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A highly hemocompatible erythrocyte membrane-coated ultrasmall selenium nanosystem for simultaneous cancer radiosensitization and precise antiangiogenesis

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Abstract

Radiotherapy is a vitally important strategy for clinical treatment of malignant cancers. Therefore, rational design and development of radiosensitizers that could enhance radiotherapeutic efficacy has attracted tremendous attention. Antiangiogenesis therapy could be a potentially effective strategy to regulate tumor growth and metastasis due to angiogenesis plays a pivotal role for tumor growth, invasion and metastasis to other organs. Herein, we have rationally designed a smart and effective nanosystem by combining ultrasmall selenium nanoparticles and bevacizumab (AvastinTM, Av), for simultaneous radiotherapy and antiangiogenic therapy of cancer. The nanosystem was further coated with red blood cell (RBC) membranes to develop the final construct, RBCs@Se/Av. The RBC membrane coating effectively prolongs the blood circulation time and reduces the elimination of the nanosystem by autoimmune responses. As expected, RBCs@Se/Av, when irradiated with X-ray demonstrated potent anticancer and antiangiogenesis response *in vitro* and *in vivo*, as evidenced by strong inhibition of A375 tumor growth in nude mice, without causing any obvious histological damage to the non-target major organs. Taken together, this study demonstrates an effective strategy for design of smart Se-based nanosystem decorated with RBC membrane for simultaneous cancer radiosensitization and precise antiangiogenesis.

Introduction

Radiotherapy (RT) is a cornerstone for clinical cancer treatment. Over 50% of the cancer patients worldwide, have received RT with curative or palliative intent.^{1, 2} However, the low therapeutic efficacy of RT fails to completely eradicate tumors, and is plagued by dose-limiting toxicities, radiation resistance and inevitable damage to normal body tissues due to its non-specific nature.^{3–5} As a result, the dose of administered radiation to the healthy tissue should be strictly limited to a tolerable level.⁶ Therefore, numerous chemical radiosensitizers have been designed to clinically augment the effect of RT, however with

suboptimal results. There is an urgent need to design and develop novel sensitizer that can optimize radiotherapeutic efficacy at a lower effective doses, and can also combine other therapeutic modalities in for enhanced elimination of tumors.^{4, 7}

Angiogenesis plays a pivotal role for tumor growth, invasion and metastasis to other organs which is responsible for majority of deaths in cancer patients.⁸ Accordingly, antiangiogenesis therapy could be a potentially effective strategy to regulate tumor growth and metastasis.^{9, 10} Bevacizumab (AvastinTM, Av) is a humanized monoclonal antibody that precisely targets the crucial angiogenic factor, vascular endothelial growth factor (VEGF), and blocks angiogenesis by inhibiting VEGF and functions as a tumor-starving (antiangiogenic) therapy to regulate the formation and growth of new blood vessels around and inside of tumor tissues.¹¹ Av binds to VEGF secreted by angiogenic tumors, thereby inhibiting its binding to the VEGF receptor in vascular endothelial cells. This in turn restrains VEGF-induced cell proliferation, survival, permeability, nitric oxide production, as well as migration and tissue factor production, ultimately leading to tumor inhibition.^{12, 13}

Red blood cells (RBCs), also known as erythrocytes, are natural delivery systems for transporting oxygen to body tissues and organs with an oval biconcave cellular morphology and without majority of cellular organelles, and continue to attract wide scientific interest for design of artificial biomimetic nanomaterials as drug delivery vehicles.^{14, 15} RBCs are endowed with diverse immunomodulatory markers such as CD47, C8-binding protein (C8bp), homologous restriction protein (HRP), CD59, etc. for self-recognition by the tissues and organs.^{16–18} In addition, as an internal delivery system, RBCs possess several excellent characteristics including 1) high biocompatibility; 2) long circulating half-life (~ 4 months); 3) flexible and stable membrane; 4) lack of most cellular organelles for augmented load-carrying capacity; 5) no tumorigenicity (owing to lack of nuclear DNA); and, 6) large superficial surface area conducive to surface modification. The semi-permeable membrane from RBCs is able to release drugs continuously.^{19–23} Therefore, RBC membrane is a promising biocompatible carrier for nanomaterials with prospective clinical applications in the near future.

In our previous study, we found that PEGylated Se nanosystems displayed significantly enhanced radiosensitization when irradiated with X-ray, and synergistically improved the antitumor effects of radiation by induction of cell apoptosis.²⁴ Recently, selenium nanoparticles (SeNPs) have garnered much attention as promising cancer therapeutics and drug carriers owing to their notable biocompatibility, anticancer efficacy and low toxicity bolstering their potential applications as therapeutic agents.^{25, 26} Here, we synthesized RBCs@Se/Av that combined PEG-SeNPs and Av antibody, encapsulated within RBCs membrane vesicles to simultaneously enhance the efficiency of cancer radiotherapy and antiangiogenesis (Figure 1a). The nanosystems passively accumulated in cancer cells and were then activated in response to X-ray. As expected, treatment with RBCs@Se/Av and X-ray induced the production of reactive oxygen species (ROS) and activated the p53 pathway to induce cancer cell apoptosis. Moreover, RBCs@Se/Av with X-ray irradiation demonstrated potent and focused *in vivo* anticancer efficacy, without causing and obvious histological damage to the major organs. Taken together, this study demonstrates a strategy

for design of smart and effective biomimetic anticancer therapy via simultaneous cancer radiosensitization and precise antiangiogenesis.

