Design of injectable agar-based composite hydrogel for multi-mode tumor therapy

Chenyao Wu\textsuperscript{a,1}, Jiulong Zhao\textsuperscript{b,1}, Fei Hu\textsuperscript{a}, Yuting Zheng\textsuperscript{a}, Hailun Yang\textsuperscript{a}, Shunjie Pan\textsuperscript{a}, Shenghua Shi\textsuperscript{a}, Xin Chen\textsuperscript{c,*}, Shige Wang\textsuperscript{a,c,*}

\textsuperscript{a} College of Science, University of Shanghai for Science and Technology, No. 334 Jinong Road, Shanghai 200093, People’s Republic of China
\textsuperscript{b} Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai 200433, People’s Republic of China
\textsuperscript{c} State Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Fudan University, Shanghai 200433, People’s Republic of China

ABSTRACT

We designed an injectable hydrogel by dissolving MoS\textsubscript{2}/Bi\textsubscript{2}S\textsubscript{3}-PEG (MBP), doxorubicin (DOX) and agar into water for the concurrent tumor photothermal and chemotherapy. The formed solution was able to be intratumorally (I.T.) administered into tumor at a relatively high temperature and automatically formed a hydrogel after cooling to body temperature. The resultant Agar/MBP/DOX (AMD) hydrogel can act as a macro-vessel to retain the MBP nanosheet and DOX and restrict their access to body fluids. Moreover, AMD hydrogel did not compromise the photoacoustic and computed tomography imaging capacity, as well as the photothermal and chemotherapy efficiency of MBP nanosheets and DOX. The heat from the photothermal transformation of MBP nanosheet can promote the drug-release from the hydrogel and thus enable an on-demand drug release. Furthermore, antibiotics were also able to be encapsulated in the hydrogel to avoid the potential wound infection during tumor surgery.

1. Introduction

As a minimally invasive tumor ablation method, photothermal therapy (PTT) has been intensively researched for the management of malignant tumor in recent years (Wang, Li, Chen, Chen et al., 2015; Wang, Zhao et al., 2017). Owing to the specific accumulation of photo-absorbing agents in tumor, the tumor temperature can be elevated selectively and accurately during PTT. Consequently, despite not being clinically provable as traditional microwave thermotherapy (Cao & Liang, 2015), ultrasound focusing thermotherapy (Wang et al., 2016), and radiofrequency thermotherapy (Vogl et al., 2014), PTT owns distinctive superiorities and helps to gain a significant insight into the selection of high efficient tumor therapy methodologies. To date, a great diversity of nanomaterials including carbon- (Zhang, Wu et al., 2015), metal- (Shi, Huang, Chen, Weng, & Zheng, 2015; Tang, Chen, & Zheng, 2014, 2015; Thomas, Gomez, Palma, Yap, & Shea, 2014; Zhao, Shi, Huang, Tang, & Chen, 2014), transition-metal chalcogenides/dichalcogenides- (Chou et al., 2013; Yang et al., 2015b; Liu, Wang, Gu et al., 2014; Yin et al., 2014), transition metal oxide- (Song, Hao et al., 2015), black phosphorus nanosheet- based nanomaterials (Shao et al., 2016; Sun et al., 2016; Wang, Li, Chen, Cai et al., 2015; Yang et al., 2015a) have been actively explored as promising and versatile platforms for cancer theranostics. The latest trend of tumor PTT is to construct intelligent agents to realize the tumor imaging, synergetic chemotherapy and hyperthermia within a single platform (Cheng et al., 2015; Yang et al., 2015). These intelligent agents were constructed by either adsorbing drugs onto the surface, active sites, coatings, pores, or channels of one nanoplateform, or establishing these configurations on the nanoplateform to introduce drug payload capacity. The driving force for such a trend is that the designed intelligent agent can not only preserve the original functions of individual ingredient, but also give birth to new functionality such as stimuli-responsive drug release to enhance the therapeutic efficacy.

Among the reported PTT therapeutic nanostructures, indocyanine green (ICG) has been approved by US Food and Drug Administration (FDA) as a kind of NIR dye for clinical uses (Sheng et al., 2013), however, the direct use of ICG as NIR photo-absorbing agent is undesirable since free ICG has inherently poor solubility, inevitable possibility of nonspecific protein binding, and lacking tumor-targeting specificity (Song, Chen, & Liu, 2015). As for inorganic nanostructures, although no obvious cytotoxicity were reported in labs, more careful studies are required to comprehend the detailed
metabolism of nanomaterials in the long run (Liu, Wang, Cui et al., 2014; Liu, Wang, Gu et al., 2014). Multifunctional nanostructures also suffered from bottlenecks such as limited drug accumulation, drug loss, multi-drug resistance and side effects toward normal tissues (Gao et al., 2011; Ma et al., 2013). Therefore, although currently reported multifunctional nanostructures have shown some tentative and impressive tumor therapy efficiency both in vitro and in vivo, there is still much room to enlarge their clinical applications. In this regard, exploring a representative therapeutic modality that can increase the utilization of photo-adsorbing agent and drug, enhance the therapeutic efficiency while coordinately decrease the side effects may hold the key to creating more opportunities for high efficient and on-demand tumor therapy.

