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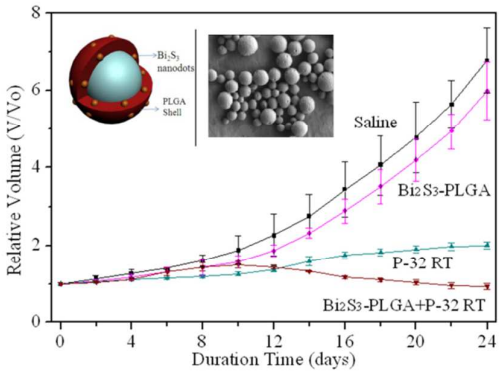
A novel multifunctional Bi_2S_3-PLGA capsule has been designed and synthesized for significantly enhancing the therapeutic efficiency of cancer brachytherapy.



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ARTICLE TYPE

Construction of Multifunctional Organic/Inorganic Hybrid Bi_2S_3-PLGA Capsules for Highly Efficient Ultrasound-Guided Radiosensitization of Brachytherapy

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The development of nanobiotechnology provides an efficient strategy to improve the therapeutic outcome of various therapeutic modalities against cancer. In this work, we successfully constructed a novel multifunctional theranostic platform based on Bi_2S_3-embeded poly (lactic-co-glycolic acid) composite capsules (Bi_2S_3-PLGA) to realize the highly efficient ultrasound-guided synergistic brachytherapy. The as-synthesized hydrophobic Bi_2S_3 nanodots were encapsulated into PLGA capsules to form Bi_2S_3-PLGA composite capsules by a simple but efficient water/oil/water (W/O/W) emulsion approach. The composite Bi_2S_3-PLGA capsules exhibit excellent contrast-enhanced ultrasound imaging performance in the prostate tumor-bearing nude mice model, which could accurately indicate the location of radiation source. More importantly, this research has demonstrated for the first time both *in vitro* and *in vivo* that Bi_2S_3-PLGA capsules could function as the efficient radiosensitizer for synergistic brachytherapy. Thus, the elaborately designed novel Bi_2S_3-PLGA capsules based on nano-biotechnology provides an efficient protocol for enhancing the therapeutic efficiency of ultrasound imaging-guided brachytherapy against cancer.

1. Introduction

Brachytherapy has been widely used as an efficient therapeutic modality against prostate carcinoma due to its unique advantage of long-term and durable radiation effect by placing the radiation source directly inside the tumor.[1-4] The implanted radiation source, containing different formations, such as colloids, seed, containers, can deliver significantly higher radiation dose to the prostate carcinoma with mitigated side-effects to surrounding normal tissues compared to the traditional external radiation therapy. To increase the accuracy of radiation source placement and ensure that the tumor can receive efficient concentration and distribution of seeds throughout the prostate tumor, the *in-situ* ultrasound imaging was generally applied by which the catheter can be monitored to guide the implanting process.[5-7] However, the proper catheter position cannot sufficiently ensure the accurate delivery of radiation sources at the desired tumor site. Thus, the development and application of an efficient ultrasound-based contrast agents to highlight the location of radiation source is highly desirable for the brachytherapy.[8-12] In addition, the brachytherapeutic efficiency strongly depends on the radiation doses, which was generated by various implanted radiation sources. The potential radiation danger to normal tissues, however, substantially restricts the administration doses of radiation sources. It is now still the great challenge to realize the efficient brachytherapy under the low administration doses of radiation sources.

Inspired by the radiosensitization effect of heavy metal element-based nanoparticles (NPs) for external radiotherapy such as Au, Gd, Bi, et al,[13-18] it is anticipated that these NPs can exhibit the similar radiosensitization effect for brachytherapy due to the same radiation mechanism for killing the cancer cells. However, no report has confirmed the sensitization effect of NPs in the brachytherapy because nanotechnology-based radiosensitization is still in its infancy. In this respect, bismuth-based NPs are the most preferred radiosensitization agent because bismuth (Bi) is the biocompatible metal element with much higher atomic number (*Z*) than traditional Au, Gd, Ag, et al.

