were extensively studied.[15, 16] However, the sol to gel phase transition of polymers triggered by temperature as stimulus was not sensitive enough to form gels rapidly in vivo, which would affect the drug diffusion.[17] Other concerns of the injectable hydrogels based drug delivery systems were the burst release and safety.[18, 19]

In recent years, low molecular weight gels (LMWGs) have attracted great interests for drug delivery. Different from polymeric hydrogels, the LMWGs were formed via the non-covalent supramolecular interactions. The gelators were self-assembled into fibers in mild conditions, the fibers further rearranged into isotropic three dimensional matrix.[20] The physical crosslink sits in the LMWGs were not fixed, which were flexible and dynamic in changing internal architectures to encapsulate therapeutics.[21] The complicated non-covalent interactions within the LMWGs including hydrogen bond, hydrophobic interaction, electrostatic interaction and π - π interaction could inhibit the free diffusion of therapeutics in the three dimensional substrates to prevent the burst release of drug. The dissociation of LMWGs in vivo was regarded as degradation and the degraded small molecule gelators were easily metabolized, thus, the LMWGs were favorable for developing as biomaterials for medical applications in biosensors, tissue engineering, 3D cell culture, biomineralization, medical diagnostics, enzyme mimics and drug delivery.[20, 22-25] Xu introduced D-amino acids in LMWGs to generate selective inhibition of local inflammation with a nonsteroidal anti-inflammatory drug (NSAID) loaded in the gels.[26] Yi prepared menthol based LMWGs to exhibit effective antimicrobial susceptibility.[27] Dastidar developed a LMWG with cetirizine drug for topical treating skin allergy in mice.[28] Patil explored vitamin B based LMWGs for targeting siRNA delivery, the released siRNA could efficiently silence gene expression and specifically target cancer cell lines.[29] Most studies on LMWGs for drug delivery were focus on the fabrication of the delivery systems and the drug loading and/or release properties in vitro, the in vivo therapeutic investigation of LMWGs based drug delivery systems were rare reported.

The purpose of this study was to study the in situ chemotherapy of phenylboronic acid modified oligopeptide based LMWGs as injectable substrates. The intratumoral injection administration of anticancer drug doxorubicin (DOX) loaded LMWGs were carried out to decrease the systemic toxicity and promote the therapeutic efficiency. The characteristics of LMWGs including gelation time and mechanism, rheological property and morphology were investigated. The in vitro antitumor activities of drug loaded LMWGs were tested in mouse fibroblasts NIH/3T3 and 4T1 breast cancer cells. The in vivo therapeutic efficacy, histological analysis, blood DOX concentration, and biodistribution of drug were evaluated in tumor bearing BALB/c mice.

EXPERIMENTAL SECTION

Materials and Measurements

3-Aminobenzeneboronic acid was obtained from Sukai Chemicals Co. Ltd. H-Gly-Gly-OH, 2, 2-dimethyl-1, 3-propanediol, di-tert-butyl dicarbonate (Boc₂O), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetra-methyluronium tetrafluoroborate (TBTU), acyl chloride, and glycine methyl ester hydrochloride were obtained from Energy Chemistry Co. Ltd. Neopentyl glycol and trifluoroacetic acid were obtained from Shanghai Aladdin Chemistry Co. Ltd. All chemicals were directly used without purification.

¹H and ¹³C NMR spectroscopy were performed on a Bruker-300 spectrometer operating at 500 MHz (¹H NMR) and 125 MHz (¹³C NMR), respectively. TMS (tetramethylsilane) was used as an internal standard. FT-IR spectra was recorded on a Bruker EQUINOX 55 spectrometer with the KBr-technique. Mass spectrometric analysis was performed on Bruker microTOF-QII HR-MS. Melting points were uncorrected and recorded on Digital Melting Point Apparatus WRS-1B. Scanning electronic microscopy (SEM) images were taken on a Nova NanoSEM 200 scanning electron microscope. UV spectra were recorded on a UV2501PC spectrometer (Shimadzu). The fluorescence spectrum was measured by HITACHI Fluorescence Spectrophotometer F-7000.