Polymer gels have been known for centuries and applied in fields as diverse as food, medicine, materials science, cosmetics, pharmacology, sanitation, etc (Sangeetha & Maitra, 2005). Recently, the design of injectable and phase-changeable polymer gel has become a research hotspot in regenerative medicine and in situ therapeutic delivery (Feng et al., 2017; Nguyen, Jeon, Krebs, Schapira, & Alsberg, 2014; Thomas et al., 2014; Zhang, Wang, Guo, & Ma, 2014). These laden polymer gels can be prepared under the activation of certain internal or external stimuli such as temperature, pH, ions, solvent, and so on (Y. Li, Rodrigues, & Tomás, 2012; Li, He, Yuan, Dong, & Chen, 2016; Zhan, Gonçalves et al., 2015). On the basis of a sol-gel phase transition of the thermosensitive poly(organophosphazene) hydrogel at body temperature, Song et al. proposed a magnetic hyperthermia mediated TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) release system for combined tumor therapy (Zhang & Song, 2017). Another kind of polymer, alginate, was also extensively studied in the construction of composite hydrogel for different biomedical applications due to the fact that alginate possesses many favorable properties required in biomaterials (Avritscher et al., 2014; Rowley et al., 1999).

However, a sufficient study of hydrogel based platform for imaging guided multifunctional tumor therapy is still lacking in literature to our knowledge.

Agar is a biocompatible polysaccharide that contains a rich variety of essential elements of human body like iodine, calcium, iron, sodium and magnesium (Wang, Dong et al., 2017). Agar can be readily dissolved into hot water and gelatinized when cooled to 37 °C. Based upon this outstanding feature, we herein designed an injectable and phase-changeable multifunctional hydrogel consisting of agar, MoS2/Bi2S3-PEG (MBP), and doxorubicin (DOX) for tumor photothermal and chemotherapy. The hydrogel was constructed by dispersing MBP nanosheets and DOX into the hot solution of agar and naturally decreasing the solution temperature to 37 °C. In this hydrogel system, the entrapped MBP nanosheets and DOX can achieve the computed tomography (CT)/photocoacoustic (PA) dual-model imaging guided tumor PITT and chemotherapy purpose, respectively. Moreover, the heat from the photothermal transforming of MBP can promote the drug permeation within the hydrogel and release to surrounding environments to realize an on-demand drug release. The I. T. injected hydrogel can act as a macro-vessel for precise and on-demand therapeutic release. Additionally, the hydrogel system can substantially decrease the side effects of MBP and DOX on normal organs by retaining the photo-adsorbing MBP nanosheet within tumor and restraining their entrance into the blood stream during the therapy. The therapeutic effectiveness of the hydrogel was evaluated via monitoring the growth inhibition efficiency of tumor that was I. T. administered with Agar/MBP/DOX solution, and the anti-recurrence effects after implanting the in vitro formed hydrogel into the wounds left after the tumor surgical excision. Finally, antibiotics (take amoxicillin (AMX) as a model) can also be wrapped into the hydrogel to avoid the potential wound infection during the tumor surgery. This research provides the first demonstration that the combination of photothermal and chemotherapy with an in situ formed biocompatible hydrogel can lead to a high tumor-synergistic therapy and exert anti-relapse as well as anti-inflammatory effects simultaneously.

2. Experimental

2.1. Preparation and characterizations of MBP nanosheets and AMD hydrogels

MBP nanosheets were synthesized according to our previous study (Wang, Li, Chen, Cai et al., 2015): briefly, 150 mg (NH4)2MoS4 and 150 mg Bi(NO3)3·5H2O were dissolved in 30 mL PEG-400 (polyethylene glycol with molecular weight of 400 Da) and magnetically stirred for 30 min. Then, 30 mL H2O was supplemented and magnetically stirred for another 30 min. After that, the resultant solution was transferred to a 100 mL polyphenylene-lined stainless steel autoclave. The autoclave was heated at 220 °C in an oven for 12 h. The resultant MBP nanosheets were thoroughly washed with monoethanolamine solution (50%, in water, v/v) once, water 3 times and dispersed in water and the Mo and Bi concentration was determined with Agilent 700 Series ICP-OES for further use. The molar ratio of Mo and Bi in the formed MBP nanosheets was also calculated based on the ICP-OES result.