Herein, a unique Bi_2S_3-embedded poly (lactic-co-glycolic acid) capsule (Bi_2S_3-PLGA) was developed, for the first time, to realize the concurrent contrast-enhanced ultrasound imaging and heavy metal element-induced radiosensitization of brachytherapy by a simple but efficient double emulsion protocol. Biocompatible PLGA capsules have been demonstrated as the excellent contrast agents for ultrasound imaging.[19-23] Additionally, PLGA capsule is considered as efficient carriers for the delivery of functional NPs, such as Gd_2O_3, iron oxide and gold NPs, which could realize multifunctional theranostic functions besides ultrasound contrast-enhanced imaging. Recently, Bi_2S_3 NPs have been employed as the contrast agents for computed tomography due to their satisfactory biocompatibility and large X-ray attenuation coefficient (Bi: 5.74, Au: 5.16, Pt: 4.99 and Ta: 4.3 cm^2/kg at 100 eV).[24-26] Especially, it is anticipated that Bi_2S_3 can be used as the radiation sensitizer because of their strong photoelectric adsorption and ejected short-range secondary electron after exposure to X-ray or gamma irradiation, which can enhance the DNA breakage and cause the death of tumor cells. The elaborate combination of organic PLGA capsules and

inorganic Bi_2S_3 NPs can construction a unique organic/inorganic hybrid multifunctional capsule with the following structural and compositional characteristics for brachytherapy. On the one hand, the administration of Bi_2S_3-embeded PLGA capsules can function as the contrast agents for ultrasound imaging, by which the radiation sources of brachytherapy can be guided into the desired position within the tumor tissues. On the other hand, the integrated Bi_2S_3 NPs within PLGA capsule can be used as the radiation sensitizer to synergistically improve the efficiency of brachytherapy. The performances of Bi_2S_3-PLGA for ultrasound-guided brachytherapy have been systematically demonstrated both *in vitro* and *in vivo* in this work.

2. Experimental

2.1 Materials

Poly (lactic-co-glycolic acid) (50:50) with the molecular weight of 15,000 MW was purchased from Polyscience, USA. The bismuth neodecanoate, octadecene, oleic acid, thioacetamide and oleylamine were obtained from Sigma-Aldrich. Methylene chloride and poly(vinyl alcohol) was obtained from Sinopharm Chemical Reagent Co. Colloidal chromium phosphate (P-32) was purchased from Beijing Yuanzi High Tech. Company. All chemicals were received without further purification. Deionized water was used in the whole experiment.

2.2 Preparation of the Bi_2S_3/PLGA capsules

The Bi_2S_3 NPs were prepared based on the previous report.[24] The prepared Bi_2S_3 NPs were dispersed into methylene chloride at the concentration of 10 mg/mL. Furthermore, Bi_2S_3/PLGA capsules were prepared by a simple but efficient double emulsion protocol. Typically, 20 mL of the methylene chloride solution was prepared containing 5 mg of PLGA and 5 mg of Bi_2S_3 NPs (0.5 mL, 10 mg/mL), which was formed by dispersing Bi_2S_3 NPs and dissolving PLGA under ultrasound treatment. Then, 4 mL of deionized water was added dropwise, and the mixture was emulsified using an ultrasonic probe with the power of 180 W at room temperature. Sequentially, the above mixture solution was added into 100 mL of poly(vinyl alcohol) solution with the concentration of 5% w/v and homogenized for 10 min at room temperature. Finally, the methylene chloride was extracted from the emulsion by gently stirring overnight at room temperature. The obtained Bi_2S_3-PLGA capsules were collected by centrifugation and washed with deionized water for three times by repeated centrifugations.

2.3 *In vivo* contrast-enhanced ultrasound imaging

The mixture of 0.05 mCi P-32 enriched gelatin chromic phosphate colloid (P-32) and 100 μg of Bi_2S_3-PLGA capsules in saline solution was intratumoral administrated into the prostate tumor bearing nude mice. The ultrasound imaging was performed before and after the injection, respectively.