General Procedure for Synthesis

The general procedure for synthesis of gelators was shown in Scheme 1, the detailed processes were described in Supporting Information.

Scheme 1. The synthesis of gelators (compound **8a**, **8b**).

Gel preparation

The gelators of compound **8a** and **8b** in distilled water (1.0 mL) with low molecular weight poly(ethylene glycol) (PEG) were placed in flasks fitted with a reflux condenser and heated to form solutions. The solutions were cooled to room temperature and transferred into closed glass vials. The gelation was simply confirmed by turning the glass vial upside down. The gelation time was noted when the gelated mass did not flow upon tilting of the vial. The critical gelation concentration (CGC) was determined by measuring the minimum amount of gelator to form a stable gel at 25 °C. The xerogels for SEM measurement were dried at room temperature in vacuum.

Four gels of G1 to G4 were prepared for in vivo antitumor efficiency evaluation. G1 and G3 were blank gels without DOX, G2 and G4 were gels loaded with DOX. G1 was prepared with 30 mg/mL of compound **8a** in the mixture solution of PEG200:H₂O=4:1, G2 was prepared with 30 mg/mL of compound **8a** and 12 mg/mL of DOX in the solution of PEG200:H₂O=4:1. G3 was 30 mg/mL of compound **8b** in the solution of PEG200:H₂O=4:1. G4 was 30 mg/mL of compound **8b** and 12 mg/mL of DOX in the solution of PEG200:H₂O=4:1.

Cytotoxicity test

Mouse fibroblasts NIH/3T3 and 4T1 breast cancer cells were incubated with the extracts of gelators to evaluate the biocompatibility and toxicity of the gels. The cells were harvested and seeded in 96-well plates with 100 µL mediums for 24 h incubation before the tests. Extracts for indirect tests were prepared in accordance with International Organization for Standardization (ISO) tests (ISO 10993-5). Different concentration of gelators (1, 5, 10, 20, 30 and 40 mg/mL) were added to complete culture medium at 37 °C for 24 h [30]. The extracts were filtered and added to NIH/3T3 cells and 4T1 breast cancer cells seeded in 96-well plates. After incubated for 48 h, the culture medium was removed and the wells were washed with PBS (pH=7.4). CCK-8 in DMEM (10%) was added to each well. After the cells were incubated for additional 4 h, the cell viability was determined by measuring the absorption at 450 nm using a microplate reader (Thermo Scientific MK3).[31]

In vivo antitumor activity

All animal experiments were performed in line with animal experiments ethical committee for care and use of research animals. Male BALB/c mice (body weight: 18-22 g) were purchased from West China Animal Culture Center of Sichuan University. 5×10^5 4T1 breast cancer cells were injected into right flank subcutaneously of BALB/c mice. After the inoculated tumor volume reached 100-200 mm³, the mice were randomly divided into eight groups. One group was injected intravenously via the tail vein with free DOX·HCl dose of 30 mg/kg body weight as control. All other seven groups were injected intratumorally with saline, PEG200, free DOX (30 mg/kg body weight), blank gels of G1 and G3, DOX-loaded gels of G2 and G4 with DOX dose of 30 mg/kg body weight. All samples were injected for one time, the body weights of mice and the tumor volumes were measured at 3-day interval. The behaviors of mice were determined by the animal

healthcare technicians. Tumor volumes were calculated using the formula $V = (a \times (b \times b))/2$, with a the largest and b the smallest diameter.

The mice in all groups were randomly picked out and sacrificed, tumor tissues were taken out at day 7, 14 and 21 after the injection, the major organs including heart, liver, spleen, lung and kidney were taken out at day 21 and fixed in 4% formaldehyde, processed routinely into paraffin, sectioned, stained with hematoxylin and eosin (H&E) and examined by a digital microscope.

Three groups of bearing tumor male BALB/c mice were injected intratumorally with free DOX (30 mg/kg), blank gels, and DOX-loaded gels (dose of DOX 30 mg/kg) for blood test. The blood of healthy mice injected with saline was set as control. Mice were sacrificed to collect the blood for serum biochemistry assay at day 7, 14 and 21 after the injection. The serum chemistry data were measured in Chengdu Lilai Biotechnology Co., Ltd.

