Research article

IVUS/IVPA hybrid intravascular molecular imaging of angiogenesis in atherosclerotic plaques via RGDfk peptide-targeted nanoprobes

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A B S T R A C T

Current intravascular imaging modalities face hurdles in the molecular evaluation of progressed plaques. This study aims to construct a novel hybrid imaging system (intravascular ultrasound/intravascular photoacoustic, IVUS/IVPA) via RGDfk peptide-targeted nanoparticles for monitoring angiogenesis in progressed atherosclerotic plaques in a rabbit model. An atherosclerotic rabbit model was induced by abdominal aorta balloon endothelialization followed by a high-fat diet. A human serum albumin (HSA)-based nanoprobe modified with RGDfk peptide was constructed by encapsulating indocyanine green (ICG) via electrostatic force (ICG-HSA-RGDfk NPs, IHR-NPs). A hybrid intravascular imaging system that combined IVUS and IVPA was self-assembled for RGDfk visualization within atherosclerotic plaques in the rabbit abdominal aorta. Through IHR-NPs and the hybrid IVUS/IVPA imaging platform, multiple comprehensive pieces of information on progressed plaques, including anatomical information, composition information and molecular information, can be obtained simultaneously, which may improve the precise diagnosis of plaque characteristics and the evaluation of early interventions for atherosclerosis.

1. Introduction

Cardiovascular disease has become a major threat to human health. Despite continuous progress in the diagnosis and treatment of cardiovascular diseases in recent years, acute cardiovascular events, such as acute myocardial infarction, still lead to more than 20 million deaths every year, which makes cardiovascular disease the leading cause of mortality worldwide [1]. Numerous studies have proved that vulnerable plaque is the main pathological cause for acute cardiovascular events, which characterized by neovascularization, inflammation, necrotic cores, superficial plaque erosion, high lipid content and spotty calcification, etc. [2–5]. The intraplaque neovascularization leading to recurrent haemorrhages, which deliver erythrocytes to the necrotic core where they degrade promoting inflammation and oxidative stress [6,7].

\textbf{In vivo} early detection of the molecular biomarkers of vulnerable plaques is crucial for predicting of the cardiovascular disease [8,9].

For the study of neovascularization of plaques at the molecular level, it is reported that integrin \(\alpha v \beta 3\) plays a prominent role in angiogenesis, which strongly upregulated at transcriptional level by pro-angiogenic growth factors or chemokines both in activated endothelial cells and atherosclerotic plaques in patients with severe carotid stenosis [10–12]. On the surface of neovascular endothelial cells, integrin \(\alpha v \beta 3\) binds specifically to the extracellular matrix of the RGD module, a short peptide containing the 3-amino acid sequence (arg-gly-asp) [13,14]. Therefore, expression of integrin \(\alpha v \beta 3\) could be an assessment of plaque vulnerability and patient prognosis concerning development of symptomatic stenosis.

Molecular imaging techniques are the ideal approach for the early detection of vulnerable plaque substrates.
detection of the progressed plaque. Our previous in vivo imaging studies for plaque vulnerability were based on noninvasive molecular imaging by optical/Magnetic Resonance Imaging (MRI)/nucleic/computerized tomography (CT) imaging platforms [15–19]. But the tiny plaque within the blood vessel in small animal was hard to get high resolution visualization, especially on molecular level. Luckily, intravascular imaging techniques with high robust imaging from the inner wall of blood vessels are of potential to obtain information more effectively and accurately [20].

Clinical well applied intravascular imaging such as IVUS and intravascular optical coherence tomography (IVOCT) could provide morphological information otherwise the molecular changes related to plaque vulnerability [21–23]. Therefore, it is highly desirable to develop a novel imaging technology for accurate detection of structural information through the whole artery wall, functional information for a certain component at the molecular level. IVPA/IVUS is a hybrid modality that has shown great potential in the detection of physiological functions such as plaque lipid composition, neovascularization and inflammatory information. In IVPA, a photoacoustic signal is generated based on the specific light absorption of biological tissue at a certain wavelength and the subsequent transient thermoelastic effect [24–26]. The in vivo lipid imaging was successfully achieved at the excitation wavelength of 1720 nm [27,28]. The IVPA application in atherosclerotic inflammation detection was proposed based on gold nanoparticles labeling on macrophage cells in vitro, demonstrating the great potential of the technology in inflammation cells detection [29–31].

