Targeted Therapy of Atherosclerosis Vulnerable Plaque By ROS-Scavenging Nanoparticles And MR/Fluorescence Dual-Modality Imaging Tracing

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Research Article

Keywords: vulnerable plaques, ROS scavengers, nano-enzyme, nanoprobe, MRI, fluorescence imaging

Posted Date: December 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1154405/v1>

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Abstract

Background: Cardiovascular diseases are currently the leading cause of death and disability worldwide, and the key pathological basis is atherosclerosis (AS). Especially, the rupture of vulnerable plaques is the main cause of acute cardiovascular and cerebrovascular events such as myocardial infarction and stroke. Thus, the early identifying and therapy of vulnerable plaques are necessary.

Results: In this study, we developed a novel multimodal imaging platform (GPRD) based on Gd doped Prussian blue (GPB) and rhodamine (Rd) to specifically target and identify the vulnerable plaques with the help of dextran sulfate (DS), one of the excellent ligands of scavenger receptor class A (SR-A). It is more important that the nano-enzyme capacity of GPRD NPs realized the elimination of the excessive production of ROS in cells, and the following reduction of ROS-induced oxidative stress, inflammation, apoptosis, and the formation of macrophage-derived foam cells, presenting an inhibition of plaque progress eventually.

Conclusions: The ROS-scavenging multimodal imaging nanoprobe provided a new avenue for the diagnosis and treatment of AS vulnerable plaques.

Background

Atherosclerosis (AS), a chronic inflammatory vascular wall lesion, is the common pathophysiological basis of cardiovascular diseases, such as coronary heart disease and stroke^[1]. The increasing burden of cardiovascular diseases worldwide has become a major public health problem. With the development and application of biological nanomaterials in recent years, researchers have designed a large number of nanomaterials for the detection or treatment of AS^[2]. AS plaque rupture is an important cause of acute cardiovascular and cerebrovascular events^[3], thus early identification and treatment of vulnerable atherosclerotic plaques are more meaningful to improve the prognosis of patients.

Researchers have discovered and applied some biological markers of vulnerable lesions for targeted detection of vulnerable AS plaques^[4]. Macrophage scavenger receptor type A (SR-A) is one of the major receptors involved in foam cell formation, mainly mediated by lipid influx into macrophages. Furthermore, SR-A mainly plays a very important role in host defense, cell activation, adhesion, and inflammatory response of cell-cell interaction^[5]. Wang et al. developed a dual-mode molecular probe for atherosclerotic vulnerable plaque. Because it can specifically recognize SR-A on the surface of macrophages, it can be effectively internalized by vulnerable plaque^[6]. Myeloperoxidase (MPO) is an inflammatory protein secreted by neutrophils and macrophages during the inflammation of atherosclerotic plaque. It has attracted more and more attention and is considered a potential target for identifying vulnerable atherosclerotic plaques. Tong Wei et al. combined superparamagnetic iron oxide nanoparticles with serotonin and cyanine 7 N-hydroxysuccinimide ester to design a new multimode imaging nanoprobe 5-HT-Fe₃O₄-Cy7 for MPO active targeting^[7]. Osteopontin (OPN) is a good biomarker for identifying vulnerable plaques, mainly secreted by macrophages and macrophages derived foam cells^[8]. Qiao

designed an OPN modified upconversion luminescent nanoprobe to specifically detect vulnerable atherosclerotic plaques *in vivo*^[12]. Except for specific detection, targeted biomarkers have also been combined with nanomaterials for the effective treatment of vulnerable atherosclerotic plaques^[1]. For example, Ma et al. developed a nanocomplex for targeted treatment of atherosclerotic plaque, where SRT1720 acted as a therapeutic drug. In their nanoformulation, OPN was selected as the targeting molecule for vulnerable atherosclerotic plaques^[1]. Manse Kim et al. developed a cRGD conjugated pluronic-based nanocarrier loaded with a multipotent anti-inflammatory cytokine, interleukin-10 (IL10). The fabricated nanomaterials could bind with $\alpha_v\beta_3$ integrin, which overexpressed in the neovasculature of the vulnerable plaque to realize the targeted treatment of atherosclerotic plaques^[1]. Nevertheless, to the urgency of the diagnosis and treatment of AS-related diseases, these studies are far from enough. Accurate treatment strategies cannot be formulated without the guidance of imaging. However, the complex structures and complicated preparation processes in many multifunctional nanoparticles lead to poor stability and hinder their long-term clinical transformation. Thus, how to realize the diagnosis and treatment of vulnerable AS plaque in an easy way is still an important issue.

AS is a kind of common chronic inflammatory vascular wall disease, which is often accompanied by the production of reactive oxygen species (ROS) in its occurrence and development. ROS can lead to macrophages activation, cell apoptosis, and oxidative damages to biomacromolecules (such as lipid, protein, DNA, etc.)^[1]. ROS and its oxidation products can activate the corresponding signaling pathways with functional signaling molecules, which mediate a series of changes in vascular cells and affect vascular function and normal structure^[1]. Based on the crucial role of ROS in the pathogenesis of AS, eliminating ROS might be an important strategy for AS treatment. What's more, vulnerable AS plaques are easy to be ignored due to their hidden early clinical manifestations. Thus, it is more necessary to identify early vulnerable lesions through the combination of multimodal imaging technology. To address these issues, we developed a theranostic nanocomplex for the diagnosis and treatment of vulnerable AS plaque by ROS scavenging and MR/fluorescence imaging. Learned from the literature^[1], PB has catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and other enzyme activities, which can effectively remove ROS in lesions and relieve oxidative response and inflammation^[1]. The nano-enzyme effect of PB has been used in the treatment of inflammatory bowel disease and stroke injury, which has achieved remarkable therapeutic effect^[1]. It is more important that PB is of interest due in part to its excellent biocompatibility, which is approved by the US Food and Drug Administration (FDA), under the brand name Radiogardase®, for treatment of patients with contamination of cesium and/or thallium^[1]. Thus, we prepared Gd-doped Prussian blue (GPB) as a multifunctional nanocarrier for MRI and ROS scavenging. Then, GPB was further modified with water-soluble polymer polyethyleneimine (PEI), fluorescence molecule rhodamine (Rd), and targeted molecule dextran sulfate (DS) which is one of the excellent ligands of SR-A^[1] step by step via electrostatic adsorption (Scheme. 1). The experimental results showed that our fabricated GPB-PEI-Rd-DS nanoparticles (GPRD NPs) not only displayed excellent MR/fluorescence dual-modality imaging of vulnerable AS plaque *in vivo*, but also effectively utilized the nano-enzyme effect of GPB to inhibit the AS progress by ROS scavenging and the following reduction of

inflammation, apoptosis, and foam cells formation, providing a new avenue for the diagnosis and treatment of vulnerable AS plaque.