Immunohistochemical analysis of CD31, Ki-67 and TUNEL

Streptavidine peroxidase methods were specially designed for Immunohistochemical (IHC) reactions and other immune detection. The paraffin-embedded tumor sections were deparaffinized, rehydrated and incubated overnight at 4 °C with primary monoclonal antibody against CD31 or Ki-67 (1:200) (British abcam-(Shanghai) trading Co., LTD). Biotinylated goat antirabbit antibodies were used as secondary antibodies at 1:200 for 30 min at room temperature and the egg protein reagent marked with horseradish peroxidase was added. The data of immunohistochemistry staining was obtained as described. IHC images were taken by the Motic Images Advanced software (Motic China Group CO., LTD.), the positive-stained integrated optical density (IOD) of the CD31 and ki-67 was scaled with each image by Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). The microvessel density (MVD) was counted by reading each section which referenced Weidner method for quantitative analysis and the CD31 positive area was expressed in endothelial cell pack. In addition, the ki-67 density in each photograph was calculated as the ratio of ki-67-positive area to total area. For terminal deoxynucleotidyl transferase mediated UTP nick end labeling (TUNEL) assay, the dewaxed and rehydrated tumor tissue sections were incubated with proteinase K at 37 °C for 15 min, rinsed with PBS twice. The TUNEL assay was performed on in situ cell death detection kit-POD (Roche Group, Switzerland) to evaluate apoptotic cells. Positive TUNEL staining was visualized by optical microscopy and the apoptotic index was formulated as the ratio of apoptotic cell number to total tumor cell number in each microscope field.

Image studies

The in vivo distribution of free DOX and DOX-loaded gel G4 in the tumors bearing male BALB/c mice was investigated using ex vivo imaging. One million 4T1 cells suspended in 100 µL PBS were injected into the same treating region of each mouse. Two or three weeks later, the mice were intratumorally injected with 50 µL aqueous solutions of DOX, DOX-loaded gels and physiological saline (as control). The injected doses of DOX were normalized as 30 mg/kg. At the selected time points, the mice were randomly picked out and sacrificed. The organs of heart, liver, spleen, lung, kidney and tumors were excised for optical imaging through Maestro In-vivo Imaging System

with the saline group as blank control. Meanwhile, the tumors were frozen and ground after being taken photos. The tumor tissues were extracted with 5 mL of chloroform/isopropanol (4:1, v/v) by vortex mixing for 120 s. After centrifugation at 10000 rpm for 5 min, the organic phase was separated and dried under nitrogen atmosphere at room temperature overnight. The residue was dissolved in 1 mL of DMSO. After centrifugation at 10000 rpm for 3 min, the supernatant was tested by fluorescence spectra. The standard curve of DOX in chloroform/isopropanol (4:1, v/v) was produced by adding different concentrations of DOX to chloroform/isopropanol (4:1, v/v). DOX distribution in the tumors was studied.[32]

Statistical analysis

Data were analyzed using Prism 4 (Graph Pad Prism 5 Software, San Diego, CA) and expressed as mean $\pm$ standard (SD) deviation. Multiple groups were compared using one-way ANOVA. Two groups were compared using T-test. Statistical significance was set at $p < 0.05$.

Results and discussion

Characteristics of Gels

The synthetic route of gelators was presented in Scheme 1. The gelators (compound **8a** and **8b**) with different length of alkyl chain exhibited excellent gelation capability in different kind of solvents such as ethanol, PEG-200, and PEG-400. As the gelation could not be formed in water as the gelator could not dissolved in water completely, therefore, the mixed solvent of water and PEG200 was used. The gels were formed in the mixed solvent of PEG200/H₂O when heated to 70 °C and cooled to room temperature. The critical gelation concentration (CGC) was depended on the solvents. The proportion of PEG200 and water affected the gelation time, the increase of the proportion of water was favorable to shorten the gelation time. For in-situ injection therapy, transparent gels were obtained by adding the gelators to the mixture solvent of deionized water and PEG-200 (PEG200:H₂O=4:1) for its promising gelation time. Drug-loaded gels were prepared by adding both DOX and gelators to the mixture solvent. The images of the sol, gel, and DOX-loaded gel were shown in Figure 1A.