With the development of molecular probes labeling angiogenesis, it is also expected that high-contrast imaging of pathological angiogenesis, a biological phenomenon closely related to the injury mechanism of vulnerable plaques, will be achieved [32,33]. In the present study, we aim to utilize integrin αvβ3 as a molecular target and apply IHR-NPs as probe carriers to label the neovascularizations within plaques on atherosclerotic rabbit models. By using a hybrid IVUS/IVPA imaging system, the molecular information of anatomic structure, plaque composition and neovascularization were obtained at the same time. To the best of our knowledge, it’s the first research for in vivo IVPA/IVUS hybrid molecular imaging of angiogenesis within plaque in atherosclerotic living animal.

2. Methods

2.1. Atherosclerosis animal modeling

All animal studies were performed according to a protocol approved by the People’s Liberation Army General Hospital Animal Care and Use Committee. After adaptive feeding for 2 weeks, 30 male New Zealand white rabbits weighing 2.5~3.0 kg were fed an atherogenic diet containing 2.5 % cholesterol, 15 % lard and 5% yolk powder (high fat and cholesterol diet group, HDF group). After 2 weeks of feeding on the atherogenic diet, the rabbits underwent abdominal aorta balloon de-endothelialization. Each rabbit was weighed and anesthetized by intravenous injection of ketamine (25 mg/kg), and the depth of anesthesia was maintained by 2–2.5 % isoflurane with 100 % oxygen [34]. The right or left femoral artery was separated and punctured, then a 5-Fr sheath (5 Fr-16 CM, plastic, Terumo, Japan) was inserted into the right femoral artery, and a 4.0 mm × 20 mm angioplasty balloon (Non-compliant Balloon, Medtronic, USA) was advanced into the abdominal aorta. The balloon was inflated with a 50 % mixture of contrast agent and saline and then pulled back three times at 18–20 atmosphere (atm). Abdominal aorta balloon de-endothelialization was implemented under the X-ray angiographic system (Optima IGS 320, General Electric, USA). To avoid postoperative infection, 2500 U/kg penicillin was given every day for 3 days after surgery. After another 20 weeks of feeding on the atherogenic diet, percutaneous abdominal aortic vascular ultrasound (40 Hz, DC-80S, Mindray, China) in vitro and blood vessel Oil Red O staining were used to verify the success of the model. 20 rabbits with conspicuous plaque were injected with IHR-NPs or ICG-HSA nanoprobes (IH-NPs) and imaged, and at last were used to harvest their arteries for histology. The other 15 rabbits in the control group (CON group) were given normal feed without undergoing de-endothelialization.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in M200 basal culture medium supplemented with low serum growth supplement and maintained in a humidified environment containing 5% CO2 and air at 37 °C. When the cells reached 90 % confluence, sterile PBS was added, and the cells were passaged by gentle rinsing. HUVECs were purchased from the American Type Culture Collection Center (Manassas, USA), and M200 basal culture medium was obtained from Gibco Biosciences (Grand Island, USA).

2.3. Preparation of nanoprobes

To prepare IHR-NPs, we followed the strategy in the schematic diagram (Fig. 1). First, 40 mg of HSA (Sigma, USA) in PBS solution and 2 mg of sulfo ICG-HSA-SMCC (Aladdin, China) were mixed and stirred at room temperature for 30 min. To remove sulfo-succinimide hexamethylene carbonate (SMCC), the HSA-SMCC solution was centrifuged three times with a 15 mL 30 KD ultrafiltration centrifuge tube at 4500 rpm at room temperature for 3 min. To prepare for sulfhydration of the RGDfk peptide, 50 mg of RGDfk peptide (Sigma, USA) in 5 mL of borac-borax buffer were mixed and oscillated at room temperature for 1 h and then kept at 4 °C for use. HSA-RGDfk was prepared by adding 1.5 mL of the obtained RGDfk active short peptide solution into 4 mL of HSA-SMCC solution, stirring and reacting at room temperature for 2 h and centrifugation with ultrafiltration. ICG (10 μL, 1 mg/10 μL in DMSO) was added dropwise to the HSA-RGDfk solution while stirring. Two hundred microliters of glutaraldehyde (10 mg/mL in PBS) was slowly added into the ICG-HSA-RGDfk solution, avoiding light and stirring for 3 h. IHR-NPs were collected by centrifugation, purified three times and then dissolved in 1 mL of PBS solution. The IH-NPs were constructed without coupling the RGDfk peptide, and the other steps were the same as those mentioned above.