Results and discussion

Synthesis and characterization of RBCs@Se/Av nanosystem

RBCs@Se/Av were synthesized successfully (outlined in the Experimental section) and characterized extensively by dynamic light scattering (DLS)-based particle sizing, atomic force microscopy (AFM) and transmission electron microscopy (TEM). As shown in Figure S1, the size of RBC nanoparticles (RBC-NP) was found to be 200 nm after extrusion, the zeta potential was -7 mV, similar to that of original RBCs. AFM image of the nanoparticles and the corresponding analytical curve demonstrated the smooth surface of RBC NPs suggesting that RBC membrane were extracted successfully. We examined the size and zeta potential of PEG-SeNPs, RBC-NPs and RBCs@Se/Av to evaluate their dispersity and stability. As shown in Figure 1b, TEM images demonstrated the ultrasmall size of PEG-SeNPs 6 nm, RBC-NPs exhibited a diameter of 79 nm and RBCs@Se/Av were highly monodisperse with uniform shape and size ~ 83 nm in diameter. Additionally, the results of HAADF-STEM clearly proved the successful coating of RBCs membrane onto the surface of PEG-SeNPs since the signals of oxygen, phosphorous and selenium were found to overlap with each other. Hydrodynamic diameters of PEG-SeNPs, RBC-NPs and RBCs@Se/Av were 106 nm, 188 nm and 358 nm, respectively (Figure 1c). In addition, the average zeta potential of RBCs@Se/Av decreased to -7.5 mV, compared to -12.2 mV of PEG-SeNPs, likely because of the reduced surface charge of RBC membranes (Figure 1d). Taken together, these results demonstrate that RBCs@Se/Av nanosystem were prepared successfully.

Enhancement of stability and hemocompatibility of RBC@Se/Av by RBCs membrane coating

It is possible that positively charged nanoparticles would be rapidly cleared from the blood circulation after systemic administration due to the potential nonspecific reactions with serum components. In this study, we found that the size of RBCs@Se/Av was slightly decreased in DMEM with 10% FBS than PBS. Moreover, the size of the nanoparticles remained stable in human serum upto 72 h, indicating attenuated binding by serum proteins (Figure 2a), and further indicating the high stability of the RBCs@Se/Av under physiological conditions.

Hemocompatibility of PEG-SeNPs and RBCs@Se/Av was evaluated to determine hemocompatible membrane properties and also to assess the potential side effects of exogenous drugs and materials.²⁷ As shown in Figure 2b, treatment only with PEG-SeNPs induced mild hemolysis after 2 h, meanwhile, treatment with RBCs@Se/Av did not trigger appreciable hemolysis of RBCs. Furthermore, no agglutination was observed in RBCs treated with PEG-SeNPs and RBCs@Se/Av for 2 h (Figure 2c).

Nanoparticles coated by RBCs membrane have been reported to be able to escape from the macrophage uptake and systemic clearance, resulting in longer blood circulation half-life

compared to naked nanoparticles.²⁸ Therefore, we investigated the blood-circulation profile of RBCs@Se/Av nanoparticles. Notably, the contents of Se in serum samples collected from SD rats injected with RBCs@Se/Av were higher than PEG-SeNPs, which exhibited that greatly prolonged blood circulation owing to the RBCs membrane coating compared to naked PEG-SeNPs nanoparticles (Figure 2d). These results demonstrate that the RBCs membrane modification could indeed prolong the blood circulation time of nanoparticles.

Radiosensitization and antiangiogenesis effects of RBCs@Se/Av

In this study, multifunctional RBCs@Se/Av nanosystem upon X-ray irradiation could induce cancer cell apoptosis and angiogenesis inhibition to restrain the growth of tumors with high efficacy. A375 melanoma cells as typical hypoxic and malignant cancer cells are employed due to their strong radioresistance.^{29, 30} We investigated the cytotoxic effects of X-ray on A375 and HUVEC cells by MTT assay. As shown in Figure S2, during individual X-ray treatment, A375 cell viability decreased only to 77% with 8 Gy X-ray irradiation as control. While HUVEC cell viability decreased to 67% when treated with 8 Gy X-ray dose. These results clearly show that X-ray treatment alone induces slight cell apoptosis. Dose-dependent radiochemotherapy was further preformed to examine the antitumor activity and angiogenesis inhibition of the RBCs@Se/Av. Under 2 Gy, 4 Gy and 8 Gy irradiation doses, the A375 cells depicted an IC₅₀ value of 9.8, 8.5 and 8.5 μ M in RBCs@Se/Av treated group, and 13.5, 12.7 and 11.6 μ M in PEG-SeNPs treated group (Figure 3a). Moreover, the IC₅₀ value of HUVEC cells was found to be 10.6, 7.5 and 7.5 μ M after co-treating with RBCs@Se/Av and 2 Gy, 4 Gy and 8 Gy irradiation (Figure 3b). Corresponding tests were also performed with Avastin only treatment group (Figure S3). Overall, the data demonstrates that radiochemotherapy by RBCs@Se/Av represses growth of the A375 and HUVEC cells, more significantly than single therapeutic regimens.