As the gelation capability and stability of the gelator would affect the formation of gel in vivo, the rheological properties of two blank gels and DOX-loaded gels were studied. The storage modulus (G') of the four gels was higher than their corresponding loss modulus (G''), revealing the formation of gels (Figure 1).[33] The frequency dependence of the dynamic storage modulus (G') and loss modulus (G'') decreased with decreasing the alkyl chain of gelators under the same frequency (Figure 1C and 1D), which demonstrated that the gelation could be further controlled with the adjustment of alkyl chain length. The gels were also formed after DOX was loaded, the storage modulus of drug loaded gels (G_2 and G_4) were similar to those of the blank gels (G_1 and G_3). Further investigation was carried out to the DOX-loaded gel (G_5) at the body temperature of 37 °C, little higher storage modulus was observed comparing to blank gel. This property ensured the injectable characteristic of the gels in subcutaneous, intratumoral and in vivo drug delivery applications. The recovery property of the gel was studied (Figure 1B). The gel was first subjected

to a large strain of 50% for 100s and the recovery was probed at the strain of 0.1% and frequency of 1 rad/s. The storage modulus G' recovered to 40% of the initial G' to 2 KPa in 300 s, and the recovery was 80% of the initial G' within 1500 s after the stress was removed. This thixotropic LMWG showed shear-thinning properties and it could quickly recover gel after the removal of the applied stress. The rapid recovery capability was mainly attributed to the easy rearrangement of the physical interactions of hydrogen bond and π - π stacking interaction within the gel. The gelation was formed in less than 15 min after subcutaneous injection as shown in Figure 1A (d, e, f), the gels could be formed both in subcutaneous and intratumoral injection.

Figure 1. The image and rheological properties of gels, (A) images of sol (a), gel (b), and DOX-loaded gel (c), gel formed after subcutaneous injection (d), DOX-loaded gel formed after intratumoral injection (e), DOX-loaded gel in tumor (f); storage and loss modulus as a function of angular frequency for the gels, (B) Recovery of gel generated by compound **8b** in the mixture solvent of PEG200:H₂O=4:1, the gel was first subjected to a large strain of 50% for 100s and then the recovery was probed at the strain of 0.1% and frequency of 1 rad/s. (C) The storage and loss modulus of G1 (30 mg/mL of compound **8a** in PEG200:H₂O=4:1) and G2 (30 mg/mL of compound **8a** and 12 mg/mL of DOX in PEG200:H₂O=4:1); (D) the storage and loss modulus of G3 (30 mg/mL of compound **8b** in PEG200:H₂O=4:1), G4 (30 mg/mL of compound **8b** and 12 mg/mL of DOX in 1 mL PEG200:H₂O=4:1) and G5 (30 mg/mL of compound **8b** and 12 mg/mL of DOX in 1 mL PEG200:H₂O=4:1, tested at 37 °C). All the samples were carried out at 25 °C except G5.

The good gelation capability was due to the chemical structure of gelators. The self-assembly of

gelators was driven by the hydrophobic interaction as well as hydrogen bonding and π - π interaction within the gelators[34] as illustrated in Figure 2. The ^1H NMR spectra of gelators with different concentrations were used to explore the formation of hydrogen bonding and π - π interaction.[35] The protons of different N-H shifted downfield within 7.5 ppm to 10 ppm revealed that the hydrogen bonding was strengthened when the concentration of gelators increased from 16.8 mg/mL (sol) to 76.0 mg/mL (gel). The ^1H NMR spectra of gelator in Figure 2A showed the gradual downfield shift of protons with increasing the concentration of gelator, the proton in O-H of PEG200 shifted downfield from 5.22 ppm to 5.37 ppm. The protons of PEG200 were involved in hydrogen bonding to accelerate the self-assembly of gelators.[36] The aromatic protons in the gelators were also gradually shifted downfield. Red shift was observed in the UV spectra of gelator with increasing concentrations, which indicated the formation of π - π stacking interaction (Figure 2B).[37] The supramolecular self-assembly fibers were observed in the SEM image after the gel was dried (Figure 2C).