2.4. Characterization of nanoprobes

The IHR-NPs and IH-NPs were diluted 100 times and dropped onto copper mesh to dry. The morphology and size distribution of nanoprobes were characterized by transmission electronic microscopy (TEM, JEM-2100, JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV. The hydrodynamic diameters and zeta potentials of these particles were determined by dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He-Ne laser (λ = 633 nm). The magnetic properties of powder samples were analyzed in a vibrating sample magnetometer (vs.M JDM-13, China). The ultraviolet absorbance values of the diluted nanoprobes were measured with an ultraviolet spectrophotometer (Perkin Elmer, USA).

2.5. Cytotoxicity assay of nanoprobes

A cell counting kit-8 (CCK8) assay was used for cell cytotoxicity analysis. After incubation with ox-Low Density Lipoprotein (ox-LDL) for 24 h, 1 × 104 HUVECs were seeded in 96-well plates under different concentrations of nanoprobes (ICG 0, 2, 4, 8, 16, 32, 64 μg/mL). After 48 h of incubation, the relative cell viability was assessed by CCK-8 assay. CCK8 solution (10 μL) was added to each well and incubated for 2 h. The absorbance of each well at 450 nm was determined by an enzyme-labeled instrument (Tecan, Switzerland). The mean absorbance of the probe group was compared with that of the control group to obtain the cell survival ratio.
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were inoculated in confocal dishes at a density of 2.6. Fluorescence imaging of nanoparticle uptake in cells connecting with the RGDfk peptide. Fig. 1.

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and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 3 min, and cell uptake was observed by confocal fluorescence microscopy (Olympus, Tokyo, Japan). The excitation wavelength of DAPI was 358 nm, and the emission wavelength was 461 nm. The excitation wavelength of the ICG excitation wavelength was 780 nm, and the emission wavelength was 825 nm.

2.7. Histological analysis

To verify the model of atherosclerosis in rabbits, all rabbits were anaesthetized after 20 weeks of de-endothelialization. The abdominal aortic arteries were harvested and opened longitudinally with the intima towards the outside. After slight washing with tap water for 5 s (sec), the arteries were first dipped into 60 % isopropanol for 3 s and then dipped into Oil Red O solution for 60 min under dark conditions at 37 °C, and then the arteries were removed and treated with 60 % isopropanol for differentiation. The differentiation was stopped when the fatty plaques in the lumen were orange or bright red. To further assess the atherosclerotic plaques, rabbit abdominal aortic arteries were removed and frozen in optimum cutting temperature (OCT) compound. Consecutive frozen aortic sections (10 μm thickness) were prepared and stained with Oil Red O for 10 min, followed by counterstaining with hematoxylin for 1–3 min at room temperature. Image J software was used to quantify the lesion areas of the entire vascular intima.

2.8. Western blot analysis

Tissue samples were extracted from the abdominal aortic arteries of rabbits. HUVECs with and without ox-LDL incubation for 24 h were harvested for Western blot analysis. Protein concentrations were determined with a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). The proteins were separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with Tris buffered saline Tween-20 containing 5 % skim milk for 30 min and then incubated with RGDfk (Sigma, San Francisco, USA) primary antibodies and monoclonal anti-β-actin antibody (1:1000, Abcam, Cambridge, UK) at 4 °C overnight. The membranes were incubated with secondary antibodies (Abcam, Cambridge, UK) conjugated with horseradish peroxidase for 1 h at 37 °C. The target proteins were detected by an enhanced chemiluminescence system (ECL; Amer sham) and quantified by Quantity One Analysis Software (version 4.5, Bio-Rad, USA).