Hydrogel was prepared as follows: 1.5 g agar powder was dissolved in 100 mL water at 90 °C in a water bath to form a homogenous solution. MBP nanosheets with Mo concentration of 200 ppm and DOX (or AMX) with concentration of 100 ppm were dispersed into the formed agar solution (the final concentration of DOX and AMX was 0.18 μM and 0.24 μM, respectively). Agar, Agar/MBP, Agar/DOX and Agar/MBP/DOX (AMD) hydrogels can be prepared by directly cooling the corresponding solutions in vitro and in vivo (solutions were I. T. administered).

2.2. In vitro photothermal performance of AMD hydrogel

NIR laser used in this study was produced by a high power multimode pump laser (wavelength = 808 nm, Shanghai Connet Fiber Optics Company). The in vitro photothermal performance of AMD hydrogel was analyzed by continuously irradiating AMD hydrogel and saline that containing AMD hydrogel with 808 nm laser. MBP concentration (Mo concentration of 0, 100, and 200 ppm), power density (0.3 W/cm², 0.6 W/cm², and 1 W/cm²), and irradiation time (0–5 min) dependent temperature increase of AMD hydrogel was monitored with FLIR™ E60 infrared camera. The starting temperature of the in vitro photothermal performance test was about 10 °C.

2.3. In vitro and in vivo CT and PA imaging

All of the tumor models used in this research were established as follows: 1 × 10⁷ HT29 cells dispersed in 150 μL serum-free RPMI-1640 medium were subcutaneously injected into the back of Balb/c nude mouse. A tumor nodule with diameter of ~0.5 cm can be found after 2 weeks feeding. For the in vitro CT imaging, 1 mL of Agar/MBP/DOX solution with Mo concentrations of 50, 100 and 200 ppm was pipetted into an eppendorf tube. For in vivo CT, HT29 tumor bearing Balb/c nude mice were I. T. injected with 50 μL Agar/MBP/DOX solution (Mo concentration = 100 ppm, solution temperature was higher than 40 °C). Mice were anesthetized 1 h after the I. T. injection to harvest the tumor into a 1.5-mL Eppendorf tube. The in vitro and in vivo CT contrast enhancements (Hounsfield units, HU) were determined with a GE CT imaging system (operated at 100 kV, 200 mA and a slice thickness of 0.625 mm). For the PA imaging, the tubes and tumors for CT scanning were analyzed to record the in vitro and in vivo PA imaging values. The in vivo PA imaging was performed at the wavelength range of 680–970 nm. The in vitro and in vivo PA imaging pictures at 808 nm were recorded using the Vevo LAZR PA Imaging System.
2.4. In vitro biocompatibility of AMD hydrogel

50 μL of Agar/MBP solution was added to 96-well cell culture plate to allow the gelatinization with the temperature decrease. The formed gel was then transferred to another 96-well cell culture plate that was seeded with L929 cells (1 x 10⁴ cells per well). The control group was added with 50 μL of saline. Cells were incubated with Agar/MBP hydrogel for 1 day and 3 days, and their viability was evaluated using CCK-8 assay according to the instructions. After that, microscopic observation using inverted phase contrast microscopy and trypan blue staining were carried out to quantitatively determine the cytocompatibility of Agar/MBP hydrogel. Before the CCK-8 assay, microscopic observation and trypan blue staining, the solid hydrogel was discarded and cells were rinsed with phosphate buffered solution (PBS) for 3 times.

2.5. In vitro DOX release and tumor synergetic therapy

The influences of pH (5.0 and 7.4) and temperature (37 °C and 60 °C) on DOX release were monitored using UV–vis spectroscopy. In brief, 5 mL acetic acid-sodium acetate buffer solution (pH = 5.0) and PBS solution (pH = 7.4) were added into a vial at 37 °C and 60 °C water bath. Then, AMD hydrogel (1 g) was added into the vial. At a predetermined time point, 1 mL of the buffer solution was removed from each vial to determine the drug concentration and 1 mL fresh buffer solution was added thereto. The amount of released DOX in the buffer solution was qualified using Lambda 25 UV–vis spectrophotometer (Perkin Elmer, USA) at 480 nm and calculated according to the concentration-absorbance standard curve at the same wavelength.