Isobologram analysis was then applied to observe the interaction between X-ray and RBCs@Se/Av. As shown in Figure 3c and 3d, the inhibitory effect of X-ray (2 Gy and 4 Gy) and RBCs@Se/Av on A375 and HUVEC cells was synergistic, as indicated by the isobologram data which were found to be far below the line defining the additive effect.³⁰ Interaction of PEG-SeNPs and X-ray on A375 and HUVEC cells was also found to be synergistic at radiation of 2 Gy and 4 Gy (Figure S4), indicating that RBCs@Se/Av maintain the radiosensitization ability of PEG-SeNPs. Cellular uptake is a main factor that affects the biological efficacy of nanoparticles. Therefore, the cellular uptake ability of RBCs@Se/Av and PEG-SeNPs by A375 and HUVEC cells was compared. As shown in Figure S5, RBCs@Se/Av exhibited higher cellular uptake than PEG-SeNPs, possibly due to longer blood circulation of the former. These results suggest that the higher cellular uptake of the nanosystems contributes to the higher activities.

We further assessed the inhibitory activities of co-treatment on colonization of A375 cells in order to reinforce the radiosensitization properties of RBCs@Se/Av. As shown in Figure 3e, images of cell colony formation demonstrated that RBCs@Se/Av improved the radiation-induced inhibition of colony formation in A375 cells. Particularly, the survival fraction of A375 cells by 4 Gy X-ray and RBCs@Se/Av alone were 56.2% and 96% respectively. However, after co-treated with X-ray and RBCs@Se/Av at 4 μ M, the survival fraction was

declined to 17.5%. These results demonstrate that RBCs@Se/Av improve the inhibitory effects of X-ray on the colony formation and survival fraction of A375 cells.

Radiosensitization and antiangiogenesis action mechanisms of RBCs@Se/Av

Studies were also carried out to examine the underlying mechanisms accounting for the radiosensitization effects of the RBCs@Se/Av. As revealed by flow cytometry in A375 cells (Figure 4a), X-ray alone caused 16.39% cell apoptosis and the RBCs@Se/Av (from 10 to 20 μ M) increased cell death from 14.32% to 34.5% as shown by the increase in the Sub-G1 cell proportion. For instance, treatment with RBCs@Se/Av (10 μ M) and X-ray increased the Sub-G1 peak from 14.32% to 30.06% of RBCs@Se/Av alone. As shown in Figure 4b (HUVEC cells), X-ray alone caused 14.95% cell apoptosis and the RBCs@Se/Av (from 10 to 20 μ M) increased cell death from 12.17% to 18.1% as shown by the increment in the Sub-G1 cell proportion. The combination of RBCs@Se/Av (10 μ M) and X-ray increased the Sub-G1 peak from 12.17% to 22.55% of nanosystem only. The results indicate caspases family of proteins play a critical role in synergistic (RBCs@Se/Av and X-ray) anticancer action through promotion of cell apoptosis.³¹ Previous report also showed the anticancer effects of SeNPs by caspase-mediated apoptosis in several cancer cells.³² Hence, we determined the expression level of caspase proteins and other related proteins by Western blot. As shown in Figure 4c and 4d, exposure of A375 and HUVEC cells to RBCs@Se/Av and X-ray activated caspase-3/-8/-9 as well as PARP cleavage which provided more evidence about that the synergistic effects of the combined treatment arising from the caspase-mediated apoptotic pathway.

RT can induce ROS overproduction to cause DNA damage and cell death. The generation of intracellular ROS was examined by the DCFH-DA probe in the cells.³³ As shown in Figure 5a and 5c, only treatment with X-ray induced slightly ROS overproduction, while the combined of RBCs@Se/Av and X-ray treatment significantly increased the ROS within 5 min of control in A375 cells. The co-treatment of RBCs@Se/Av and X-ray also markedly induced the generation of ROS within 5 min that remained constant upto 30 min in HUVEC cells (Figure 5b and 5d). These data indicate that the RBCs@Se/Av and X-ray elevate ROS generation synergistically in both A375 and HUVEC cells. Moreover, the combined treatment induced dramatically ROS-mediated mitochondrial fragmentation compared to X-ray or RBCs@Se/Av alone (Figure 6a and 6b).

To the best of our knowledge, high level of ROS could trigger DNA damage, which has been considered a pivotal factor of cell apoptosis.³⁴ Therefore, we carried out Western blot analysis of the proteins related to DNA damage for verification. As shown in Figure 6c and 6d, the RBCs@Se/Av and X-ray induced a drastic increment in p53 phosphorylation at Ser15. Moreover, the levels of Ser139-Histone H2A.X (a DNA damage marker), p-Chk1 and p-ATR were elevated in the cells that were exposed to the RBCs@Se/Av and X-ray. These outcomes further indicate that RBCs@Se/Av enhance radiation induced apoptosis by inducing DNA damage and activation of p53 phosphorylation pathway.

Previous study has shown the radiation-induced sublethal DNA damage to tumor cells, however, such damages were found to be repaired by the cells via increased expression of X-ray repair cross complementing protein 1 (XRCC1) and other related proteins.³⁵ As shown

in Figure 6e and 6f, co-treatment with RBCs@Se/Av and X-ray could efficiently inhibit the expression of XRCC1 in A375 and HUVEC cells. Moreover, the expression level of XRCC1 was escalated when treated with X-ray alone, but was markedly repressed by RBCs@Se/Av plus X-ray irradiation. These data further demonstrate that combined treatment triggers irreparable DNA damage in cells.