2.9. IVPA/IVUS imaging platform

The overall architecture of the IVPA/IVUS system is illustrated in Fig. 2A. The system consists of a pulsed laser for IVPA signal excitation, an attenuator for adjusting the excitation intensity, an optical lens for beam shaping, a fiber coupler for coupling the laser into an optical fiber, an optical/electrical slip ring for transmitting optical and electrical signals between the catheter and the imaging system, an ultrasound pulser/receiver for triggering ultrasonic signal generation and receiving ultrasonic and photoacoustic signals, and a computer for receiving, saving, processing and displaying the imaging data [32,37]. During the data processing, the digitized photoacoustic and ultrasonic signals were processed by voltage conversion, digital filtering, envelop demodulation, and finally saved by a data acquisition card (ATS9325, Alazar, Canada). A time delay of 3 us is set between photoacoustic and ultrasound signals for distinguishing the two signals to obtain the IVPA/IVUS images simultaneously [38].

The hybrid IVUS/IVPA imaging catheter was designed as described in a previous study [37]. An optical fiber with 105 μm cores, a 0.5 mm-sized gradient-index lens and a mirror were applied for laser transmission, focus and reflection, respectively. An ultrasound transducer (40 MHz, Blatek) was used for ultrasonic signal generation and ultrasonic/photoacoustic signal detection. All the small optical and ultrasonic elements were aligned in a line in a stainless housing, whose outer diameter was kept at 0.9 mm. (Fig. 2B). A plastic tube (material: PA 12) with outer diameter of 1.2 mm and one end sealed using UV glue was applied to protect the catheter from contamination of the surrounding blood during in vivo experiment. The space between the catheter and the plastic tube was filled with saline. The imaging catheter was connected to a torque coil with an outer diameter of 0.8 mm and length of approximately 1 m, which allowed the imaging catheter to move freely in space and rotate smoothly during imaging [22].

2.10. Ex vivo IVPA/IVUS imaging of lipid components in atherosclerotic plaques

We used a laser (OPOTEK) at a wavelength of 1720 nm for lipid photoacoustic signal excitation. The repetition rate of 10 Hz and 200 A-lines/frame lead to the frame rate of 1 frame/20 s. The pullback step was fixed at 150 μm and the total pullback length was 1.5 mm (10 B-scans were record for one pullback). The excitation intensity was fixed at 80 μJ. The aorta samples excised from the rabbits in the control group and HFD group were used for ex vivo imaging. During the imaging process,
heavy water filled the excised aorta to improve the transmission of the laser and assure the imaging contrast of lipids. During this experiment, the plastic tube depicted in section 2.9 was not applied to assure more laser energy achieving the artery wall.

2.11. In vivo IVUS/IVPA molecular imaging

During molecular imaging, a laser (Innolas) with a repetition rate of 20 Hz and wavelength of 780 nm was used for photoacoustic signal excitation. For the imaging of 200 A-lines/frame, the frame rate was 1 frame/10 s. The pullback step was fixed at 150 μm and the total pullback length was 1.5 mm (10 B-scans were record for one pullback). The excitation intensity was fixed at 25 μJ. Two rabbits in the HFD group were first anesthetized by ketamine (25 mg/kg) via a marginal ear vein and maintained by 2–2.5 % isoflurane, and then in vivo images were taken 1 h after IHR-NPs (2 mg ICG/kg) or IH-NPs were injected into rabbits via a marginal ear vein. After the rabbits were anesthetized, the abdominal aortic artery was surgically exposed. A 5 French catheter sheath was inserted into the aortic artery, and then the IVPA/IVUS catheter was introduced through the sheath. Along with the flushing technique, we achieved volumetric IVPA/IVUS imaging of the aorta.

2.12. Quantification of photoacoustic and ultrasound signals in in vivo and ex vivo imaging

For photoacoustic signals, we chose two regions with same area from the same image: the first region enclosing the photoacoustic signals in the image (yellow square as shown in Fig. S1) as the region of interest (ROI) for photoacoustic signal quantification; the second region without the effective photoacoustic signal from targets (red square as shown in Fig. S1) as the region for noise quantification. The standard deviation of photoacoustic signals from region 2 were averaged as the background noise ($A_{\text{noise}}$), while the photoacoustic signals with values above the noise in region 1 were averaged as the effective photoacoustic signal ($A_{\text{signal}}$). At last, the SNR was calculated according to the following formula [39]:

$$\text{SNR} = 20 \times \log_{10} \frac{A_{\text{signal}}}{A_{\text{noise}}}$$

For ultrasound signals, each group of data was measured 3 times. All IVUS images were shown in gray scale, where each pixel in the image was normalized by the maximum pixel value using Image J software (Image J 1.8, NIH, USA). The fold increase of echointensity (%) was calculated.