For the in vitro tumor synergetic therapy, 50 μL of agar, Agar/MBP or Agar/MBP/DOX solution was added to 96-well cell culture plate. The above solution will automatically gelatinize with the temperature decrease. The formed gel was then transferred to another 96-well cell culture plate that was seeded with HT29 cells (1 x 10⁴ cells per well). Wells added with Agar/MBP or AMD gel were irradiated with NIR laser (1 W/cm²) for 5 min. After incubated for another 24 h, the viability of Agar/MBP hydrogel was evaluated using the MTT assay, microscopic observation with inverted phase contrast microscopy, and trypan blue staining.

2.6. In vitro Mo ion release and in vivo biodistribution and histocompatibility

The release behaviors of Mo ions from the hydrogel under the acidic pH that can mimic the micro-environment of the tumor were measured by Agilent 700 Series ICP-OES. Specifically, of the AMD hydrogel (1 g) was placed in a vial containing 5 mL acetic acid-sodium acetate buffer solution (pH = 5.0, n = 3). The vials were then placed at 37 °C in a water bath. At the predetermined time point, 1 mL of the solution was taken from each vial, and 1 mL corresponding fresh solution was added. The Mo ion concentration was measured with an Agilent 700 Series ICP-OES. For the in vivo biodistribution of Mo ions, HT29 xenografted tumor bearing Balb/c nude mice were euthanized at 1 h, 1 day, and 3 days post I. T. injection (50 μL Agar/MBP/DOX). Major organs (heart, liver, spleen, lung, and kidney) were digested by aqua regia solution overnight and the total amounts of Mo were quantified using an Agilent 700 Series ICP-OES. The longer term in vivo biosafety of AMD hydrogel was evaluated with standard H&E staining. KM mice were subcutaneously injected with 50 μL Agar/MBP/DOX and euthanized after 28 days’ feeding to harvest the heart, liver, spleen, lung, and kidney. Standard H&E staining was performed according to instructions with untreated KM mouse as control. The images were recorded using a Leica DM IL LED inverted phase contrast microscope.

2.7. Combined in vivo tumor therapy

HT29 xenografted tumor bearing mice with tumor diameter of ~0.5 cm were randomly divided into 4 groups (group I-IV, n = 7 per group). Then, mice were I. T. administered with 50 μL saline (control, group I), 50 μL Agar/MBP/DOX solution (groups II & IV), and 50 μL Agar/MBP solution (group III), respectively. After 1 h to allow the solution thoroughly gelling, mice in group I, III & IV were continuously irradiated with NIR laser for 5 min (1 W/cm²). The tumor temperature and thermal images of mice in groups I & IV were recorded using the FLIR™ E60 camera. After PTT, one tumor in each group was harvested and fixed with 10% neutral buffered formalin for further CD31, Ki67, and TUNEL staining to assess the tumor therapy efficiency. These immunohistochemical images were recorded using a Leica DM IL LED inverted phase contrast microscope. Relative tumor volume (V/V₀, where V₀ represents the initial tumor volume at day 0) and tumor appearance of the left 6 mice in each group were measured on live animals using the vernier caliper and recorded using Nikon D5300 digital camera (Nikon, Japan). The anti-recurrence outcome of AMD hydrogel was assessed as follows: tumor of the grouped HT29 mice were surgically excised when tumor diameter was ~1 cm. Then, 0.5 g different hydrogel constructed in vitro was implanted into the wounds left after the tumor surgical excision (group I, agar gel; group II, AMD gel; group III, Agar/MBP gel; group IV, AMD gel). Mice in group I, III & IV were continuously irradiated with NIR laser for 5 min (808 nm, 1 W/cm²). The tumor temperature and thermal images of mice in groups I & IV were recorded using the FLIR™ E60 camera. Tumor appearance in different groups was recorded using Nikon D5300 digital camera.

2.8. Statistical analysis

One way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. 0.05 was selected as the significance level, and the data were indicated with (*) for probability less than 0.05 (p < 0.05), (**) for p < 0.01, and (***) for p < 0.001, respectively. Unless specified, the sample size was 3 (n = 3).