As cell migration and invasion are very important for tumor growth, metastasis, and angiogenesis,³⁶ we carried out a wound-healing, Transwell invasion and tube formation assay to measure the antiangiogenic activity of the RBCs@Se/Av. As shown in Figure 7, S6 and S7, the RBCs@Se/Av markedly suppressed the migration triggered by VEGF at nontoxic concentrations (10 μ M) after pre-treatment with 10 μ g/mL mitomycin C for 2 h to inhibit proliferation before creating a scratch gap. Moreover, invasion and tube formation triggered by VEGF were dramatically decreased at nontoxic concentrations (10 μ M). While equal concentration of Avastin only slightly inhibited HUVEC cell migration, invasion and tube formation (Figure 7 and S7). VEGF and VEGFR2 act as an index for measurement of angiogenesis in tumors.^{37, 38} As shown in Figure S8, the expression levels of VEGF and VEGFR-2 were decreased upon RBCs@Se/Av treatment in HUVEC cells. These data clearly indicate that the main antiangiogenic action of RBCs@Se/Av occurs via suppression of VEGFR-2 signaling pathway.

***In vivo* radiosensitization and antiangiogenesis effects of RBCs@Se/Av**

We also examined the therapeutic efficacy of RBCs@Se/Av on A375 xenografts. RBCs@Se/Av dramatically inhibited the growth of the A375 tumors, as verified by decrease in the relative tumor volume and relative tumor growth ratio in the treated group. For example, the relative tumor growth ratio decreased from 100 % to 17.4 % after combined treatment with X-ray (4 Gy) and 1 mg/kg RBCs@Se/Av (the concentration was calculated based on Se content). In comparison, the relative tumor growth ratios were 32.7 and 60.1 % respectively, in X-ray only or RBCs@Se/Av (1 mg/kg) only treatment groups (Figure 8a). The relative tumor volume of combined treatment group was 2.5 after treatment for 21 day, significantly smaller than that of X-ray-treated (4.7) and RBCs@Se/Av-treated (8.7) groups (Figure 8b). As shown in Figure S9a, compared with untreated control, combined treatment markedly decreased the tumor weight (0.95 g and 0.14 g, respectively). These data suggest that the antitumor activity of X-ray *in vivo* was markedly enhanced after radiosensitization of the tumors by RBCs@Se/Av nanosystems. Fluctuation of body weights is a direct indicator of toxicity *in vivo*. Neither death of animals nor a significant change in body weights was observed in both the combination treatment and control groups over a span of 21 days (Figure S9b), indicating that RBCs@Se/Av combined X-ray treatment exhibited no significant side effects on the mice.

Moreover, MR imaging was also employed to confirm the anticancer activity of RBCs@Se/Av *in vivo*. T2-weighted MR images were obtained from tumor-bearing nude mice after 21-day treatment. Slow ADC (ADCs) and standard ADC (ADCst) values could reflect the density and activity of cells in the tumor area. As shown in Figure 8c, strong inhibition was observed in tumors after combined treatment compared with control group. ADCst and ADCs values were markedly increased to 0.000995 and 0.000900 in tumors

exposed to RBCs@Se/Av with X-ray (Figure S10), while X-ray and RBCs@Se/Av treatments caused little change compared to the control group. These results indicate that combined treatment markedly reduces the activity and density of cancer cells in the tumor tissue. CD34 is a marker for small vessel endothelium and hematopoietic progenitors. RBCs@Se/Av + X-ray treatment markedly activated p53 phosphorylation at Ser15 site and inhibited the expression level of CD34, as demonstrated by H&E staining, which may contribute to the antiangiogenic action of our developed nanosystem (Figure 8d). Taken together, these results indicate that the combined treatment significantly inhibits A375 tumor growth *in vivo* by suppressing cell proliferation and inducing apoptosis.

***In vivo* toxicity evaluation of RBC@Se/Av and radiotherapy**

For development of new therapeutic paradigms, balance between toxicity and anticancer efficiency is critical. Consequently, the acute toxicity of the combination therapy was examined. 21 days after injection, the mice were euthanized and their tumor, major organs and blood were collected for histology analysis and blood biochemistry. Compared with the healthy nude mice (without A375 xenografts), the tumor-bearing nude mice showed obvious acute adverse effects to the major organs, as reflected by the change in the tested hematological indices (Figure 9a). Interestingly, combination treatment of effectively alleviated the damage to the major organs induced by tumor xenograft growth, as reflected by reversion of the blood biochemical values, such as serum globulin (GLB), serum albumin/serum globulin (A/G), glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), and uric acid (UA), towards normalcy. Moreover, in the histological analysis, no noticeable damage in the major organs was observed in the mice (Figure 9b) which demonstrated the high *in vivo* biocompatibility of RBCs@Se/Av. Taken together, this study supports the promising potential of RBCs@Se/Av as effective and safe radiosensitizer for radiotherapy of human malignant melanoma.

Conclusion

Radiotherapy is a vitally important strategy for clinical treatment of malignant cancers. Therefore, rational design and development of radiosensitizers that could enhance radiotherapeutic efficacy have attracted more and more attention. In this study, we have rationally designed and synthesized a smart nanosystem (RBCs@Se/Av), antiangiogenic therapy and radiosensitization for effective radiotherapy were seamlessly combined into a single highly efficient, biomimetic nanosystem. The RBC membrane coating effectively prolonged the blood circulation times and reduced the elimination of our designed nanosystem by autoimmunity. As expected, RBCs@Se/Av with X-ray irradiation demonstrated potent anticancer and antiangiogenesis efficacy *in vitro* and *in vivo*, as evidenced by enhanced inhibition of A375 tumor growth in athymic nude mice model, without causing obvious histological damage to the major non-target organs. Taken together, this study demonstrates a promising, facile strategy for design of smart Se-based nanosystems decorated with RBC membrane for simultaneous cancer radiosensitization and precise antiangiogenesis in melanoma cancer models.