Figure 2 (A) The ^1H NMR spectra of the gel (Compound **8a**) with different concentrations in the mixture solvent of PEG200:D₂O=4:1, which demonstrated the hydrogen bonding and the π - π interaction; (B) the UV spectra of gelators in ethanol with increasing the concentration; (C) the SEM image of the gel.

In vitro cytotoxicity

In vitro cytotoxicity tests of the gels were evaluated using NIH/3T3 fibroblasts and 4T1 breast cancer cells. The results were shown in Figure 3. The cell viabilities of medium were used as control. The cell viabilities were beyond 90% for all gelators' extracts, even the concentration of

gelator was as high as 40 mg/mL. It implied that the gels were non-toxic to cells.

Figure 3. The cytotoxicity of gelators incubated with (A) NIH/3T3 cells and (B) 4T1 breast cancer cells. The data were expressed as mean $\pm$ standard deviation (n=5).

In vivo antitumor activity

The in vitro release of DOX from the DOX-loaded gels was investigated in PBS with different pH values as shown in Fig. S1. The cumulative release rate of DOX from the gels increased with decreasing the pH values of buffered solution. A total of 76.9% of the loaded DOX was released in buffered solution at pH 5.5 in 108 h, whereas only 25.5% DOX at pH 6.8, 15.6% DOX at pH 7.4, and 8.2% DOX at pH 8.2 were released in the same time. The gels exhibited promising sustained release of DOX at the mimic physiological environment (pH 7.4) and weak acidic tumor microenvironment (pH 6.8). The sustaining release of DOX from the drug-loaded gel was closely related to the π - π stacking interaction between DOX and gelators, which was characterized by fluorescence spectra (Fig. S2). The fluorescence spectra of DOX with different concentrations were measured (Figure S2A), the increase of DOX fluorescence was attributed to the increase of DOX concentration from 7.5 to 50 μ g/mL when excited at 480 nm. The fluorescence quenching occurred once the concentration of DOX reached 75 μ g/mL. As for the mixed solution of gelators and DOX·HCl, the intensity of DOX fluorescence increased with increasing the concentration of the mixture till 35 μ g/mL (Figure S2B). The quenching occurred when the concentration was higher than 50 μ g/mL. The quenching of fluorescence indicated the π - π stacking interaction between drugs and gelators was formed after DOX was encapsulated in the gels due to the π conjugated moieties in both DOX and gelators. Attributed to these π - π stacking interaction between DOX and gelators, the drug-loaded gel exhibited sustaining release of DOX not only to maintain the concentration for chemotherapy, but also to reduce the side effect.

As the gel exhibited excellent sustaining release of DOX without burst release, large dose of DOX of 30 mg/kg was administrated to investigate the therapeutic effect (Fig. 4). Eight groups of tumor-bearing BALB/c male mice were treated with different formulations (saline, PEG200, blank G1, blank G3, DOX-loaded gel G2, DOX-loaded G4, and free DOX) in intratumoral injection or tail intravenous injection. The tumor inhibition rate of these groups was showed in Fig. 4A. The saline and PEG200 groups did not show any inhibition effect. The tumor volume increased rapidly.

Both blank gels of G1 (compound 8a) and G3 (compound 8b) showed a little inhibition effect possibly due to the oppression of gels around tumor tissue resulting in cells death. Both DOX-loaded gels (G2, G4) exhibited promising inhibition efficiency, which were much better than those groups of free DOX·HCl injected intratumorally or intravenously via the tail vein. The inhibition effect of G4 was the best in the six therapeutic groups. The ideal therapeutic effect of drug-loaded gels was because that the drug was limited around tumors by gel to enhance the anticancer activity in vivo.