3. Results and discussions

3.1. Preparation and characterization of AMD hydrogel

Homogeneous Agar/MBP/DOX solution was prepared by dissolving MBP and DOX into the hot agar solution, and the mixed solution can solidify upon the temperature (T) decreasing (Fig. 1a). As a dominant factor for the minimally invasive I. T. administration, the syringeability of the hot agar solution was well preserved after the encapsulation of MBP and DOX. The resultant Agar/MBP/DOX solution can be readily filled into a standard 1 mL 21-gauge syringe and smoothly pumped out from the needle pinpoint (Fig. 1b). Additionally, Agar/MBP/DOX solution remained semi-liquid at a higher temperature (i.e. 42 °C, Fig. 1c left and middle), however, its viscosity gradually increased (Fig. 1f) and the solution swiftly solidified upon cooling to 37 °C (Fig. 1c right). The gelling process was irreversible and once it has solidified, the semi-solid hydrogel can stay at its original location in the vial and not flow again within a broad temperature range (30–60 °C, Fig. 1d, e). The AMD hydrogel only showed a mass loss of ~5% after 20 days immersing in acetate buffer solution (pH = 5.0, Fig. S1), which illustrates that it can maintain the structural integrity for a long period of time. Moreover, the AMD hydrogel swelled in aqueous solution and the swelling ratio (SR) was significantly increased with the temperature-increasing. As shown in Fig. 1g, the SR increased from 8.5 ± 0.5%, 11.4 ± 2.7%, and 10.7 ± 1.1% to 13.2 ± 4.0%, 13.9 ± 3.7%, and 14.6 ± 4.6% after 10, 20 and 30 min immersing in citrate buffer (pH = 5.0), respectively. In PBS, the hydrogel swelling was more evident, which increased from 12.1 ± 2.8%, 15.1 ± 2.9%, and 13.7 ± 3.3% to 40.7 ± 8.4%, 43.5 ± 2.7%, and 43.7 ± 2.6% after 10, 20 and
3.2. In vitro photothermal performance

The high photothermal conversion efficiency of MBP nanosheets inspired us to pursue the photothermal performance of MBP nanosheet-contained AMD hydrogel. The AMD hydrogel exhibited an apparent photothermal transformation capacity. When the doped Mo concentration was 200 ppm, the temperature increase ($\Delta T$) of AMD hydrogel after 5 min irradiation was $\sim 50.6 \, ^\circ C$, 33.2 $^\circ C$, and 16.6 $^\circ C$ when the laser power density was 1 W/cm$^2$, 0.6 W/cm$^2$ and 0.3 W/cm$^2$, respectively (Fig. 3c, d). Since the starting temperature of the in vitro photothermal performance test was $\sim$ 10 $^\circ C$, the highest $\Delta T$ of 50.6 $^\circ C$ (i.e., a maximum final temperature of 60.6 $^\circ C$) will not liquefy the hydrogel (Fig. 1d, right bottle).

After immersing the AMD hydrogel in saline, a physiological simulated microenvironment, the temperature of hydrogel can still be increased about 40.5 $^\circ C$, 26.7 $^\circ C$ and 11.3 $^\circ C$ when the power density of NIR laser was 0.8, 0.6, and 0.3 W/cm$^2$, respectively (Fig. 3e, f). Although the $\Delta T$ decreased to $\sim$ 46.6 $^\circ C$ and 14.7 $^\circ C$ when the Mo concentrations were lower to 100 and 20 ppm at the laser power density of 1 W/cm$^2$, the temperature changes were still very fast and can reach the maximum temperature almost within the first 30 s irradiation and then kept equilibrium (Fig. 3c, d). In marked contrast, the temperature of agar hydrogel increased only by $\sim 2 ^\circ C$ even over a period of 5 min irradiation at the powder density of 1.0 W/cm$^2$, hence implying that the MBP nanosheet is the high priority for the photothermal effect of AMD hydrogel. Based on the in vitro photothermal study result, AMD hydrogel with Mo doping concentration of 200 ppm and laser power density of 1 W/cm$^2$ were selected in the following studies unless otherwise specified.

3.3. In vitro biocompatibility

Being one of the most important factors of its biomedical applications, the biocompatibility of AMD hydrogel was systematically studied by monitoring the metabolic activity and morphology of L929 cells that were incubated with the hydrogel for 1 day and 3 days. It should be noted that DOX may excert harmful effects on L929 cells, therefore, Agar/MBP hydrogel was selected as an alternative for the in vitro biocompatibility assay. Compared with untreated cells, the viability of Agar/MBP hydrogel treated L929 cell was still higher than 90%, regardless of the doped Mo concentrations and the culture duration (Fig. S4a, b). Qualitatively consistent with the CCK-8 assay results, cell...
morphology observation also indicated that there was no morphology difference between Agar/MBP hydrogel and PBS treated L929 cells (incubation time: 3 days, Fig. S4c, d), suggesting that Agar/MBP hydrogel will not influence the cell-skeleton integrality. Moreover, no obvious blue stained PBS and Agar/MBP hydrogel treated cells can be detected from trypan blue staining which can positively stain dead cells (Fig. S4e, f). Putting quantitative and qualitative cellular viability results together, the AMD hydrogel possessed an excellent in vitro biocompatibility, therefore is expected to create a promising future for diverse biomedical applications.