Results

Preparation and characterization of GPRD NPs

Gd-doped GPB was prepared by a one-step hydrothermal method with a simple and stable structure. TEM images demonstrated the formation of uniform GPB and GPRD NPs with cube morphology. The average sizes were 88.7 ± 6.3 and 99.4 ± 6.7 nm for GPB and GPRD NPs, respectively (Fig. 1a). Element mapping confirmed the composition of GPRD NPs with C, N, Fe, Gd, and K elements (Fig. 1b), and the five elements were distributed uniformly in the structure of GPRD NPs. As shown in Fig. 1c, the hydrodynamic diameter was increased accordingly after each step of assembly. They were 142 nm for GPB, 190 nm for GPB-PEI, and 220 nm for GPRD NPs. In addition, the zeta potential change also witnessed this assembly process. The potential value of GPB was -14.4 mV, and after PEI assembly, it was changed to 29.4 mV due to the positive charged PEI. With the addition of DS and Rd, the zeta potential of the GPRD NPs became -17.6 mV (Fig. 1d). Fourier transform infrared spectroscopy (FT-IR) of GPRD NPs showed the typical absorbance peaks of -CN- stretching of GPB and S=O stretching of DS at approximately 2089 and 1262 cm^{-1} (Fig. 1e), respectively, indicating the successful assembly of DS. The UV-vis-NIR absorbance spectrum of GPRD NPs discovered an obvious characteristic GPB peak at 706 nm and the red-shift absorbance spectrum from 527 nm to 553 nm (Fig. 1f), coming from the Rd coating. The above results proved the successful synthesis of GPRD NPs well.

Dual-modality imaging and stability features of GPRD NPs

The MRI and fluorescence imaging ability of GPRD NPs were first evaluated. As shown in Fig. 1g, GPRD NPs solution emitted a bright yellow-green light upon irradiation with a UV lamp (365 nm). The emission wavelength of GPRD NPs was 550 nm with an excitation wavelength of 490 nm. Furthermore, as the concentration of GPRD NPs increased, the fluorescence intensity displayed a gradual increase. The MR images of GPRD NPs were conducted by a previously described MR system. As shown in phantom images and pseudo-color images (Fig. 1h), the MR signal intensity of GPB and GPRD NPs increased along with the increase of Gd concentration, and the T_1 relaxivity (r_1 value) of GPRD NPs were calculated to be $32.06 \text{ mM}^{-1} \text{ s}^{-1}$ (Fig. 1i). The T_1 relaxivity of GPRD NPs was probably 8 times comparing with that of Gd-DTPA ($4.21 \text{ mM}^{-1} \text{ s}^{-1}$)^[31], indicating an enhanced MR contrast ability of GPRD NPs.

The prepared GPRD NPs should possess good physiological stability for their biomedical application. Thus, we investigated the colloid stability of GPRD NPs by determining the changes of hydrodynamic diameter with the time passing. As shown in Fig. 1j, their hydrodynamic diameters kept stable at about 220 nm in PBS buffer without significant changes for even 7 days standing. More importantly, no noticeable signal changes of MR and fluorescence intensities were observed during the 7 day-period (Fig. 1k, l), demonstrating the good stability of GPRD NPs in aqueous conditions.

ROS-Scavenging Capacity of GPRD NPs

PB was reported to possess the ability to eliminate ROS^[32], and act as a variety of nano-enzymes to limit tumor and inflammation^[33,21]. Thus, we suspected that GPRD NPs should preserve the ROS-scavenging capacity of PB. Therefore, their catalase (CAT)-like, peroxidase (POD)-like, and superoxide dismutase (SOD)-like activities of GPRD NPs were first evaluated. As shown in Fig. 4a, the addition of H₂O₂ into the GPRD NPs solution could generate a number of observable bubbles, which were verified to be O₂ by a dissolved oxygen electrode. It might be attributed to the decomposition of H₂O₂ into H₂O and O₂ by the CAT-like activity of GPRD NPs. Moreover, GPRD NPs induced H₂O₂ consumption displayed a time- and concentration- dependent manner (Fig. 2a-c). The CAT-like activity can be shown in the following reaction.

Then, we further studied the POD-like activity of GPRD NPs. A commonly used natural POD substrate, TMB, was selected to investigate their POD-like activity. The absorbance of TMB_(oxidized) at 650 nm was recorded with a Microplate Reader. As shown in Fig. 2d and 2e, the absorbance was increased after the addition of GPRD NPs, indicating GPRD NPs could catalyze H₂O₂ to oxidize the toxic TMB into nontoxic substances. Furthermore, such oxidation was also concentration- and time-dependent. The POD-like activity can be shown in the following reaction.

Lastly, we evaluated the O₂^{•-}-scavenging capability of GPRD NPs by SOD-like activity. We used the xanthine/xanthine oxidase system to induce the generation of O₂^{•-} by SOD Assay kit. The absorbance of 550 nm was recorded after the addition of the GPRD NPs into the xanthine/xanthine oxidase system 45 min later. With the O₂^{•-}-scavenging, the color of the reaction solution became lighter due to the nitrite reduction, which indicates a high O₂^{•-}-scavenging capacity of GPRD NPs (Fig. 2f). Moreover, GPRD NPs eliminated O₂^{•-} in a dose-dependent profile. The SOD-like activity can be shown in the following reaction.

Hence, the above results suggested that the prepared GPRD NPs can act as nano-enzymes to efficiently change the detrimental ROS into H₂O and O₂, possessing the potentials to avoid oxidative damages to biomacromolecules and block some ROS mediated inflammatory signaling pathways. Studies have shown that PB NPs have CAT-like, SOD-like, and POD-like activities, which may be due to their various forms, such as Prussian yellow, Prussian white, and Prussian blue. Therefore, they have rich redox potential and become powerful electron transporters^[23]. Therefore, we believe that GPRD NPs have great potential for the treatment of oxidative stress in vulnerable atherosclerotic plaques.