2.4 *In vitro* evaluation of the synergistic radiosensitization effect

A human prostate carcinomas cells line, PC3 cells were cultured in the RPMI 1640 medium with 10% FBS, and 1% penicilin–streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 incubator. To evaluate the *in vitro* enhancement of Bi_2S_3-PLGA in P-32 radiation, PC3 cells were cultured in a 96-well plate with the density of 1×10^5/well. After incubation overnight, the medium was replaced by fresh medium with 0.03 mCi P-32 and different concentration of Bi_2S_3-PLGA nanocapsules. Then the cells were cultured for another 24 h and 48 h, respectively. The cell viabilities were evaluated by the typical MTT assay. In addition, the apoptosis levels of PC3 cells after each treatment were demonstrated by the flow cytometry analysis.

2.5 *In vivo* evaluation of the synergistic radiosensitization effect

The nude mice prostate carcinomas xenograft was firstly established for *in vivo* evaluations. The P-32 radiation treatment was performed when the tumor diameter reached about 1 cm. The prostate tumor-bearing nude mice (n = 12) were randomly divided to 4 groups (n = 3) and intratumorly received the treatment of saline solution, Bi_2S_3-PLGA capsules, P-32 radiation therapy (RT) and Bi_2S_3-PLGA capsules combined with P-32 radiation therapy, respectively. The amount of administrated Bi_2S_3-PLGA capsules is 100 μg/mouse and the radiation dose of P-32 is 0.05 mCi. The tumor volume was measured every two days. After the 24th day treatment, the tissue section was dissected for Ki 67 immunohistochemisty analysis according to the manufacture's protocol.

2.6 Characterizations

Transmission electron microscopy (TEM) image was acquired on a JEM-2100F electron microscope operated at 200 kV. Scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM) were obtained on a field-emission Magellan 400 microscope (FEI Company). Ultrasound imaging was acquired on a VisualSonics Vevo2100, MS-400 with the parameter of B-mode and 30 MHz frequency. The corresponding average gray values of ultrasound imaging were determined by the analysis with SONOMATH-DICOM software. All the animal procedures were performed under the guidance approved by the University Animal Care and Use Committee of Nanjing Medial University.

3. Results and discussion

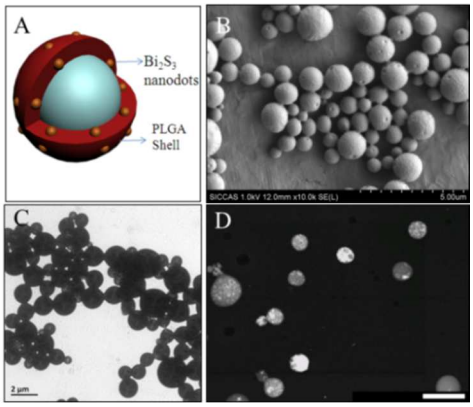

Figure 1. (A) Schematic illustration of the structural characteristics of Bi_2S_3-PLGA capsules; SEM (B), bright-field TEM (C) and dark-field TEM (D) images of Bi_2S_3-PLGA composite capsules. The scale bar of D is 1 μm.

As shown in **Figure 1A**, Bi_2S_3/PLGA composite capsules exhibit the typical spherical morphology. The shell of such composite capsule was composed of PLGA with embedded Bi_2Si_3 nanodots. The as-synthesized monodispersed Bi_2S_3 nanodots were hydrophobic (**Figure 2**), which could be easily encapsulated into the hydrophobic tails of PLGA molecules during the typical double emulsion process. The well-defined spherical morphology of Bi_2S_3/PLGA capsules can be directly observed in SEM image (**Figure 1B**), which shows that such capsules possess the smooth surface with the diameter in the range of 500 - 1000 nm. Bright-field TEM images exhibit the similar spherical morphology of Bi_2S_3/PLGA capsules, but the presence of Bi_2S_3 nanodots within PLGA capsule is hardly to distinguish. To give the further information of the distribution of Bi_2S_3 nanodots within PLGA capsules, dark-field TEM image of B_2S_3/PLGA composite capsules was acquired (**Figure 1D**). Based on the contrast differences between Bi element of Bi_2S_3 nanodots and C element of PLGA capsule, dark-field TEM image shows that Bi_2S_3/PLGA capsules possess the typical "sesame ball" microstructure with the uniform distribution of Bi_2S_3 nanodots (much brighter dots in **Figure 1D**) within the PLGA matrix. These electron microscopic characterizations give the strong evidence that Bi_2S_3 nanodots have been successfully encapsulated into PLGA capsules to achieve a unique organic-inorganic hybrid composite capsule with multifunctions for further imaging-guided brachytherapy.