3.4. In vitro and in vivo imaging capacity

MBP nanosheets possessed an excellent photothermal performance and high X-Ray attenuation capacity (Wang, Li, Chen, Cai et al., 2015). We hypothesized that the MBP nanosheet-containing AMD hydrogel can also be used for PA/CT tumor dual-modal imaging. To prove this hypothesis, the in vitro PA and CT imaging of AMD hydrogel with different Mo ions doping levels were recorded (Fig. 4a, b, and e–h). There existed a typical linear relationship between the HU intensity and Bi experimental concentration (Fig. 4a, according to the molar ratio of Mo and Bi in MBP nanosheets, the Mo concentration was about 0.054 M, 0.11 M, 0.16 M, and 0.22 M). The CT imaging contrast enhancement can be obviously visualized even under a Bi concentration of 0.05 M and its brightness gradually increased with Bi concentration (0.05–0.20 M, Fig. 4b). To prove the in vivo CT imaging feasibility, HT29 xenografted tumor bearing mouse with I. T. hydrogel administration was anesthetized to harvest the tumor. Obviously, the CT signal intensity and brightness were significantly improved after I. T. administration of AMD hydrogel (104.8 ± 13.7 v.s. control: 21.5 ± 16.1, p < 0.05, Fig. 4c, d).

Apart from the CT imaging, AMD hydrogel also exhibited a concentration-dependent PA signal enhancement effect within the experimental laser wavelengths and concentration ranges (Fig. 4e). The PA contrast can be observed within the experimental Mo concentration ranges (50–200 ppm, Fig. 4e), and the brightness was deepening with the increasing of Mo concentration (Fig. 4f–h). Due to the gelling of the I. T. administered Agar/MBP/DOX solution, the PA signal around the HT29 xenografted tumor was significantly enhanced when compared with the tumor administered with saline (Fig. 4i, j), which obviously indicates that the PA contrast enhancing capacity of MBP nanosheets was well preserved after the gelling in vivo. With the aid of this superior CT and PA dual-modal imaging feature, the accurate location of AMD hydrogel within tumor can be exactly monitored and therefore the tumor therapy efficacy can be promoted to a great extent.

3.5. Synergetic in vitro tumor therapy

In this research, DOX molecules were physically blended and entrapped within the hydrogel during the automatic cooling of agar solution. It would thus seem natural to conclude that the DOX loading can reach a high efficiency provided that the drug can be readily dissolved into the agar solution. These entrapped DOX exhibited an obvious sustained release from the hydrogel regardless of the pH and temperature, and the release rate can be appreciably accelerated via enhancing the temperature and acidity of the surrounding solution. As
shown in Fig. 5a, the cumulative release ratios at equilibrium were about 4.8% (37 °C, pH = 7.4), 6.1% (37 °C, pH = 5.0), 14.7% (60 °C, pH = 7.4), and 23.2% (60 °C, pH = 7.4). The sustained drug release can be ascribed to the retardant effect of hydrogel on the DOX molecules, whereas the temperature and pH dual-stimuli responsive drug release may originate from the thermal induced hydrogel matrix swelling (Fig. 1g) and accelerated thermal motion of DOX molecules, and the solubility increase of DOX in acidic medium, respectively.

Together with the excellent photothermal performance of MBP nanosheets, the designed AMD hydrogel exhibited a combined tumor photothermal and chemotherapy capacity. Agar/DOX, Agar/MBP + NIR treated HT29 cell viability decreased to 28.3 ± 3.1%, and 45.1 ± 6.4% respectively, which indicates that the tumor inhibition efficiency of the DOX and MBP nanosheet was well preserved after entrapping with the hydrogel (p < 0.01, Fig. 5b). Owing to the co-existence of MBP and DOX, the proliferation of AMD hydrogel treated cells was significantly suppressed with the viability decreased to less than 5% after NIR irradiation (p < 0.05, versus Agar/MBP + NIR treated group, and p < 0.001, versus Agar treated cells, Fig. 5b). Further trypan blue staining results consistently confirmed the combined tumor therapy, in which a small portion of cells were blue stained in Agar/DOX and Agar/MBP + NIR treated cells, while AMD + NIR treated cells.
treated HT29 cells were almost totally counterstained with trypan blue (Fig. 5c–f).