Targeted dual-modality imaging for activated RAW264.7 cells

Atherosclerotic plaques are easy to be ignored due to their hidden early clinical manifestations. Thus, it is more necessary to identify early vulnerable lesions through the combination of multimodal imaging technology. At present, MRI and fluorescence imaging are widely used in AS. MRI is used to obtain the anatomical structure of the diseased blood vessels. Meanwhile, the nature and composition of plaque are preliminarily analyzed. The molecular and cellular information can be obtained by fluorescence imaging with its high sensitivity. The advantages of bimodal imaging are integrated to provide a more accurate imaging mode for vulnerable plaque.

Before we applied GPRD NPs for targeted dual-modality imaging *in vitro*, an MTT assay was first performed to assess their cytotoxicity against RAW264.7 and HUVEC cells. As shown in Fig. 3a, even at a high concentration of 400 $\mu\text{g/mL}$, more than 90 % of the cells still survived after 24 h incubation with GPRD NPs for both RAW264.7 and HUVEC cells, suggesting the low toxicity and excellent biocompatibility of the prepared GPRD NPs. Then, we used LPS to induce macrophage activation and evaluated the expression of SR-A in activated macrophages by Western Blot assay. As shown in Fig. 3b, compared with unactivated macrophages, LPS-treated activated macrophages overexpressed SR-A strongly, showing the potential of SR-A as a target to recognize activated macrophages.

We then demonstrated the targeting ability of GPRD NPs towards activated and unactivated RAW264.7 cells using CLSM. As shown in Fig. 3c and Fig. S1a, there was strong red fluorescence from GPRD NPs in activated cells, but weak red fluorescence from GPR NPs. But for the unactivated RAW264.7 cells, only a faint red fluorescence could be observed in cells no matter in the presence of GPRD NPs nor GPR NPs, indicating very limited binding of NPs with unactivated RAW264.7 cells. To further detect the uptake of GPRD NPs by activated RAW264.7 cells, fluorescence imaging was performed at different time points and different concentrations of GPRD NPs. As shown in Fig. 3d and Fig. S1b, with the concentration increased, more and more GPRD NPs gathered in the cells. Such binding-induced uptake was also time-dependent. With 10 $\mu\text{g/mL}$ GPRD NPs incubation, only a few GPRD NPs were observed in activated cells at the first 10 min, but with the time passing by, more and more GPRD NPs were accumulated in cells (Fig. 3e, Fig. S1c). Hence, the CLSM results indicated that the DS-targeted GPRD NPs were more easily recognized and swallowed by activated RAW264.7 cells to be visualized by fluorescence imaging *in vitro*.

Later, the target MR imaging ability of GPRD NPs towards activated RAW264.7 cells was also evaluated by a 3.0T MR scanner. DS as the target molecules could selectively bind to SR-A, which overexpressed in activated RAW264.7 cells. Similar to the results of fluorescence imaging, a stronger signal intensity was observed in activated cells than that in unactivated cells after incubation with GPRD NPs (Fig. 3f, Fig. S1d). Nevertheless, they both displayed weak signal intensity with GPR NPs incubation, showing that GPRD NPs not only had a good MR imaging ability but also had an excellently targeted ability towards activated RAW264.7 cells.

GPRD NPs enhances ROS-scavenging and anti-inflammatory effect in activated RAW264.7 cells

AS is a kind of common chronic inflammatory vascular wall disease, which is often accompanied by the production of ROS in the occurrence and development of AS. ROS can promote the development of AS in

a variety of ways. For example, ROS can lead to macrophages activation, cell apoptosis, and oxidative damages to biomacromolecules (such as lipid, protein, DNA, etc.)^[17,34]. In addition, ROS and its oxidation products activate the corresponding signaling pathways with functional signaling molecules, which mediate a series of changes in vascular cells and affect vascular function and normal structure^[35]. Based on the crucial role of ROS in the pathogenesis of AS, eliminating ROS might be an important strategy for AS treatment. Therefore, we would like to explore the ROS clearance ability of GPRD NPs to achieve AS progress inhibition.

Nanozymes are artificial enzymes based on nanomaterials. Nanozyme systems have successfully become direct substitutes for traditional enzyme catalysis by effectively mimicking the catalytic sites of natural enzymes^[21]. Zhang's team found that PB NPs have many enzyme activities, such as POD, CAT, and SOD, and can effectively eliminate a variety of ROS. Furthermore, they indicated that the multi-enzyme-like activities of PB NPs were likely caused by the abundant redox potentials of their different forms, making them efficient electron transporters. Inspired by this, we first confirmed whether GPRD NPs can inhibit ROS generation in activated RAW264.7 cells. As expected, LPS treated RAW264.7 cells produced more ROS, presenting a stronger green fluorescence signal in cells (Fig. 4a-b, control and model group). After GPR NPs and GPRD NPs introduced, the green fluorescence emissions from ROS were reduced and the presence of DS in GPRD NPs favored such ROS clearance, showing the strong inhibition ability of GPRD NPs in ROS production in activated RAW264.7 cells (Fig. 4a-b, GPR and GPRD group).

AS is a chronic inflammatory disease and ROS overexpressed in activated macrophages in vulnerable plaque involves inflammatory signal transmission pathways to produce excessive inflammatory factors, such as IL-1 β , MCP-1, and TNF- α . The ROS-scavenging ability of GPRD NPs stimulated us to evaluate whether GPRD NPs could indirectly reduce the production of inflammatory cytokines. As shown in Fig. 4c-e, after treatment with LPS for 12 h, inflammatory cytokines (IL-1 β , MCP-1, and TNF- α) were all overproduced in the model group. With GPR NPs or GPRD NPs further treated, the overproduced inflammatory cytokines were all reduced. In addition, such reduction was more significant in the GPRD group, mainly due to the targeted recognition of activated macrophages by GPRD NPs.

Inhibition of foam cell formation and oxidation-induced apoptosis by GPRD NPs in activated RAW264.7 cells

The continuous accumulation of ROS and inflammatory cytokines in atherosclerotic vulnerable plaque areas can aggravate the injury of endothelial cells and the expression of chemokines. In addition, it can also promote the expression of SR-A in macrophages and its own activation, resulting in the acceleration of the intake of ox-LDL by macrophages and the formation of foam cells^[36,37]. The above results indicated that GPRD NPs could reduce ROS activated cells and reduce the release of inflammatory cytokines. Thus, we further studied the following influence of GPRD NPs on the inhibition of foam cell formation and oxidation-induced apoptosis in activated RAW264.7 cells. As shown in Fig. 4f, in the model group (LPS + ox-LDL treatment), the level of total cholesterol (TC) in cells was the highest. The TC levels went down in the GPRD NPs group and the GPR NPs group, indicating that the NPs could inhibit ox-LDL

uptake. Moreover, TC levels in the GPRD NPs group were even lower than the non-targeted GPR NPs group due to the target of DS. Subsequently, the formation of foam cells was further compared by Oil Red O staining (ORO). Similarly, without LPS activation, few foam cells were formed in the control group even with ox-LDL incubation (Fig. 4g-h). However, a large number of foam cells were observed in the model group (LPS + ox-LDL treatment), which could be reduced in the presence of GPR NPs and GPRD NPs. The fewer foam cells in the GPRD NPs group showed that GPRD NPs had a strong role in the inhibition of cellular internalization of ox-LDL and foam cell formation with the help of DS.