Other than inhibition rate, less side effect was also important to evaluate the therapeutic efficacy. Although group h, which was administrated with free DOX·HCl via tail intravenous injection in situ injection, exhibited good tumor inhibition efficiency, serious side effects including hydroadbomen and intestinal adhesion were observed in the groups due to the high dose of DOX administration (Fig. S3), which resulted in high mortality of 80% within 10 days (Fig. 4C). However, to the group f treated with DOX-loaded gel G4, partial tumor-bearing BALB/c male mice were cured after three weeks (Fig. S4). The sustaining release of DOX from gel not only maintained the concentration for chemotherapy but also reduced the side effect to enhance the therapeutic efficacy greatly.

The body weight and survival rate of the tumor bearing mice were monitored. Less body weight shift was found in the BALB/c male mice administrated with gels formulations, saline and PEG200 (Fig. 4B). This suggested that the mice showed better drug tolerability to gels formulations. About 20% and 50% body weight decreases were observed in free DOX·HCl formulation groups via intratumoral and intravenous injection, respectively. These results revealed that the gels could reduce the systemic toxicity of large dose of DOX. The survival rates of the mice of the eight groups were also measured (Fig. 4C). All the mice of the group treated with DOX·HCl by intravenous injection via the tail vein died in 24 days. Only 25% of the mice in the group treated with DOX·HCl by intratumoral injection were survival within 27 days, and the survival ratios of both the groups treated with blank G1 or blank G2 were 50%. The survival ratios of the groups treated with DOX-loaded G3 and DOX-loaded G4 were 75% and 87.5%, respectively. The survival rates of the groups treated with gels were higher than other treatments. The gels loaded with large amount of anticancer drug exhibited excellent therapeutic efficacy both in tumor inhibition and survival rate.

Figure 4. The in vivo anticancer activity of drug loaded gels, A: the volumes of tumors treated with drug loaded gels; B: body weights of the mice treated with drug loaded gels; C: survival rate of tumor-bearing mice; p values at 21 days. ($P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***). a Group: saline; b Group: PEG200; c Group: blank G1; d Group: blank G3; e Group: DOX-loaded G2; f Group: DOX-loaded G4; g Group: DOX·HCl; h Group: DOX·HCl (intravenous injection), DOX equivalent (dose of 30 mg DOX/kg mice). G1 was compound **8a** in PEG200:H₂O=4:1 (30 mg/mL), G2 was compound **8a** (30 mg/mL) and DOX·HCl (12 mg/mL) in PEG200:H₂O=4:1, G3 was compound **8b** in PEG200:H₂O=4:1 (30 mg/mL), G4 was compound **8b** (30 mg/mL) and DOX·HCl (12 mg/mL) in PEG200:H₂O=4:1.

Serum biochemistry assays were also performed to assess the potential toxicity of DOX-loaded gels in Fig. 5.[38] Mice were sacrificed to collect the blood for serum biochemistry assays at day 7, 14 and 21 after injection. Blood of healthy mice injected with saline was set as control. Blood routine were investigated including red blood cell count (RBC), white blood cell count (WBC), blood platelet count (PLT), and so on. There was no obvious damage of DOX-loaded gels on the tumor-bearing mice. The WBCs of saline group, PEG200 group and blank gels groups were out of the normal range, indicating the mice had severe inflammation and bleeding symptoms. These results implied that DOX-loaded gels could effectively inhibit inflammation and symptoms of congestion.

Figure 5 Test of blood routine of a Group: saline; b Group: PEG200; c Group: blank G1; d Group: blank G3; e Group: DOX-loaded G2; f Group: DOX-loaded G4; g Group: DOX·HCl; h Group: DOX·HCl (intravenous injection), DOX equivalent (dose of 30 mg DOX/kg mice). All the groups except h group were injected via intratumor. The data were expressed as mean $\pm$ standard deviation (n=5) (P<0.05 *, P<0.01 **, P<0.001 ***)