3.6. In vitro and in vivo hemo- and histo-compatibility of AMD hydrogel

The next step towards the in vivo tumor therapy of AMD hydrogel is the study of in vivo hemo- and histo-compatibility. The probe into the hemo-compatibility of AMD hydrogel was of high importance since it can contact blood when I. T. administered into tumor. The hemo-compatibility of AMD hydrogel was studied from aspects of hemolysis and routine blood tests of mouse red blood cells (mRBCs). It was worth noting that DOX has an apparent absorption at 541 nm and can affect the measurement of hemoglobin (hemoglobin has a characteristic absorption at 541 nm). Therefore, Agar/MBP hydrogel was selected for the measurement. Compared with mRBCs which were exposed to saline and the hemolytic ratio was set as zero, the hemolysis of Agar/MBP hydrogel treated mRBCs was not obvious (hemolytic ratio = 1.9 ± 0.5%). In sharp contrast, mRBCs contacted with distilled water were severely damaged (with a hemolytic ratio of 100%, Fig. S5a). The hemo-compatibility was further qualitatively evaluated by visualizing the mRBCs structural integrity with Wright's staining. It was found that the mRBCs morphology was well maintained after being incubated with saline and Agar/MBP hydrogel for 2 h (Figs. S6 and SSb). The in vivo routine blood test was then performed to further ascertain the interactions between the mRBCs and hydrogel. As expected, no statistical difference of various blood parameters was detected between the saline and AMD hydrogel administered mice (Fig. S5c–k), further proving the admirable blood cell safety of Agar/MBP hydrogel.

The histo-compatibility of hydrogel was performed to comment on its long-term safety so as to highlight its translational prospect in depth. MBP nanosheets were confined within tumor during the swift phase change of agar solution and hence have little opportunity for entering the body fluid circulation stream. As a consequence, the release of Mo ions into the surrounding buffer (Fig. 6a) and the Mo enrichment in major organs were not obvious (Fig. 6b). Compared with previous report in which a substantial amount of the injected MBP nanosheets were captured by spleen and liver (Wang et al., 2016), the hydrogel designed in this study can act as a macro-vessel to restrict the access of MBP nanosheets and DOX to body fluid circulation, and therefore benefit the utilization improvement of MBP nanosheets and DOX. Further H & E staining strategy was carried out to evaluate the long-term tissue safety of the hydrogel on the 28th day post hydrogel administration. Compared to control group, no detectable tissue damage or pathology abnormality of AMD hydrogel treated mice was found (Fig. 6d). Moreover, there existed negligible abnormal body weight fluctuate of hydrogel treated KM mice within 28 days’ feeding (Fig. 6c), further confirming few adverse effects of AMD hydrogel on the health of mice.

3.7. Combined in vivo tumor therapy

In continuation of in vitro and in vivo biocompatibility assays that have proved the safety of AMD hydrogel as a platform for the co-entrapment of MBP and DOX, the in vivo synergistic antitumor studies were performed via I. T. injected Agar/MBP/DOX solution into tumor (Fig. 7a). Benefited from the excellent photothermal efficiency of MBP nanosheets, AMD hydrogel induced a fast tumor temperature increase to 44.5 °C and 58.2 °C after 30 s and 5 min irradiation, respectively.
Fig. 6. (a) Cumulative Mo release amount of AMD hydrogel in acetate buffer solution; (b) biodistribution of Mo in heart, liver, spleen, lung, and kidney at different time points after the hydrogel embedding (N.D. means not detectable); (c) body weight change of KM mice that were I. T. injected with saline (control) or AMD hydrogel; (d) H & E-staining results of heart, liver, spleen, lung, and kidney sections of mice I. T. injected with saline (control) and AMD hydrogel on day 28 (magnification: 100×).

Fig. 7. (a) Scheme of in vivo phase transformation, hyperthermia, and NIR-triggered DOX release of AMD hydrogel; (b) tumor temperature change of mice treated with saline or AMD hydrogel and 5 min laser; (c-d) in vivo photothermal images of mice treated with saline or AMD hydrogel and 5 min laser, respectively; (e) tumor growth profiles of HT29 xenografted tumors after different treatments; (f) representative photos of HT29 tumor bearing mice on day 0 and day 13 after different treatments.
The photothermal effect of mice treated with saline, by contrast, was not obvious (with $\Delta T$ of only 3.04 °C after 5 min of NIR irradiation, Fig. 7b, c). Along with the chemotherapy efficiency of DOX, tumors with I. T. AMD hydrogel administration and NIR irradiation shrank significantly and almost disappeared after 13 days’ feeding. Comparatively, tumor growth of mice received Agar/DOX and Agar/MBP + single NIR treatments was only partly inhibited (tumor volume expanded about 4.42, and 3.35 times, respectively after 13 days' feeding, Fig. 7e, f), implying that single photothermal or chemotherapy was powerless in absolutely restraining the malignant proliferation of tumor. Moreover, the heat from the photothermal transformation of MBP nanosheet can promote the releasing of drug molecules from the hydrogel (Figs. 7a and 5a) and thus improve the chemo-therapeutic efficacy. The in vitro synergistic antitumor performance was further verified by performing immuno-histochemical staining. The tumor received photothermal and chemotherapy presented the highest suppression of tumor cell proliferation and thereby showed the lowest CD31 expression level. Ki 67 antibody and TUNEL staining also intuitively indicated a more apparent inhibition of tumor malignant proliferation than conducting PTT or chemotherapy only (Fig. S7).