In atherosclerotic plaques, oxidative stress induced by ROS can promote the apoptosis of cells. Then, the necrotic core of the plaque becomes larger and the plaque is prone to rupture. Thus, ROS-scavenging by GPRD NPs might inhibit macrophage apoptosis. Apoptosis detection kit and flow cytometry were used to determine whether GPRD NPs could inhibit the apoptosis of macrophage induced by H₂O₂. As shown in Fig. 4i, after 300 μ M H₂O₂ injury for 24 h, about 27.4 % of the cells were apoptotic, while only about 10.69 % of untreated cells were apoptotic. But with GPRD NPs or GPR NPs pre-intervention, the apoptosis rates induced by H₂O₂ were inhibited to be 14.91 % and 21.35 %, respectively. The results indicated that GPRD NPs could effectively inhibit macrophage apoptosis induced by peroxidation.

In vivo safety evaluation

Blood compatibility is an important safety index of nanoprobe. We used mice blood to study the blood compatibility of the GPRD NPs. Visual hemolysis images showed no obvious hemolysis at different concentrations of GPRD NPs (Fig. S2a). There was no significant difference in OD value between the GPRD NPs group and the negative control group. The results showed that GPRD NPs had good biocompatibility in blood and did not cause hemolysis.

Then, the *in vivo* safety was also systematically tested by H&E staining of major organs, blood biochemical analysis, and routine blood analysis. As shown in Fig. S2b, histological analysis of major organs collected at 1, 7, and 21 d was performed to determine the biocompatibility of GPRD NPs *in vivo*. Compared with the control group, no significant inflammation or damage was observed in the major organs of the mice injected with GPRD NPs at all time points. For routine blood indexes and blood biochemical indexes analysis, no significant difference between GPRD NPs groups (1d, 7d, and 21d groups) and the control group in all above indicators, suggesting that there was little effect on major organ functions of GPRD NPs (Fig. S2c).

After 8 weeks of a high-fat diet, the ApoE ^{-/-} mice were intervened with saline, GPR NPs or GPRD NPs from week 9 (Fig. 5a). The weights of mice were measured once a week and no significant difference was observed among the mice in all groups (Fig. S3a), indicating that GPRD NPs had no significant effect on the bodyweight of mice. All of these data suggested the good biocompatibility and safety of GPRD NPs for the diagnosis and treatment of AS vulnerable plaques.

In vivo targeted dual-modality imaging of vulnerable plaques

After 8 weeks of a high-fat diet, Oil Red O staining of the aorta of ApoE^{-/-} mice was first performed to show the well-developed atherosclerotic plaques. Immunofluorescence results showed that the pink fluorescence-labeled SR-A was significantly expressed in the plaque area of the thoracic aorta, confirming the presence of vulnerable plaques in our constructed ApoE^{-/-} mice (Fig. 5b). Then, we tried to realize the targeted MR and fluorescence imaging of ApoE^{-/-} mice bearing vulnerable plaques with GPRD NPs. The mice were divided into two groups and injected with GPRD NPs or GPR NPs, respectively. MR images were collected at different time points before and after injection of NPs using a 3.0 T MR scanner. As shown in Fig. 5c-d, the T₁ signal in vulnerable plaque brightened after 30 min injection of NPs, and then gradually increased to the maximum intensity at 2 h. Subsequently, the signal gradually decreased over time within 24 hours. Moreover, the T₁-MR signals in the vulnerable plaques of the GPRD NPs group was brighter than those in the GPR NPs group, displaying the stronger targeting ability of GPRD NPs to vulnerable plaques. Besides, the accumulation of GPRD NPs and GPR NPs in the gallbladder with the time passing by, indicating their possible excretion route through the hepatobiliary system (Fig. S3b-c), which was in accordance with the literature determined by the sizes of nanomaterials [38].

Furthermore, after intravenous injection of GPRD NPs or GPR NPs 2 hours later, the isolated entire aorta showed significant fluorescence in the aortic arch and thoracoabdominal aorta in the GPRD NPs group (Fig. 5e-S4a). However, the fluorescence emission in the aorta in the GPR NPs group was much weaker. Subsequently, the fluorescence emissions in the heart, liver, spleen, lung, and kidney were carried out and the strongest fluorescence was observed in the gallbladders in both NPs groups (Fig. S4b-c), indicating the possible clearance route of the NPs by the hepatobiliary system once again.

The targeting ability of GPRD NPs against vulnerable plaques was further confirmed by immunofluorescence analysis. ApoE^{-/-} mice bearing vulnerable plaques in two groups were injected with GPRD NPs or GPR NPs, respectively. Two hours later, the immunofluorescence analysis was performed in the thoracic aorta. It was showed that SR-A was abundant in the vulnerable plaque area, and the colocalization of GPRD NPs with SR-A could be found in the vulnerable plaque area (Fig. 5f-S4d). In the GPR NPs group, only a small amount of GPR NPs accumulated in the SR-A region, demonstrating the excellent targeting ability of GPRD NPs to SR-A in vulnerable plaques.

In vivo targeted treatment of vulnerable plaques

Afterward, we investigated the therapeutic effect of GPRD NP *in vivo*. ApoE^{-/-} mice were randomly divided into four groups: control group (normal diet for mice without treatment), model group, GPRD NPs group, and GPR NPs group. After 2 months of high-fat diet, the latter three groups were given saline, GPRD NPs, and GPR NPs twice a week for 5 weeks, respectively. After 5 weeks, all the aortas were collected and stained with Oil-red O staining. A lower ORO-positive region was found in the control group and the highest ORO-positive region was found in the model group (Fig. 6a). Especially, the ORO-stained regions of aortas in the GPRD NPs and GPR NPs group were significantly smaller than those of the model group, and the smallest ORO-stained region was observed in the GPRD NPs group. Further quantitative analysis was performed and the plaque areas in the control group, model group, GPR and GPRD-treated

mice were $10.2 \pm 1.4 \%$, $44.5 \pm 3.0 \%$, $35.9 \pm 1.7 \%$, and $24.1 \pm 0.9 \%$, respectively (Fig. S4e). The results suggested that GPRD NPs can efficaciously attenuate the development of AS.