Experimental section

Materials

Bevacizumab (Roche Diagnostics GmbH., Mannheim, Germany, Av), acetic acid, nitric acid, perchloric acid, sodium chloride, potassium chloride and magnesium chloride were purchased from Guangzhou Chemical Reagent Factory. Thiazolyl blue tetrazolium bromide (MTT), DMSO, phosphate buffer, DMEM media, ascorbic acid, Na_2SeO_3 , ethylenediaminetetraacetic acid (EDTA) and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were procured from Sigma-Aldrich. EDTA-free mini protease inhibitor tablet was purchased from Thermo Scientific. The ultrapure water from Milli-Q water purification system (Milipore) was used in all experiments.

Preparation of RBC-NPs

Whole blood from human was centrifuged at 3000 rpm for 10 minutes to separate the plasma and erythrocytes. The collected erythrocytes were washed thrice with pre-cooled $1 \times$ PBS buffer. After that, the washed erythrocytes were suspended in pre-cooled $0.25 \times$ PBS buffer in an ice bath for 30 min to hemolysis and then were centrifuged at 3000 rpm for 10 minutes at 4°C to remove the released hemoglobin. Further, the pellets were collected and washed for three times with $1 \times$ PBS buffer. The pellets designated as RBC ghosts (devoid of cytoplasmic contents) were suspended in $1 \times$ PBS and sonicated in a capped glass vial for 10 min using a bath sonicator before centrifugation at 20000 g for 30 min. The supernatant was transferred to another tube and centrifuged again at 100,000 g for 30 min. The final pellets were then suspended in PBS and extruded through 200 nm polycarbonate porous membranes with an Avanti mini extruder (Avanti Polar Lipids).^{39, 40}

Preparation of RBCs@Se/Av

PEG-SeNPs were prepared as described in our previous study. Briefly, 20 mg Se powder was dissolved in 10 mL PEG 400 solution at 290°C for 1 h under magnetic stirring. The product was then mixed with water in a 1:6 ratio.²⁹ 700 μL of PEG-SeNPs at Se content of 2736 μM and 42 μL (25 mg/mL) Av was mixed with RBC-NPs derived from 1.5 mL of whole blood. The resultant mixture was subsequently extruded for 20 times through a 400 nm polycarbonate porous membrane using an Avanti mini extruder, the mechanical force produced by the extruding facilitates the fusion of RBC membranes cover nanoparticles, yielding the RBC-membrane-encapsulated PEG-SeNPs and Av (RBC@Se/Av).^{41, 42}

Characterization of RBC-NPs, PEG-SeNPs and RBC@Se/Av

The as-synthesized RBC-NPs, PEG-SeNPs and RBC@Se/Av were then characterized by a Zetasizer Nano ZS particle analyzer (Malvern Instruments Limited) to determine its size and zeta potential. TEM and AFM were carried out to study its morphology and high resolution TEM was carried out to analyze its elemental composition. The concentration of Se in the nanoparticles was determined using an atomic fluorescence spectrometer (AF-2200, Beijing Rayleigh analysis instrument co. LTD).

Stability and hemocompatibility of RBC@Se/Av

Approximately 0.5 mL of PBS, 0.5 mL of DMEM with 10% fetal bovine serum (FBS) or 0.5 mL human serum was added to 0.5 mL RBC@Se/Av to examine the stability of nanoparticles. After different incubation periods, the size of nanoparticles was determined by Zetasizer Nano ZS particle analyzer.

Different concentrations of RBC@Se/Av were pre-mixed with heparinized RBCs, and then incubated for 2 h at 37 °C. Thereafter, RBCs were centrifuged to collect the supernatant and the absorbance was measured for the collected supernatant by spectrophotometry at 540 nm and the hemolysis was determined based on the following equation (1).

$$\text{Hemolysis (\%)} = \frac{A_{\text{sample}} - A_{\text{NC}}}{A_{\text{PC}} - A_{\text{NC}}} \times 100 \% \quad (1)$$

Where A_{sample} is the absorbance of sample at 540 nm, A_{NC} is the absorbance of negative control at 540 nm, A_{PC} is the absorbance of positive control at 540 nm.

The collected cells were then investigated further for agglutination, each sample was placed on a glass slide, under a cover slip and observed under a phase contrast microscope (Life technologies, EVOS FL auto).⁴³

Cell lines and cell culture

All human cell lines including A375 (human melanoma cells) and HUVEC (human vascular endothelial cell), were purchased from American Type Culture Collection (Manassas, VA). A375 cells were incubated in DMEM media and HUVEC cells in F12 media supplemented with penicillin (100 units/mL), streptomycin (50 units/mL) and fetal bovine serum (10%) at 37 °C in a humidified incubator with 5% CO₂ atmosphere.

In vitro anticancer efficacy of RBC@Se/Av

The cytotoxicity of RBC@Se/Av on cancer cells and HUVEC cells were evaluated by MTT assay as reported.⁴⁴

Cellular uptake of RBC@Se/Av in A375 and HUVEC cells

A375 and HUVEC cells were seeded into 10 cm dish at 1×10^5 cells/mL and allowed to attach for 24 h. Different concentration of PEG-SeNPs and RBC@Se/Av were added and incubated for 1, 2, 4, 6 and 8 h. The cells were collected for measuring the concentration of Se by an atomic fluorescence spectrometer.