2.13. Immunohistochemistry staining

After hybrid imaging in vivo was performed, the abdominal aortic arteries in the control and HFD groups were located under X-ray, removed, embedded in paraffin and sectioned. Hematoxylin-eosin (H&E), Masson’s trichrome and Sirius red staining were performed to analyze the histology of the atherosclerotic artery. Immunohistochemistry staining including von Willebrand factor (vWF, Servicebio, Wuhan, China, GB11020, dilution ratio 1:800) and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31, Servicebio, Wuhan, China, GB12063, dilution ratio 1:500) was applied to identify the presence of new blood vessels in atherosclerotic plaques. Integrin αvβ3 immunohistochemistry staining was applied to analyze the expression of integrin αvβ3 in atherosclerotic plaques. Images were taken by confocal fluorescence microscopy (Nikon E100, Japan).

2.14. Statistical analysis

All statistical tests were performed using the Statistical Package for the Social Sciences software version 19.0 (SPSS Inc., Chicago, IL, USA). Continuous variables that approximated the normal distribution were
shown as mean ± standard deviation (SD). The histogram was used to describe the distribution characteristics of nanoprobes size. The graph was used to describe variable trends of hydrodynamic diameters and dispersive stability over time. Simultaneously, categorical variables were compared with use of chi-square analysis. \( P < 0.05 \) was considered statistically significant. GraphPad Prism5 statistical software was used for all data analyses.

3. Results

3.1. Characterization of IHR-NP and IH-NP probes

The schematic structure of IHR-NPs is shown in Fig. 1. TEM images showed that IH-NPs and IHR-NPs were circular in shape with uniform particle sizes of \( 42 \pm 0.44 \) nm and \( 51 \pm 0.62 \) nm, respectively. The nanoprobes were scattered without agglomeration (Fig. 3A and B). As shown in Fig. 3C and D, the distribution of the hydrated particle size of IHR-NPs and IH-NPs was roughly between \( 90 \pm 14 \) nm and \( 110 \pm 18 \) nm, and the system was relatively stable (Fig. 3E and F) and not prone to agglomeration. Fig. 3H shows the ultraviolet absorption spectra of the nanoprobes. The characteristic absorption peaks of HSA and ICG were measured at 278 nm and 780 nm, respectively, indicating that all components were present in the nanoscale system. Fig. 3G shows the fluorescence emission spectra of the IHR-NPs and IH-NPs under laser irradiation at different wavelengths. It is suggested in the figure that the fluorescence intensity of the two probes reached a peak between 810–830 nm, which conformed to the fluorescence spectrum characteristics of ICG, indicating that ICG was successfully encapsulated in the probe. The zeta potential of the IH—NP nanoprobes was \(-16.7 \pm 0.75\) mV, which changed to \(-47.2 \pm 1.2\) mV after coupling with RGDfk (Fig. 3I).

3.2. Cellular uptake of IHR-NP probes

As shown in Fig. 4A and B, the intracellular red fluorescence was the signal of ICG in the nanoprobes, and the blue fluorescence was the signal of the nuclei stained by DAPI. Fig. 4A and B proved that the nanoparticle fluorescent signal was concentrated in the cytoplasm, and the uptake of IHR-NPs in ox-LDL group cells was significantly higher than that of other nonactivated cells and nontargeted nanomaterials. After pre-addition of RGDfk peptide, the uptake of IHR-NPs into the endothelial cells was significantly reduced, which indicated that RGD active short peptide has a good ability to recognize activated endothelial cells, thus verifying that the IHR-NPs in this study can accurately identify activated endothelial cells in vulnerable atherosclerotic plaques (VASPs). Fig. 4B shows the statistical analysis of the fluorescence signal intensity in each group, which also indicated that the fluorescence signal intensity in the ox-LDL + IHR-NP group (0.14 ± 0.001 a.u.) was significantly higher than that in the other groups (\( P < 0.05 \)).

3.3. Cytotoxicity of IHR-NP probes on HUVECs

Fig. 4C shows the cytotoxicity of IH-NP and IHR-NP probes. The CCK-8 assay demonstrated that the viability of HUVECs incubated with IHR-NP nanoprobes was not significantly different from that of HUVECs incubated with IH-NP nanoprobes in the concentration range of ICG from 2 to 64 \( \mu \)g/mL (\( P > 0.05 \)), indicating low cytotoxicity of the resulting probes. Both the CCK-8 assay and flow cytometry demonstrated that there was an insignificant influence of the two kinds of nanoprobes on the apoptosis of HUVECs; therefore, they were safe for in vivo studies.