The area of plaque and the stability of plaque were the two main aspects to evaluate the effectiveness of treatment for AS. Thus, histological and immunohistochemical analyses of atherosclerotic plaques in the thoracic aorta were performed (Fig. 6b). H&E staining showed that the plaque was the largest in the model group among all the groups (Fig. 6c). GPR NPs and GPRD NPs could reduce the plaque area, but the GPRD group had a better effect. This result indicated that GPRD NPs could effectively reduce the plaque area, which was consistent with the results of Oil-red O staining of the whole aortas. The infiltration of macrophages was positively correlated with the development of vulnerable plaques. And another cause of vulnerable plaques was the degradation of extracellular matrix and collagen by matrix metalloproteinases. Thus, we chose CD68 and MMP-9 to evaluate the number of macrophages and the expression of matrix metalloproteinases in plaques after therapy. The results showed that GPRD NPs could effectively reduce the number of macrophages and the expression of MMP-9 in aortic plaques (Fig. 6d-e). Smooth muscle cells were the main components in AS plaques. α -SMA (smooth muscle cell labeling) immunohistochemical analysis was performed on aortic sections of mice from different groups. As shown in Fig. 6f, a large number of smooth muscle cells were distributed in AS plaques in the model group and GPR NPs group, which was significantly reduced in the plaque of the GPRD NPs group. The experimental results showed that GPRD NPs could effectively prevent the progression of AS and played a vital role in the treatment and stabilization of vulnerable plaques.

Conclusion

In this study, an integrated MR/fluorescence dual-modal imaging and therapeutic nanoprobe has been constructed to target and treat AS vulnerable plaques. Based on the MR and fluorescence imaging ability of GPB and Rd, the nano-enzyme capability of GPB as well as the targeting ability of DS, the fabricated GPRD NPs displayed successfully *in vivo* imaging of AS vulnerable plaque and the inhibition of plaque progress. The ROS-scavenging strategy to inhibit the AS plaque progress and stabilize the vulnerable plaques possesses promising potentials for AS treatment because of the important role of ROS in the formation and progress of AS. The ROS clearance brought a series of ROS-related changes, such as the reduction of oxidative stress, inflammation, apoptosis, and the formation of macrophage-derived foam cells, which all favored the therapy of AS plaque. The dual-modal imaging and therapy of AS vulnerable plaques by our fabricated GPRD NPs might promote the early identification and treatment of AS plaques.

Methods

Materials

Polyvinylpyrrolidone (PVP, K30), potassium ferricyanide ($K_3[Fe(CN)_6]$), gadolinium nitrate hexahydrate [$Gd(NO_3)_3 \cdot 6H_2O$] and Rhodamine 6G were purchased from Dalian Meilun Biotechnology Co., LTD (Dalian, China). Polyethyleneimine (PEI) was obtained from Aladdin Chemical Reagent Co., Ltd. Dextran sulfate

(DS) and Oil Red O stain kit were obtained from Solarbio Chemical Reagent Co., Ltd. Reagents for cell cultures including fetal bovine serum, trypsin, and DMEM were supplied by Gibco (Thermo Fisher Scientific Co., Ltd., Shanghai, China). The TNF- α , IL-1 β and MCP-1 ELISA Kits were purchased from Solarbio Chemical Reagent Co., Ltd. Total Cholesterol (TC) Content Assay Kit was purchased from Shanghai cablebridge biotechnology Co., Ltd. SOD Activity Assay Kit was received from Shanghai bestbio biological reagent Co., Ltd. Annexin V-FITC/PI cell apoptosis assay kit was obtained from Jiangsu Keygen Biotechnology Co. Ltd. ROS Fluorescent Probe-DCFH-DA was purchased from Dalian Meilun Biotechnology Co., LTD (Dalian, China). Oxidized low-density lipoprotein was received from Shanghai Yuanye Biotechnology Co., Ltd. All aqueous solutions were prepared with ultrapure water ($\geq 18\text{ M}\Omega$, Milli-Q, Millipore).

Preparation of GPRD NPs

GPB nanoparticles were synthesized according to the literature with some modifications^[1]. In brief, 32 mg $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 1 g PVP were added into 10 mL HCl solution (0.01 M) to obtain a clear solution A by stirring. 1 g PVP and 60 mg $\text{Gd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ were added to 10 mL HCl solution (0.01 M) to obtain a clear solution B. Subsequently, solution A and solution B were slowly mixed with magnetic stirring for 3 h. Place the mixed solution in an oven (80°C for 24 hours). After centrifugation at 16000 rpm for 30 min, the precipitate was washed with distilled water three times and ethanol one time to obtain GPB nanoparticles.

Then, 2 mL PEI aqueous solution (10 mg/mL, pH=5.0) was added slowly into 2 mL of GPB (5 mg/mL). The obtained solution was stirred at 1000 rpm for 4 h^[1]. After centrifugation at 14000 rpm for 30 min, GPB-PEI were washed with distilled water three times and freeze-dried. GPRD NPs were then prepared via electrostatic absorption^[1]. 1 mL DS (10 mg/mL) and 1 mL Rd (0.5 mg/mL) were added slowly to 1 mL GPB-PEI (1 mg/mL), and the mixture was stirred under an ice rocker for 4 h. After centrifugation at 13000 rpm for 30 min, the precipitate was washed with distilled water three times to obtain GPRD NPs.

Characterization of GPRD NPs

The morphology, size, and composition of the GPRD NPs were characterized by transmission electron microscopy (TEM, TEC-NAI G2, USA) and EDS-mapping. A DSL analyzer (Zetasizer Nano ZS90) was used to measure the size distribution and zeta potential during the preparation process. The UV-vis absorption spectra of the GPB, Rd, and GPRD NPs were recorded by a HITACHI UH4150 Spectrophotometer (HITACHI, Japan). The absorbance peaks of GPB and DS were detected by a fourier infrared spectrometer (FT-IR, Bruker, Germany). The concentration of gadolinium in GPB and GPRD NPs was determined by inductively coupled plasma-optical emission spectroscopy (ICP-MS, Thermal Elemental). Fluorescence emission of GPRD NPs was measured by LS55 Fluorescence Spectrometer (PerkinElmer, America). The 3.0T MR imaging system (GE discovery 750W) with a small animal coil was used to measure the MR signal value of GPRD. The particle size, fluorescence, and MRI signal intensity of GPRD NPs were measured at 0, 1, 3, 5, 7 days to demonstrate the stability of GPRD NPs.