Clonogenic assays

A375 cells were seeded into 6-well plates at 1500 cells/mL (2 mL) and allowed to attach for 24 h. After 4 h incubation with RBC@Se/Av at different concentrations, the cells were then exposed to X-ray (4 Gy) and then incubated for another 5 days. After that the cells were

fixed with 4% paraformaldehyde (v/v), and then stained with 0.5% crystal violet (w/v). The survival fraction of the clones was used to evaluate the effect of different treatments.

Flow Cytometric Analysis

The effects of the RBCs@Se/Av on the cell cycle distribution were analyzed by flow cytometry as previously described. The proportion of A375 and HUVEC cells in G0/G1, G2/M, and S phases was shown in a DNA histogram. The number of apoptotic cells with hypo-diploid DNA content was measured by quantifying the sub-G1 peak.

Measurement of ROS Generation

RBCs@Se/Av-induced intracellular ROS accumulation in the treated A375 and HUVEC cells was measured by staining the cells with DCFH-DA as described previously.⁴⁵

Mitochondria fragmentation

Mito-Tracker Red, a fluorescent dye which can stain mitochondria in living cells, was used in this assay. Firstly, A375 and HUVEC cells were pretreated with 10 μ M RBCs@Se/Av at 37 °C for 4 h, followed by irradiation by X-ray (4 Gy) and incubation at 37 °C for 24 h. The cells were stained with Mito-Tracker (final concentration of 100 nM) for at least 2 h. Mitochondria fragmentation and nuclear condensation induced by RBCs@Se/Av with X-ray was observed and detected by a fluorescence microscope.

Western blot analysis

Western blot analysis was used to analyze the expression levels of various proteins related to different signaling pathways following treatment with the RBCs@Se/Av and X-ray.⁴⁶

Examination of cell migration, invasion and tube formation

Migration assay were investigated as literature reported, 10 μ g/mL mitomycin C was added for pre-treatment 2 h to inhibit proliferation before creating a scratch gap.⁴⁷ Invasion and tube formation assays on HUVEC cells were conducted as our previous study.^{30, 48}

Retention of RBCs@Se/Av in rats

Six male SD rats (7 weeks old) were randomly separated into two groups and administered with PEG-SeNPs and RBCs@Se/Av respectively, via intravenous injection with equivalent dose of 1 mg Se/kg. Then blood samples were collected from the retro-orbital plexus of the eye at specific time-points (0, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h). After that, the blood samples were nitrified and the selenium contents were determined by atomic fluorescence spectrometer.

In vivo antitumor activity of RBCs@Se/Av with X-ray irradiation

All animal studies were approved by the Animal Experimentation Ethics Committee of Jinan University. A375 cells at a density of $1.0 \times 10^6 \text{ mL}^{-1}$ were suspended in DMEM (150 μ) and subcutaneously injected into the armpit of female nude mice to establish neoplasia. Once the mean tumor volume reached approximately 125 mm³, the mice were divided into four groups stochastically. The mice were administered with (1) saline (200 μ L); (2) X-ray (4

Gy); (3) RBCs@Se/Av (200 μ L, concentration of Se: 0.1 mg/mL and (4) RBCs@Se/Av (200 μ L concentration of Se: 0.1 mg/ml) + X-ray through intravenous injection and monitored for 21 days. The body weights of mice were measured regularly every two or three days.

After 21 days, T2-weighted MRI was carried out on a 1.5 T Signa HDxt superconductor clinical MR system (GE medical, Milwaukee, WI). The blood of mice was collected for measurement of biochemical parameters including serum globulin (GLB), serum albumin/serum globulin (A/G), glucose (GLU), blood urea nitrogen (BUN), creatinine (CREA), and uric acid (UA) followed by euthanization. Tumor and other major organs including heart, liver, spleen, lung and kidney were collected and fixed in 4% paraformaldehyde, 2 mm sections of the fixed tissues were then subjected to H&E staining and immunohistochemistry, and observed under a light microscope (Nikon ni-u, Japan).

Statistical analysis

All experiments in this study were examined in triplicate. Data were represented as mean $\pm$ standard deviation (SD). Difference between control and experimental groups were analyzed by two-tailed student's t-test method. Differences indicated as $P < 0.05$ (*) or $P < 0.01$ (**) were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Joiner MC, Van der Kogel A. Basic clinical radiobiology. CRC press; 2016.
2. Delaney G, Jacob S, Featherstone C, Barton M. Cancer. 2005; 104:1129–1137. [PubMed: 16080176]
3. Song G, Liang C, Gong H, Li M, Zheng X, Cheng L, Yang K, Jiang X, Liu Z. Advanced Materials. 2015; 27:6110–6117. [PubMed: 26331476]
4. Cheng L, Shen S, Shi S, Yi Y, Wang X, Song G, Yang K, Liu G, Barnhart TE, Cai W. Advanced functional materials. 2016; 26:2185–2197. [PubMed: 27110230]
5. Chen Q, Feng L, Liu J, Zhu W, Dong Z, Wu Y, Liu Z. Advanced materials. 2016; 28:7129–7136. [PubMed: 27283434]
6. Her S, Jaffray DA, Allen C. Advanced drug delivery reviews. 2017; 109:84–101. [PubMed: 26712711]
7. Zhong X, Yang K, Dong Z, Yi X, Wang Y, Ge C, Zhao Y, Liu Z. Advanced Functional Materials. 2015; 25:7327–7336.
8. Narita Y. Therapeutics and clinical risk management. 2015; 11:1759. [PubMed: 26664126]
9. Lai H, Zhang X, Feng P, Xie L, Chen J, Chen T. Chemistry–An Asian Journal. 2017; 12:982–987.
10. Jain RK. Cancer cell. 2014; 26:605–622. [PubMed: 25517747]
11. Mukherji S. American Journal of Neuroradiology. 2010; 31:235–236. [PubMed: 20037132]