Fig. 3. Characterization of IH-NPs and IHR-NPs. TEM micrograph (A, B), size distribution (C, D) and hydrodynamic diameters and dispersive stability (E, F) of IH-NPs and IHR-NPs. (G) ICG fluorescence spectra of IH-NPs and IHR-NPs. (H) Ultraviolet absorption spectra of IH-NPs and IHR-NPs. (I) The zeta potential of IH-NPs and IHR-NPs.
3.4. Verification of atherosclerosis in rabbit model

Fig. 5A shows the balloon de-endothelialization of the abdominal aortic artery in rabbits. To validate the rabbit atherosclerosis model, plaque formation of the abdominal aorta in HFD group rabbits was first examined by ultrasound and compared to rabbits in the control group. In the HFD group, long strip hyperechogenicity was observed in the intima of the common carotid artery near the bifurcation with uneven intensity and no blood flow signals. In the control group, the lumen of the vessel was uniform in thickness, the intima of the vessel wall was continuous, and no obvious intima lesion was observed (Fig. 5B). For further confirmation, the lipid-rich plaques in the HFD group were observed by gross Oil Red O staining and frozen section Oil Red O staining compared to the control group (Fig. 5C and D). Atherosclerotic plaque formation was evaluated by quantitative analysis of the plaque area in the total aorta with Oil Red O staining (the ratio of plaque area to whole artery area: $87.90 \pm 2.6\%$ in the HFD group vs. $14.52 \pm 2.9\%$ in the control group, $P < 0.01$) (Fig. 5E).
3.5. Evaluation of αvβ3 integrin expression in activated endothelial cells and atherosclerotic plaques

To evaluate αvβ3 integrin expression in activated endothelial cells, HUVECs were exposed to Ox-LDL to induce the formation of activated endothelial cells [40]. As shown in Fig. 4E, the expression of αvβ3 integrins in Ox-LDL-stimulated endothelial cells was much higher than that in untreated HUVECs. The quantitative western blot results further confirmed the upregulated expression of αvβ3 integrins in vulnerable atherosclerotic plaques, a western blotting of the abdominal aortas of rabbits in the two groups was carried out. αvβ3 integrin expression in the abdominal aorta of rabbits in the HFD group was much higher than that in the HFD group (Fig. 5F). The quantitative western blot results further confirmed the upregulated expression of αvβ3 integrins in the abdominal aorta of rabbits in the HFD group (Fig. 5F), with αvβ3 integrin/β-actin values up to 45.2 ± 1.4 %, in contrast to 20.3 ± 0.81 % for the control, with P < 0.05. These results indicated that αvβ3 integrins are a potential target for neovascular detection of vulnerable atherosclerotic plaques.

3.6. IVUS/IVPA imaging of the lipid composition of atherosclerotic plaques

To verify the lipid core inside the arterial plaque, IVUS/IVPA imaging of the abdominal aorta of the rabbits was performed in the HFD and control groups (Fig. 6). Fig. 6C and D show the IVUS images of the abdominal aorta, and clear outlines of the vascular intima and fibrous plaque were visible in both groups. The ultrasound signal of plaques in HFD groups was significantly stronger than that of control group. The quantitative analysis of ultrasound signal, as shown in Fig. 6G, also support this conclusion with a HFD/CON echo intensity ratio of 1.32 ± 0.07 (p < 0.05). The IVPA image results showed that the photoacoustic signal in the abdominal aorta of the control group was weak, while the photoacoustic signal intensity in the HFD group was significantly enhanced (Fig. 6A and B). The quantitative analysis of the photoacoustic signal in Fig. 6H shows that the SNR of the HFD group was significantly higher than that of the control group (35.62 ± 0.54 vs. 20.61 ± 0.49, P < 0.05). This result indicated that there was a large lipid core in the abdominal aortic plaque of the HFD group but not in the control group. Fig. 6E and F showed the IVUS/IVPA fusion images of the abdominal aorta in the two groups. For further confirmation, the lipid-rich plaques in the HFD group were observed by frozen section Oil Red O staining compared to the control group (Fig. 6I and J).