T₁ Relaxivity Measurements of GPB and GPRD NPs

The T₁-weighted relaxivity of GPB and GPRD NPs was determined under a 3.0 T MR system. T1 images of GPB and GPRD NPs suspensions with different concentrations were obtained by 3.0 T MRI. The scanning parameters were as follows: Field of View = 18 cm×18 cm, TE = Min Full, TR = 425 ms, Matrix size = 384×224, Slice spacing = 1.5 mm, Slice thickness = 3.0 mm.

Determination of ROS-Scavenging Capability

Different concentrations of GPRD NPs (0, 10, 30, 50, 80, 100 µg/mL) were cultured in 2mL 0.01M PBS (pH = 7.4) containing 500mM H₂O₂ for 2h to evaluate H₂O₂ scavenging ability. Subsequently, the residual H₂O₂ was measured by a UV-vis-NIR spectrometer at 240 nm. In addition, 100 µg/mL of GPRD NPs were added into 2 mL of 0.01 M PBS (pH = 7.4) containing 500 mM H₂O₂ for different times (from 0 to 4 h), and the residual H₂O₂ amount was measured similarly. The eliminated H₂O₂ amount was calculated accordingly.

Commonly used natural POD substrate, 3,5,3',5'-tetramethylbenzidine (TMB) was selected as a substrate to investigate the POD activity of GPRD NPs. H₂O₂, TMB and GPRD NPs with different concentrations were added to the buffer solution (pH = 5.0) to observe the color change (Reactant ratio GPRD : 30% H₂O₂ : 10 mg/mL TMB : pH = 5.0 buffer system = 1 : 3 : 1 : 20). The POD-like activity of GPRD NPs was evaluated by recording the absorption value at 650 nm of the oxidation state TMB generated in the reaction system with a UV spectrophotometer. Also, we detected the absorption value at 650 nm of the oxidation state TMB at different time points.

SOD activity of GPRD NPs with different concentrations (10, 30, 50, 80, 100 µg/mL) was determined by a SOD Assay kit (BestBio, Shanghai) according to the introduction.

Cell Culture

RAW264.7 macrophages and human umbilical vein endothelial cells(HUVEC)were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RAW264.7 cells were cultured in a DMEM medium (Gibco) containing 10% FBS, 1% penicillin (100 mg mL⁻¹), and streptomycin (100 mg mL⁻¹). HUVEC cells were propagated in RPMI 1640 medium (Gibco) containing 10% FBS-containing, 1% penicillin (100 mg mL⁻¹), and streptomycin (100 mg mL⁻¹). All cells were cultured in a 5% CO₂ saturated humidity incubator at 37 °C.

Cell Cytotoxicity Evaluation

In this study, MTT assays were employed to evaluate the cytotoxicity of GPRD NPs *in vitro*. Briefly, RAW264.7 and HUVEC cells (1×10⁵ cells each well) were cultured in 96-well plates for 24 h at 5% CO₂, 37 °C. Then, 100 µL fresh medium containing different concentrations of GPRD NPs (0, 10, 30, 60, 90, 150, 200, 300, 400 µg/mL) was used to replace the culture for another 24 hours. After removing GPRD NPs

and washing with PBS buffer, DMEM medium containing 1 mg/mL MTT was added to each well and cultured for 4 h. The medium was then removed and 100 μ L of dimethyl sulfoxide (DMSO) was added to dissolve formalin crystals for another 15 minutes. The absorbance at 490 nm was measured by a microplate reader (Multiskon MK3, USA).

Cell uptake and targeted fluorescence imaging

To study the possibility of targeted imaging with GPRD to activated macrophages, the overexpression of SR-A in activated macrophages was first evaluated by Western Blot (WB) assay. The RAW 264.7 cells incubated with the fresh medium were used as a normal control group, and the macrophages treated with medium containing lipopolysaccharide (LPS) were used as model cells. After 12 h, the cells in two groups were collected for the WB assay.

The targeting ability of GPRD NPs towards the activated RAW264.7 cells was observed by confocal laser scanning microscopy (CLSM). RAW264.7 cells were cultured in 6-well plates (1×10^5 cells per well) and incubated for 12 h. The unactivated cells were cultured in the medium for 12 h, and the activated cells were stimulated with LPS for 12 h. Then they were incubated with GPRD NPs and GPR NPs at 10 μ g/mL, respectively. After 20 minutes of culture, RAW264.7 cells were washed and stained with DAPI. Then, the fluorescence images were obtained by CLSM. Similarly, cell internalization was also studied at different times (10, 20, 40, 90 min) and different doses of GPRD NP (10, 30, 50 μ g/mL).

***In vitro* cell targeted MR Imaging**

RAW264.7 cells were cultured in 6-well plates (1×10^5 cells per well) and incubated for 12 h. The unactivated cells were cultured in the fresh medium for 12 h, and the activated cells were stimulated with LPS for 12 h. Cells were incubated with GPRD NPs or GPR NPs at 50 μ g/mL, respectively. Subsequently, the cells were washed with PBS, trypsinized centrifuged to collect cells. Then the cells were resuspended and solidified with 300 μ L 1% agarose solution. Finally, MR imagings for activated and unactivated RAW264.7 cells were performed on a 3.0 T MR scanner.

Intracellular ROS Generation

RAW264.7 cells were cultured in 6-well plates at 1×10^4 per well overnight to clearly show the cells. The control group was cultured in fresh medium, and the model group was activated with LPS (10 ng/mL) for 30 min. Then the cells in two groups were incubated with GPRD NPs or GPR NPs with a concentration of 50 μ g/mL for 4 h, respectively. Afterward, cells were rinsed and treated with 2,7-Dichlorodi - hydrofluorescein diacetate (DCFH-DA, 10 μ M) in PBS for 30 min. After washing with PBS, the production of intracellular ROS in cell culture medium was observed by Inverted Fluorescence Microscope.

Intracellular inflammatory cytokines measurement

RAW264.7 cells were cultured in 12-well plates at 1×10^5 per well and incubated overnight. The control group was cultured in fresh medium, and the model group was activated with LPS. The cells in two

experimental groups were incubated with GPRD NPs or GPR NPs with a concentration of 50 µg/mL for 24 h. Afterward, supernatants in all groups were harvested and assayed for IL-1β, MCP-1, and TNF-α using corresponding ELISA kits.