12. Cohen MH, Shen YL, Keegan P, Pazdur R. The oncologist. 2009; 14:1131–1138. [PubMed: 19897538]
13. Ferrara N, Hillan KJ, Novotny W. Biochemical and biophysical research communications. 2005; 333:328–335. [PubMed: 15961063]
14. Hu CMJ, Fang RH, Zhang L. Advanced healthcare materials. 2012; 1:537–547. [PubMed: 23184788]
15. Tan S, Wu T, Zhang D, Zhang Z. Theranostics. 2015; 5:863. [PubMed: 26000058]
16. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Science. 2000; 288:2051–2054. [PubMed: 10856220]
17. Kim DD, Miwa T, Kimura Y, Schwendener RA, van Lookeren Campagne M, Song W-C. Blood. 2008; 112:1109–1119. [PubMed: 18524992]
18. Zhou H, Fan Z, Lemons PK, Cheng H. Theranostics. 2016; 6:1012. [PubMed: 27217834]
19. Gao W, Zhang L. AIChE J. 2015; 61:738–746.
20. Luk BT, Zhang L. Journal of Controlled Release. 2015; 220:600–607. [PubMed: 26210440]
21. Wu Z, Li T, Gao W, Xu T, Jurado-Sánchez B, Li J, Gao W, He Q, Zhang L, Wang J. Advanced Functional Materials. 2015; 25:3881–3887.
22. Luk BT, Fang RH, Hu CMJ, Copp JA, Thamphiwatana S, Dehaini D, Gao W, Zhang K, Li S, Zhang L. Theranostics. 2016; 6:1004. [PubMed: 27217833]
23. Dehaini D, Wei X, Fang RH, Masson S, Angsantikul P, Luk BT, Zhang Y, Ying M, Jiang Y, Kroll AV. Advanced Materials. 2017:29.
24. Yu B, Liu T, Du Y, Luo Z, Zheng W, Chen T. Colloids and Surfaces B: Biointerfaces. 2016; 139:180–189. [PubMed: 26709976]
25. Zhang J, Wang X, Xu T. Toxicological sciences. 2007; 101:22–31. [PubMed: 17728283]
26. Zeng L, Chen J, Ji S, Chan L, Zheng W, Chen T. Journal of Materials Chemistry B. 2015; 3:4345–4354.
27. Neu M, Germershaus O, Behe M, Kissel T. Journal of Controlled Release. 2007; 124:69–80. [PubMed: 17897749]
28. Kroll AV, Fang RH, Zhang L. Bioconjugate chemistry. 2016; 28:23–32. [PubMed: 27798829]
29. Chang Y, He L, Li Z, Zeng L, Song Z, Li P, Chan L, You Y, Yu XF, Chu PK. ACS nano. 2017; 11:4848–4858. [PubMed: 28460158]
30. Hu H, You Y, He L, Chen T. Journal of Materials Chemistry B. 2015; 3:6338–6346.
31. Wang N, Feng Y, Zeng L, Zhao Z, Chen T. ACS applied materials & interfaces. 2015; 7:14933–14945. [PubMed: 26107995]
32. Liu T, Lai L, Song Z, Chen T. Advanced Functional Materials. 2016; 26:7775–7790.
33. Fan C, Zheng W, Fu X, Li X, Wong Y-S, Chen T. Oncotarget. 2014; 5:2853. [PubMed: 24797310]
34. You Y, He L, Ma B, Chen T. Advanced Functional Materials. 2017:27.
35. Nguyen GH, Murph MM, Chang JY. Cancers. 2011; 3:1232–1252. [PubMed: 21603589]
36. Xie L, Luo Z, Zhao Z, Chen T. Journal of medicinal chemistry. 2016; 60:202–214. [PubMed: 28001402]
37. Nagy VM, Buiga R, Brie I, Todor N, Tudoran O, Ordeanu C, Virag P, Tarta O, Rus M, Balacesu O. Rom J Morphol Embryol. 2011; 52:53–59. [PubMed: 21424032]
38. Qi X, Liu G, Qiu L, Lin X, Liu M. Biomedicine & Pharmacotherapy. 2015; 75:58–66. [PubMed: 26463632]
39. Gao W, Hu CMJ, Fang RH, Luk BT, Su J, Zhang L. Advanced Materials. 2013; 25:3549–3553. [PubMed: 23712782]
40. Rao L, Bu LL, Cai B, Xu JH, Li A, Zhang WF, Sun ZJ, Guo SS, Liu W, Wang TH. Advanced Materials. 2016; 28:3460–3466. [PubMed: 26970518]
41. Hu CMJ, Zhang L, Aryal S, Cheung C, Fang RH, Zhang L. Proceedings of the National Academy of Sciences. 2011; 108:10980–10985.
42. Piao JG, Wang L, Gao F, You YZ, Xiong Y, Yang L. ACS nano. 2014; 8:10414–10425. [PubMed: 25286086]

43. Liu T, Zeng L, Jiang W, Fu Y, Zheng W, Chen T. Nanomedicine: Nanotechnology, Biology and Medicine. 2015; 11:947–958.
44. Nie T, Wu H, Wong KH, Chen T. J Mater Chem B. 2016; 4:2351–2358.
45. Huang Y, He L, Liu W, Fan C, Zheng W, Wong YS, Chen T. Biomaterials. 2013; 34:7106–7116. [PubMed: 23800743]
46. Chen T, Wong YS. The international journal of biochemistry & cell biology. 2009; 41:666–676. [PubMed: 18718551]
47. Mahapatra C, Singh RK, Lee JH, Jung J, Hyun JK, Kim HW. Acta biomaterialia. 2017; 50:142–153. [PubMed: 27940193]
48. Song Z, Liu T, Chen T. Journal of Materials Chemistry B. 2018

Figure 1.