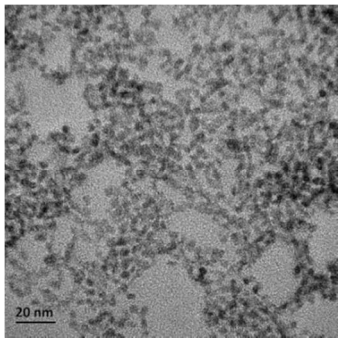

Figure 2. TEM image of as-synthesized hydrophobic Bi_2S_3 nanodots.

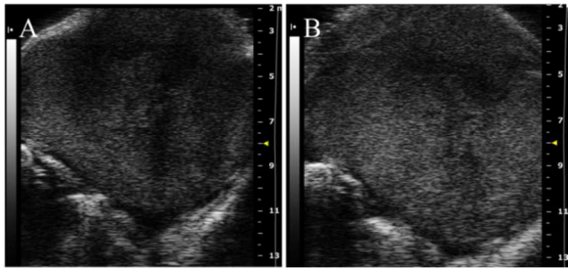

Figure 3. Ultrasound image of the tumor tissue before (A) and after (B) administration of Bi_2S_3-PLGA capsules.

Imaging-guided brachytherapy is very important for the guidance of implanting process for radiation sources because such guidance can assist the determination of the accurate implanting sites to guarantee the high radiation efficiency and mitigate the side-effects of radiation sources. Ultrasound has been proved to function as an efficient imaging modality to achieve this goal due to its portable and operational features. PLGA capsules with varied particle sizes have demonstrated their excellent performance to enhance the contrast-enhanced ultrasound imaging performance for ultrasonography.[19] To demonstrate the feasibility of Bi_2S_3/PLGA capsules as the contrast agents for ultrasound-guided brachytherapy, the ultrasound imaging of tumor-bearing mice before and after administration of Bi_2S_3-PLGA capsules was conducted. As shown in **Figure 3**, the contrast-enhance ultrasound imaging of tumor tissues **(Figure 3B)** shows the significantly enhanced positive ultrasound signals after the injection of Bi_2S_3-PLGA capsules compared to the initial tumor tissue (**Figure 3A**). The corresponding grey value of Bi_2S_3/PLGA-treated tumor tissue shows a great increase from the initial 27.6 dB to 46.2 dB, which further gives a strong evidence of the contrast-enhanced ultrasound imaging performance and is expected to be potentially used for the ultrasound-guided brachytherapy.

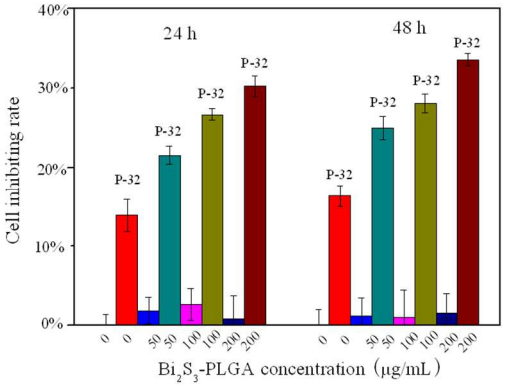

Figure 4. (A) The PC3 cell inhibiting rate after incubation with 0.03 mCi P-32 colloids and Bi_2S_3-PLGA capsules for 24 h and 48 h, respectively. The concentrations of Bi_2S_3-PLGA capsules are in the range of 0 to 200 μg/mL. The P-32 RT treated groups were highlighted in the figure.