Inhibition of Foam Cell Formation

The foam cell formation was evaluated by the determination of intracellular TC levels and Oil Red O staining. RAW264.7 cells were cultured in 6-well plates at 1×10^5 per well and incubated overnight. The control group was cultured in fresh medium, the other groups(model group, GPR group, GPRD group)were stimulated with LPS for 12h. The GPR group and GPRD group were treated with GPRD NPs or GPR NPs at 50 µg/mL for 2 h. Then, 50 µg/mL ox-LDL was added to all four groups of cells and incubated for 48 h. Subsequently, the contents of RAW264.7 cells were extracted via ultrasound to detect the TC levels by TC assay kits. Similarly, the treated cells, in the same way, were stained with Oil Red O staining for 40 min and rinsed carefully three times with double distilled water. Finally, the Oil Red O staining of cells was observed by optical microscope.

Anti-Apoptosis Activity of GPRD NPs in Macrophages

RAW264.7 cells were cultured in 6-well plates (1×10^5 cells per well). After 12 h, the GPRD group and the GPR group cells were incubated with medium containing 50 µg/mL GPRD NPs or GPR NPs for 2 h. Then fresh medium containing 200 µM H₂O₂ was added to the cells for 24 h. The model group was incubated in fresh medium containing 200 µM H₂O₂ for 24 h. The control group was incubated only in fresh medium. Finally, the cells were collected and stained with an annexin V-FITC / PI apoptosis detection kit. And apoptosis was assayed via flow cytometry (AutoMACS Pro, Miltenyi Biotec).

Animal experiments

Animal experiments were conducted according to the guidelines of the National Institutes of Health on the use of animals in research. All animal experiments were approved by the Animal Care Committee of Xuzhou Medical University (L20210226113). C57BL/6 mice (female, 6 weeks old) were obtained from the Animal Center of Xuzhou Medical University. Apolipoprotein E-deficient (ApoE^{-/-}) mice (female, 6 weeks old) were purchased from the Animal Center of Xuzhou Medical University. ApoE^{-/-} mice were given a high-fat diet containing 21.2% lard, 49.1% carbohydrate, 19.8% protein, and 0.2% cholesterol for 2 months to construct animal models of atherosclerosis.

***In vivo* safety evaluation**

As mentioned, the hemolysis of GPRD nanoparticles was detected by direct contact method *in vitro*^[1]. 1 ml of mouse blood was washed three times with 1.25 ml of isotonic sodium chloride solution. Dilute 200 µl of red blood cells with 10 ml of isotonic sodium chloride solution. Then, 0.1 ml of diluted blood samples were added to different concentrations of GPRD NPs solutions (10, 50, 100, 150, 200, 300, 400, 500 µg/mL), and cultured at 37 °C for 1 h, and then centrifuged at 3000 rpm for 5 min. The absorbance

of the samples was measured at 540 nm by a microplate reader to determine the hemoglobin released by dissolved red blood cells. In addition, the samples incubated with isotonic sodium chloride solution and double distilled water were used as negative and positive controls.

In vivo toxicity study was conducted on C57BL/6 mice (female, 6 weeks). Mice were randomly divided into two groups and then injected with normal saline and GPRD NPs (10 μ mol/kg Gd). Blood samples were collected at 1st, 7th, and 21st days after injection for biochemical analysis and routine blood analysis, such as AST, ALT, Cr, TP, WBC, RBC, MCV, PLT, and so on. After the experiment, the heart, liver, spleen, kidney, and lung were collected, fixed with 10% formalin, sectioned, and stained with H&E for histological analysis. Finally, the histological samples were observed using an optical microscope.

The expression of SR-A in the plaque of ApoE^{-/-} mice

ApoE^{-/-} mice were given a high-fat diet for 2 months, the aortas were dissected and the thoracic aortas were selected. The sections were fixed in ice-cold acetone for 5 min and then blocked with goat serum. Subsequently, the sections were labeled with anti-mouse SR-A monoclonal antibody overnight and labeled with pink-conjugated secondary antibody for 1 h. Finally, the sections were stained with DAPI and fixed, and then observed by confocal laser scanning microscope.

***In vivo* targeting performance**

The ApoE^{-/-} mice were divided into two groups. GPRD NPs or GPR NPs (10 μ mol/kg Gd) were injected through the tail vein, respectively. Two hours later, the aortas were dissected and the thoracic aortas were selected. The sections were fixed in ice-cold acetone for 5 min and then blocked with goat serum. Subsequently, the sections were labeled with anti-mouse SR-A monoclonal antibody overnight and labeled with pink-conjugated secondary antibody for 1 h. Finally, the sections were stained with DAPI and fixed, and then observed by confocal laser scanning microscope.

***In vivo* targeted Imaging**

The ApoE^{-/-} plaque-bearing mice were injected with GPRD NPs or GPR NPs (10 μ mol/kg Gd) for *in vivo* imaging. And the images were performed on the 3.0 T MR scanner pre- and post-administration at various periods (0.5, 2, 6, 12, 24 h).

The ApoE^{-/-} plaque-bearing mice were injected with GPRD NPs or GPR NPs (10 μ mol/kg Gd) for *in vivo* fluorescence imaging. Two hours later, the aorta and major organs were dissected and fluorescence images were captured using an *in vivo* imaging system.

***In vivo* targeted treatment**

The ApoE^{-/-} mice were divided into four groups, (a) control group, (b) model group, (c) GPR NPs group (d) GPRD NPs group (n=8 each group). The mice in the control group were fed normal diets. The mice in the other three groups were fed with high-fat diets for 2 months. Then, 200 μ L GPR (10 μ mol/kg Gd) or GPRD

(10 $\mu\text{mol/kg}$ Gd) were administered intravenously (group c, d), whereas 200 μL of saline was administered intravenously (group b). Mice were treated twice a week and their body weight was recorded once a week. After 5 weeks of administration, all mice were euthanized, and the aortas were collected for Oil Red O staining. The efficacy of different treatments was evaluated by Oil Red O staining of atherosclerotic plaque. In addition, the quantitative analysis of atherosclerotic plaque was determined by Image-J.

For histological analysis, thoracic aortas of four groups were collected for H&E staining. For immunohistochemistry analysis, sections of thoracic aorta were incubated with antibodies, including CD68, MMP-9, and $\alpha\text{-SMA}$, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software (Chicago, Illinois). All data in this study are expressed as mean value $\pm$ SD. The statistical significance of each group was compared by one-way analysis of variance (ANOVA) and Dunnett test. Student t-test was used to evaluate the statistical significance between the two groups. In all cases, $P < 0.05$ was considered statistically significant.