The histological tissues slides of tumor in three phases as well as heart, liver, spleen, lung and kidney of the tumor-bearing mice were presented in Fig. 6. The samples of DOX-loaded G4 and DOX injected intratumorally showed much higher tumor inhibition effect (tumor images in f and g, Fig. 6A) as the tumor necrosis with hemorrhage appeared in most areas. In addition, with the increase of treatment time, the area of the tumor necrosis area decreased rapidly (tumor images in I, II and III, Fig. 6A). However, compared to gels formulations, the formulations of free DOX·HCl injected intratumorally and intravenously appeared heart toxicity for the observation of necrosis (heart image in g and h, Fig. 6B) with acute inflammatory cells infiltration in epicardium. congestion between myocardial cells and acute inflammatory cells infiltration (liver image in g and h, Fig. 6B) were observed in hepatic sinus and blood vessels. Simultaneously, in saline group, PEG group and blank gels groups, little necrosis (tumor image in a, b, c and d, Fig. 6A) area in center of neoplastic cells was observed. Especially, in saline group, with the treatment time increasing, the area of tumor necrosis area increased rapidly (tumor images in a I, II and III, Fig. 6A). The disappearance of white pulp atrophy and splenic corpuscle were observed in spleen (spleen images in b and c, Fig. 6B). Multifocal metastases of cancer in serosa of lung were observed (lung images in a, b, c, d and h, Fig. 6B). Other groups were in weak inflammation. Hyperemia bleeding of renal interstitial appeared in kidney (kidney images in a, b, c, d, g and h,

Fig. 4B) in two free DOX·HCl groups, saline group and blank gels groups. All the organs of heart, liver, spleen, lung and kidney in DOX-loaded gels groups were normal in the histological tissue slides, indicating the non-toxicity of gels formulations.

Figure 6. Histological analysis of different organs in tumor-bearing mice with A: tumor image in eight groups. (I : 7th day; II : 14th day; III: 21th day) B: major organs in tumor bearing mice. a Group: saline; b Group: PEG200; c Group: blank G1; d Group: blank G3; e Group: DOX-loaded G2; f Group: DOX-loaded G4; g Group: DOX·HCl; h Group: DOX·HCl (intravenous injection), DOX equivalent (dose of 30 mg DOX/kg mice).

Immunohistochemical analysis of CD31, Ki-67 and TUNEL

Invasion and metastasis always accompanies with angiogenesis in the process of growth for solid tumors.[39] To investigate the antitumor efficacy and the tumor suppression mechanism mediated by DOX-loaded gels, the CD31 and Ki-67 antigen staining were performed to assess the antitumor efficacy on tumor cell proliferation, and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was employed to detect apoptotic programmed cell death as shown in Fig. 7. The CD31 and Ki-67 immunohistochemical staining studies showed that the DOX-loaded G4 resulted in the least number of microvessel density (MVD) and Ki-67-positive cells compared to other seven groups, which suggested that the cell proliferation in DOX-loaded

gels treated group was less active, and the anticancer activity was considerable higher than that of free DOX injection groups. In addition, from the CD31 and Ki-67 immunohistochemical staining studies, it showed that the number of MVD and Ki-67-positive cells decreased with increasing treatment time (I , II and III in e, f, g and h, Fig.7). However, the CD31 and Ki-67 immunohistochemical staining studies in saline group and PEG200 group showed the opposite results with the increase of treatment time (I , II and III in a and b, Fig. 7). About 49% of apoptotic cells in 7 day and 73% of apoptotic cells in 21 days were obtained in the tumor-bearing mice treated with DOX-loaded G4 gels, it was the highest apoptosis among all the eight groups. In addition, only ~38% of apoptotic cells in 7 days and ~57% of apoptotic cells in 21 days were observed in the tumor-bearing mice administrated with DOX injected intravenously. This result revealed the formulation of DOX-loaded G4 gels was more effectively to induce the apoptosis of cancer cells. The apoptotic index of saline and PEG200 group decreased gradually with the increase of treatment time (I , II and III in a and b, Fig.7). The inhibition of cancer cell proliferation as well as induction of apoptosis was the pathway of DOX-loaded gels to achieve significant anticancer activity.