It has been reported that heavy metal elements can be used as the sensitizer for synergistic radiotherapy because of its strong photoelectric absorption and generated secondary electron under the radiation exposure.[17, 18] Different from most reported heavy metal element-based nanoparticles for radiotherapy, we evaluated the radiosensitization effect Bi_2S_3 nanodots within PLGA capsules in brachytherapy. Bi_2S_3-PLGA capsules were homogenously mixed with 0.03 mCi P-32 enriched gelatin chromic phosphate colloid (P-32). Then, Bi_2S_3-PLGA capsules with different concentrations were employed for *in vitro* assessments against PC3 cancer cells. The mixtures were added into the 96-well plate containing PC3 cells and co-incubated for 24 h and 48 h, respectively. As shown in the **Figure 4**, no obvious cell toxicity was found for the Bi_2S_3-PLGA capsules groups without P-32 radiation. Comparatively, the obvious cell toxicity was found when performing P-32 radiation, and increased with concentration increment of capsules. In addition, it was also found the apoptotic percentage of PC3 cells after the combined treatment of Bi_2S_3-PLGA capsules and P-32 RT was 13.1 % (**Figure 5**), which is obviously higher than 8.8 % of the P-32 RT group. Both the above *in vitro* MTT results (**Figure 4**) and apoptosis data (**Figure 5**) indicate the obvious sensitization effect of Bi_2S_3-PLGA in the brachytherapy.

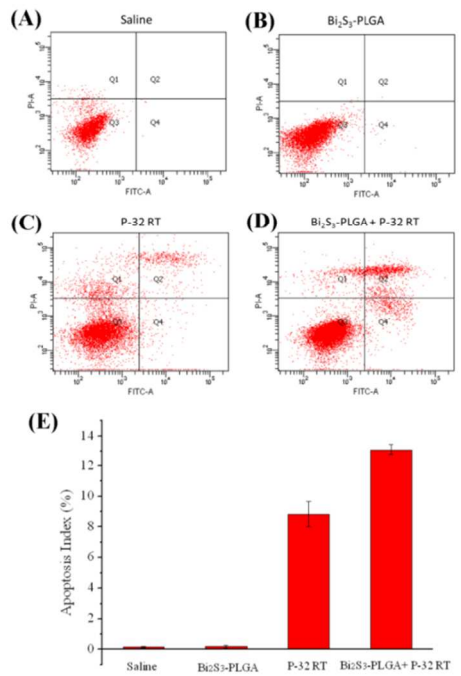

Figure 5. (A-D) The apoptosis profile of PC3 cells demonstrated by the flow cytometry analysis after treatment of saline solution (A), Bi_2S_3-PLGA capsules (B), P-32 RT (C) and Bi_2S_3-PLGA capsules combined with P-32 RT (D), respectively. The cells were stained by Annexin V-FITC/PI staining; (E) The apoptosis index (AI) of PC3 cells after treatment of saline solution, Bi_2S_3-PLGA capsules, P-32 RT and Bi_2S_3-PLGA capsules combined with P-32 RT, respectively.