Abbreviations

AS: atherosclerosis; GPB: Gd doped Prussian blue; Rd: rhodamine; DS: dextran sulfate; SR-A: scavenger receptor class A; ROS: reactive oxygen species; CAT: catalase; POD: peroxidase; SOD: superoxide dismutase; GPRD NPs: GPB-PEI-Rd-DS nanoparticles; PEI: polyethyleneimine; PVP: Polyvinylpyrrolidone; DMSO: dimethyl sulfoxide; LPS: lipopolysaccharide; ApoE $^{-/-}$: Apolipoprotein E-deficient

Declarations

Acknowledgments

Not applicable.

Author Contributions

Yue Dai, Jingjing Li, and Kai Xu conceived and designed the experiments. Yue Dai, Xuan Sha, Xiaoxi Song, and Xiuli Zhang performed the experiments. Yue Dai, Mengyuan Xing, Siwen Liu analyzed the data. Yue Dai, Xuan Sha, and Jingjing Li wrote the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (8217071424), the Natural Science Foundation of Jiangsu Province for the Excellent Young Scholars (BK20170054), Qing Lan Project, the Peak of Six Talents of Jiangsu Province (WSN-112), Jiangsu Provincial Medical Youth Talent (QNRC2016776), Six one project of Jiangsu Province (LGY2018083). Postgraduate Research & Practice

Innovation Program of Jiangsu Province (KYCX21_2737). Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX21_2724). Postgraduate Research & Practice Innovation Program of Jiangsu Province KYCX20_2448.

Ethics approval and consent to participate

Animal experiments were conducted according to the guidelines of the National Institutes of Health on the use of animals in research. All animal experiments were approved by the Animal Care Committee of Xuzhou Medical University (L20210226113).

Competing interests

The authors declare that they have no competing interests.

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Scheme

Schemes 1 is available in the Supplemental Files section.

Figures

Figure 1

(a) TEM image and size distribution profile of GPB and GPRD NPs. (b) Corresponding STEM-EDS elemental mappings of GPRD NPs. Characterization of the assembly process of GPRD NPs with hydrodynamic diameter (c) and zeta potential determination (d). (e) FT-IR spectra of GPB (black line), DS (blue line), and GPRD NPs (red line). (f) The UV-Vis spectra of GPB (black line), Rhodamine (blue line), and GPRD NPs (red line). (g) The photoluminescence emission of GPRD NPs with different

concentrations with excitation wavelengths of 490 nm. (h) T_1 -weighted MR images of GPB and GPRD NPs with different concentrations. r_1 relaxivity curves of GPRD NPs (i). The stability characterizations of GPRD NPs with hydrodynamic diameter (j), fluorescence intensity (k), and MRI T_1 signal intensity (l).

Figure 2

Measurement of CAT-like activity of GPRD NPs with different concentrations (a) and different time points (b). (c)The capacity of GPRD to remove H_2O_2 in a dose-dependent profile. Measurement of POD-like activity of GPRD NPs with different concentrations (d) and different time points(e). (f) Evaluation of the SOD-like enzyme activity of GPRD.

Figure 3

(a) Cell viability of RAW264.7 cells and HUVEC cells after exposure to various concentrations of GPRD NPs, determined by MTT assay. (b) Detection of the expression of SR-A in LPS activated macrophages. The targeting ability to cells and bimodal imaging of GPRD NPs. (c) The CLSM images of activated RAW 264.7 cells and untreated RAW264.7 cell incubated with GPR NPs or GPRD NPs for 20 min. The cell nucleus was stained by DAPI. The CLSM images of activated RAW 264.7 cells treated with different GPRD NPs concentrations (d) and different times (e). (f) T₁-weighted and T₁ mapping image of LPS activated RAW 264.7 cells and unactivated RAW 264.7 cells incubated with GPR NPs or GPRD NPs. (scale bar = 50 μm). In CLSM images, the red color emissions were from NPs and blue color emissions were from cell nucleus.

Figure 4

4 (a-b) Fluorescence images showing the intracellular ROS generation after treatment with GPR NPs and GPRD NPs and the quantitative analysis. (c-e) Typical inflammatory cytokines TNF- α , IL-1 β , and MCP-1 secreted by RAW264.7 cells by ELISA after different treatments. (f) The level of total cholesterol in RAW264.7 cells after different treatments. (g-h) Optical microscopy images showing oxLDL-induced foam cell formation in macrophages cells and the quantitative analysis. (i) Inhibiting the apoptosis of RAW264.7 induced by H₂O₂ after different treatments. (scale bar = 50 μ m). * P < 0.05, ** P < 0.01, *** P < 0.001

Control: RAW264.7 cells without LPS (unactivated cells)

Model: RAW264.7 cells with LPS (activated cells)

GPR: activated RAW264.7 cells co-incubation with GPR NPs

GPRD: activated RAW264.7 cells co-incubation with GPRD NPs

Figure 5

(a) Schematic illustration of atherosclerotic mouse model development and treatment with various formulations. (b) The aorta oil red O staining in the atherosclerotic model mice and the expression of SR-A in vulnerable plaque (pink SR-A, blue DAPI). (c-d) MR imaging of atherosclerotic model mice aorta plaque pre-injection and after injection of GPR or GPRD NPs at different time point and quantitative analysis of T₁ signal intensity. (e) Fluorescence imaging of atherosclerotic model mice aorta plaque after injection of GPR or GPRD NPs at 2h. (f) Images of colocalization between GPR or GPRD NPs and SR-A in vulnerable plaque post-injection for 2h (scale bar = 100 μm). **P* < 0.05, ***P* < 0.01, ****P* < 0.001

Figure 6

(a) ORO stained aorta tissues collected from atherosclerotic mice after treatment with various formulations. (b-f) Representative photographs and quantitative analysis of thoracic aorta sections stained by H&E, CD68 antibody, MMP-9 antibody, and α -SMA antibody after treatment with various formulations (scale bar = 200 μ m). * P < 0.05, ** P < 0.01, *** P < 0.001

Control: mice fed with normal diets without treatment

Model: mice fed with high-fat diets and treated with normal saline

GPR: mice fed with high-fat diets and treated with GPR NPs

GPRD: mice fed with high-fat diets and treated with GPRD NPs

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Scheme1.png](#)
- [renamed2e335.pdf](#)