Figure 7. The CD31, Ki-67 and TUNEL immunohistochemical (IHC) staining of tumor tissues (all tissues: $\times 400$). The brown areas indicated CD31-positive, TUNEL-positive, or Ki-67-positive staining. Representative photomicrographs of 4T1 breast cancer cells harvested at the end of study from mice with various treatment: a Group: saline; b Group: PEG200; c Group: blank G1; d Group: blank G3; e Group: DOX-loaded G2; f Group: DOX-loaded G4; g Group: DOX·HCl; h Group: DOX·HCl (intravenous injection), DOX equivalent (dose of 30 mg DOX/kg mice). All the groups except h group were injected via intratumor. The CD31 positive area was expressed in endothelial cell pack and counted capillary number by reading each section (MVD). The Ki-67 density in each image was calculated by Ki-67-positive area/total area. The apoptotic index was calculated as a ratio of the apoptotic cell number to the total tumor cell number in each field of view. Data were presented as mean $\pm$ SD (n = 5). (P<0.05 *, P<0.01 **, P<0.001 ***)

The fluorescence image studies

The ex vivo images of organs of tumor-bearing mice injected with free DOX and DOX-loaded G4 intratumorally were carried out to study the in vivo drug release (Fig. 8A). [40, 41] DOX was found accumulated in tumor after 1 h post-injection in the sample of DOX-loaded G4, the strong fluorescence intensity signal was maintained for more than 12 h. To free DOX group, much weaker fluorescence was observed (Fig. 8A). The average fluorescence intensities of the ex-vivo images were available in Fig. 8B. In addition, the total amount of drug in tumors was further tested (Fig. 8C). 90.1% of DOX was retained in tumor in the DOX-loaded G4 group, and only 50% of DOX was retained in tumor in the free DOX group at 1h after post-injection. 30.7% of DOX was maintained in tumor after 12h in the free DOX group, and more than 76.2% of DOX was maintained in the DOX-loaded G4 group. Free DOX injected into tumor might diffuse into

surrounding tissues and was cleared rapidly by liver and kidney during the blood circulation. The encapsulation of gels could retained the clearance of DOX and the DOX could be retained within gels to maintain sustaining drug release in effective concentration to kill cancer cells.[42]

Figure 8. (A) The ex vivo fluorescence images. The mice bearing tumors were injected one time with free drug DOX, DOX-loaded G4 (DOX equivalent, dose of 30 mg DOX/kg mice) and saline (as control). At the selected time points of post-injection, the mice were sacrificed, the organs (heart, liver, spleen, lung and kidney) and tumors (n = 3) were excised from mice for ex vivo image in sequence. All the organs and tumors were placed in the order of heart, liver, spleen, lung, kidney and tumor. I to IV represents 1 h, 4 h, 8h, and 12h, respectively. For all the images from I to IV, the organs and tumors were placed in the order of control, DOX-loaded gels and free DOX groups from top to bottom. (B) The average intensities of signal of DOX-loaded gel group and DOX group were counted using Maestro in vivo imaging system. (C) The amount of drug in tumors. The mice bearing tumors were injected one time with free drug DOX, DOX-loaded organogel (DOX equivalent, dose of 30 mg DOX/kg mice) and saline (as control). At the selected time points of post-injection, the mice were sacrificed, and tumors (n = 3) were excised from mice for extracting DOX in sequence.

Conclusion

Biocompatible gels using phenylboronic acid gelators were fabricated in the mixture solvent of deionized water and PEG-200 (PEG200:H₂O=4:1) for in-situ injection chemotherapy. The rheological properties and recovery capability of both blank gels and DOX-loaded gels ensured

the rapid formation of gels in vivo. The DOX-loaded G4 exhibited the best therapeutic efficiency, some tumors even vanished in tumor-bearing mice with intratumor injection. The ex vivo imaging study revealed that DOX could sustainingly be released from gels and maintain effective concentration for chemotherapy. The systemic toxicity of DOX was reduced greatly with the encapsulation of gel and the side effect of DOX was weakened significantly. This phenylboronic acid-based in-situ-forming gel is a promising drug delivery system for local chemotherapy.

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