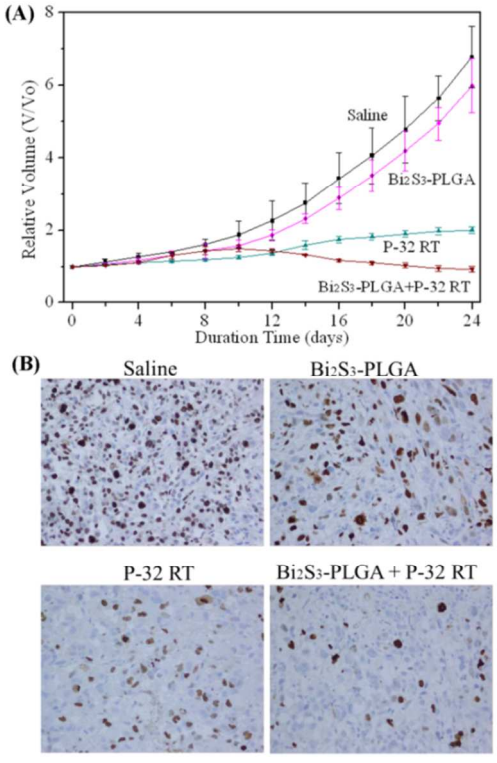

Figure 6. (A) The tumor growth curve of prostate tumor-bearing nude mice after treatment of saline solution, Bi_2S_3-PLGA capsules, P-32 RT and Bi_2S_3-PLGA capsules combined with P-32 RT; (B)The immunohistochemical staining analyses for Ki67 of tumor cells after treatment of saline solution, Bi_2S_3-PLGA capsules, P-32 RT and Bi_2S_3-PLGA capsules combined with P-32 RT, respectively.

To preliminary evaluate the radiosensitization effect of Bi_2S_3-PLGA capsules *in vivo*, nude mice bearing PC3 prostate carcinomas tumor xenograft was established for the assessment. The tumor-bearing mice were administrated with saline solution (group I), Bi_2S_3-PLGA capsules (group II), 0.05 mCi P-32 RT (group III) and Bi_2S_3-PLGA capsules combined with 0.05 mCi P-32 RT (group IV), respectively. It was found the tumor growth curves exhibited no obvious difference between the saline solution (I) and Bi_2S_3-PLGA capsules (II) group in the 24th day (**Figure 6A**), further demonstrating the negligible biological effect and high biocompatibility of fabricated composite capsules. Importantly, an obvious eradication of the tumor by about 30 % reduction in the tumor volumes was recorded in the combined (IV) group, in comparison to the P-32 RT (III) group with nearly double growth, which strongly demonstrates the high synergistic therapeutic efficiency of Bi_2S_3-PLGA composite capsules for brachytherapy. To further reveal the synergistic mechanism, we analyzed the immunohistochemical results for Ki67 of tumor cells, which reflects the cell proliferation condition after each treatment. Obvious lowest expression of Ki67 in combined (IV) group can be found compared to P-32 RT (III) group, saline group (I) and Bi_2S_3-PLGA group (II) (**Figure 6B**), indicating the significantly suppressed activity of tumor cells and improved synergistic efficiency after the introduction of Bi_2S_3-PLGA composite capsules combined with radiation therapy for brachytherapy. Based on the tumor growth curve and the analysis of Ki67 of tumor cells, it can be concluded that the fabricated Bi_2S_3-PLGA capsules can function as the efficient *in vivo* radiation sensitizer for enhancing the therapeutic efficiency of brachytherapy. In addition, the in-situ intratumor administration was adopted because brachytherapy typically implants the radiation source into the tumor clinically,[1-4] which can maximally accumulate the Bi_2S_3-PLGA capsules and P-32 radiation source within the tumors. Thus, the administration dose can be much lower than traditional systemic administration, such as intravenous injection, causing the significantly reduced toxicities to mice.

4. Conclusions

In summary, a novel organic-inorganic hybrid Bi_2S_3-PLGA composite capsule was successfully developed for ultrasound-guided synergistic brachytherapy based on nano-synthetic chemistry and nano-biotechnology. The Bi_2S_3-PLGA capsules with high dispersity and spherical morphology exhibit excellent contrast-enhanced ultrasound-imaging performance confirmed by the *in vivo* imaging evaluation against mice xenograft. Importantly, due to the presence of heavy metal element bismuth, high radiosensitization effect of Bi_2S_3-PLGA capsules in the P-32 interstitial radiation therapy was confirmed by systematic *in vitro* and *in vivo* assessments. Thus, it is anticipated that the elaborately designed Bi_2S_3-PLGA capsules could be used as an efficient sensitizer in the ultrasound imaging-guided brachytherapy against cancer, which also provides an alternative

approach to enhance the therapeutic efficiency of brachytherapy rather than the traditional increase of dangerous radiation doses.

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Notes and references

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