The morphological analysis of RBCs@Se/Av. (a) Schematic illustrating of the preparation of RBCs@Se/Av for induction of cancer and HUVEC cells apoptosis *in vitro* and inhibition of cancer growth and angiogenesis *in vivo*. (b) The TEM images of PEG-SeNPs, RBC-NPs and RBCs@Se/Av nanoparticles. Bottom row: HAADF-STEM images of an individual RBCs@Se/Av nanoparticle. (c) Average hydrodynamic diameters and (d) zeta potential of PEG-SeNPs, RBC-NPs and RBCs@Se/Av. Each value represents means $\pm$ SD (n = 3).

Figure 2.

Hemocompatibility of RBCs@Se/Av. (a) The stability of RBCs@Se/Av in PBS, DMEM with 10% FBS and human serum within 72 h. (b) Hemolysis induced by RBCs@Se/Av after 2 h. (c) Agglutination of red blood cells observed by phase microscopy after 2 h of incubation with PEG-SeNPs and RBCs@Se/Av. (d) Time-concentration curves of naked PEG-SeNPs and RBCs@Se/Av in the blood, based on concentration of Se in serum at different time points post injection. Each value represents means $\pm$ SD ($n = 3$).

Figure 3.

Synergistic radiotherapy of RBCs@Se/Av *in vitro*. Cytotoxic effects of PEGSeNPs and RBCs@Se/Av with X-ray irradiation on (a) A375 cells and (b) HUVEC cells. Isobologram analysis of the synergistic anti-proliferative effect of combination RBCs@Se/Av + X-ray treatment on (c) A375 and (d) HUVEC cells. The data points in the isobologram correspond to the 50% growth inhibition of cells with combined treatment RBCs@Se/Av + X-ray. (e) Clonogenic assay was performed in 6-well plates cultured up to 5 days after treatment with RBCs@Se/Av and X-ray on A375 cells. Each value represents mean $\pm$ SD ($n = 3$).

Figure 4. *In vitro* anticancer and antiangiogenic action mechanism. (a, b) Flow cytometric analysis: cells were treated with different concentrations of RBCs@Se/Av and X-ray for 72 h. (c, d) Western blot analysis for the expression of caspase-9, caspase-8, caspase-3, and PARP in cells treated with different concentrations of RBCs@Se/Av and X-ray for 72h. β -Actin was used as loading control.

Figure 5.

Induction of ROS by RBCs@Se/Av and radiation. (a, b) The ROS generated by 10 μ m RBCs@Se/Av x-ray irradiation were analyzed by DCF-DA assay. (c, d) Fluorescence microscopy images of the generation of ROS detected by DCF-DA staining. Each value represents mean $\pm$ SD (n = 3).

Figure 6. Induction of ROS-mediated mitochondrial dysfunction. The morphological change of mitochondria in cells after treatment with RBCs@Se/Av and X-ray irradiation for 24 h on (a) A375 and (b) HUVEC cells. The cells were pre-stained with Hoechst 33342 and Mito-Tracker Red, and observed by fluorescence. *In vitro* anticancer and antiangiogenic action mechanisms. (c, d) RBCs@Se/Av enhances radiation-induced activation of p53 signaling pathway and (e, f) RBCs@Se/Av and X-ray downregulates the expression of VEGFR2 and XRCC1. β-Actin was used as loading control.

Figure 7. RBCs@Se/Av inhibited VEGF-induced angiogenesis. (a) Wound healing assay, 10 µg/mL mitomycin C was added for pre-treatment 2 h to inhibit proliferation before creating a scratch gap; (b) anti-invasion effect and (c) tube formation assay of RBCs@Se/Av on HUVEC cells induced by VEGF.

Figure 8.

In vivo therapeutic effects of RBCs@Se/Av with X-ray irradiation in A375 tumor-bearing mice, (a) Relative tumor growth ratio and (b) Relative tumor volumes. T2-weighted MR images of A375 tumor-bearing mice after treatment for 21 days, (c) T2-weighted images, DWI MB, ADCst and ADCs. The third and fourth row of each figure is the pseudocolor image of the tumor site, (d) Histological analyses of the tumor tissue following treatment with RBCs@Se/Av and X-ray. Each value represents mean $\pm$ SD ($n = 3$). Significant difference between control and treatment groups is indicated at $P < 0.05$ (*) or $P < 0.01$ (**) level.

Figure 9.

In vivo toxicity evaluation, (a) Blood biochemistry data including liver-function markers: GLB, A/G, kidney-function markers: BUN, CREA, UA and blood glucose: GLU. (b) H&E-stained images of major organs. Each value represents means $\pm$ SD (n = 3). Bars labeled with different characters show statistically significant difference at $P < 0.05$ level.