Although surgical treatment of early stage malignant tumor was widely accepted in clinical, it is still likely to suffer recurrence due to the inadequate resection of tumor tissue. As a result, developing a platform that can not only suppress the malignant proliferation, but also prevent its recurrence may hold the key to enhancing the tumor therapeutic efficiency. To demonstrate the advantage of AMD hydrogel in preventing tumor recurrence, the AMD hydrogel was constructed in vitro and then filled in the wound left after the surgical excision. After filling with AMD hydrogel, a swift tumor temperature increase by 15.6 °C was obtained after 25 s, and the tumor temperature increased by 18.2 °C and 25.8 °C after laser irradiation for 1 min and 5 min (1 W/cm²), respectively (Fig. S8a,b). Combined with the chemotherapy activity of DOX, the AMD hydrogel can totally inhibit the tumor recurrence after 5 min of NIR irradiation. However, tumor recurrence was found in mice filled with agar (blank), AMD hydrogel but without NIR irradiation, and Agar/MBP + NIR (Fig. 8c) after they had received tumor surgical excision and fed for 28 days, which suggests that the synergistic tumor therapy effects of AMD hydrogel can also efficiently prevent the tumor recurrence.

3.8. In vitro anti-inflammatory therapy efficacy

In addition to the risk of recurrence, postoperative infections occurring in patients with solid malignancy was another issue that was largely unengaged (Avritscher et al., 2014). In this research, the drug loading can be extended to the encapsulation of other water-soluble antibiotic such as AMX. Similar to DOX, the release of AMX from hydrogel was also characterized with a sustained manner due to the retardation of the gel. About 26% of the loaded AMX released within the first 12 h and this low-rate sustained release can maintain for 48 h (Fig. S8a). The in vitro antibacterial activity of the AMX-loaded hydrogel both in liquid and on solid medium was inquired by selecting S. aureus as a model. A total bacterial inhibition can be obtained under the studied AMX concentration (50–400 ppm, Fig. S8b). It was noteworthy that pure agar showed no anti-bacterial activity, which implies that the microbial growth was solely killed by the released AMX. The bacterial inhibition efficacy of the Agar/MBP/AMX hydrogel was further confirmed on solid medium by either attaching the hydrogel on the medium or removing the Agar/MBP/AMX hydrogel from the solid medium after 4 h AMX releasing. Obviously, the bacterial growth was effectively inhibited on solid medium in both cases and the inhibition zones were basically similar in size after 1 d, 2 d, and 7 d of culture (Fig. S8c, d), clearly demonstrating that the agar hydrogel will not reduce the antibacterial activity of AMX. Since there exists the risk of infection after the tumor surgical resection in clinical, the anti-inflammatory effect of the antibiotic laden hydrogel will contribute to the promotion of tumor therapy efficiency.

4. Conclusions

In summary, an injectable and phase-changeable multifunctional hydrogel was facilely designed by cooling the hot agar solution that containing photo-adsorbing MBP nanosheets and drug. Thereinto, the in situ formed agar gel can serve as a macro-vessel to retain the MBP and DOX and integrate CT/PA dual-modal imaging-guided tumor PTT and chemotherapy capacity. Such a material design strategy based on the phase-transformation of agar is highly effective and versatile in treating the malignant tumor. On the one hand, it can not only preserve the CT/PA dual-model imaging-guided tumor PTT and chemotherapy efficiency, but also generate novel on-demand drug release functionality due to the photothermal transformed heat can promote the drug permeation within the hydrogel. On the other hand, the hydrogel can efficiently reduce the side effect of MBP nanosheets and DOX towards normal tissue since it can restrict their entrance into body fluid circulation. In addition, antibiotic was likewise encapsulated with the hydrogel to avoid the potential wound infection during the tumor surgery. We believe that this research will provide a good background on the in situ formation of biocompatible hydrogel for tumor photothermal, chemo-, and anti-inflammatory therapy.

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Fig. 8. (a) Laser-induced temperature changes of mice which were received tumor removing and I. T. agar (blank) and AMD hydrogel filling; (b) in vivo photothermal images of mice corresponded to (a); (c) representative photos of HT29 tumor bearing mice on day 0 and day 28 after tumor removing, hydrogel filling and NIR irradiation.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.10.024.

References