3.7. Molecular imaging of angiogenesis with IHR-NP nanoprobes in vivo

IVUS/IVPA imaging of the abdominal aorta in the HFD groups injected with IH-NPs or IHR-NPs is shown in Fig. 7. Fig. 7C and D show the IVUS images of the abdominal aorta. The arterial plaque load in both groups was heavy, and the lumen area was significantly reduced, but there was no significant difference between two groups (echo signal intensity of HFD/ that of CON: 1.05 ± 0.08, p > 0.05, Fig. 7G). The photoacoustic imaging showed obvious photoacoustic signals on several consecutive B-scan images along the pullback direction in the IHR-NPs group (Fig. S1). A representative B-scan image of the abdominal aorta in Fig. 7B presented obvious photoacoustic signal with SNR of 20.34 dB at 8 o’clock in the cross section, while no photoacoustic signal was found in the abdominal aorta in the IH-NPs group (Fig. 7A). Fig. 7H shows the quantitative analysis of the photoacoustic signal intensity of the abdominal aortic plaques in the two groups of rabbits, which also

![Fig. 6.](image-url)
indicated that the SNR of the IHR-NPs group was significantly higher than that of the control group (20.34 ± 0.71 vs. 0).

3.8. Histopathological and immunohistochemistry staining

To verify the features of the targeted vulnerable plaques, H&E, Masson’s trichrome and Sirius red staining were performed. The results showed that the vascular wall was thin, the intima was smooth, and without hyperplasia, no obvious atherosclerotic plaque was found in the control group (Fig. 8A). In contrast, the vascular wall was relatively thickened, the intima structure was disordered, and significant atherosclerotic plaque formation was observed in the HFD group (Fig. 8B). Furthermore, tubular cavity tissue surrounded by a monolayer of endothelial cells was found, and there was an accumulation of oval cell tissue in the middle membrane of the artery in the HFD group, as marked with a red square (Fig. 8B), which was not found in the control group (Fig. 8A).

To further verify the possibility of neovascularization in atherosclerotic vulnerable plaques, vWF, CD31 and integrin αvβ3 immunohistochemistry staining was performed. Fig. 9A shows the vWF, CD31 and integrin αvβ3 immunohistochemistry staining in the control groups. No significant immunostaining was found in the section of the blood vessel even if the vessel was enlarged (Fig. 9A). In contrast, significant immunostaining was found in the HFD groups. In addition, the immunofluorescence results confirmed the abundant expression of integrin αvβ3 (yellow staining) in the section of the vessels imaged by IVUS/IVPA.

![Image](https://example.com/image.png) Fig. 7. Intravascular photoacoustic and ultrasonic imaging of abdominal aortic vessels in HFD groups with the injection of IH-NPs (A, C, E) and IHR-NPs (B, D, F). (G) Ultrasonic signal intensity of abdominal aortic vessels in the HFD groups injected with IH-NPs and IHR-NPs. (H) Photoacoustic signal intensity of abdominal aortic vessels in the HFD groups injected with IH-NPs and IHR-NPs.

![Image](https://example.com/image.png) Fig. 8. Representative macroscopic images of H&E, Masson’s trichrome staining and Sirius red staining in the control (A) and HFD (B) groups. The red arrows showed the neovascularization (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).
4. Discussion

The high-risk plaques is an end stage of a chronic process of injury including endothelial cell injury, macrophage activation, angiogenesis formation and is prone to be rupture followed by acute events. In vivo imaging of plaque molecular events could help to elucidate the mechanisms of plaque progression and rupture [41-43]. However, the in vivo noninvasive imaging of artery plaques is limited in terms of the detailed characteristics that can be obtained due to the resolution, accurate localization and quantitative visualization in the thin inner wall of the vessel [11,44].

In present study, we construct a RGD targeted photoacoustic HSA-ICG nanoprobe which was capable of labeling the integrin \( \alpha v \beta 3 \) in atherosclerotic rabbit model through the hybrid IVUS/IVPA imaging system platform. Meanwhile, the lipid content was also detected through 1720 nm wavelength by IVPA, which also were confirmed by pathological staining.

The biocompatibility, targeting and low cytotoxicity of nanoprobes are most critical for molecular imaging. Numerous studies have demonstrated that HSA is a major component of serum proteins and has attracted wide interest as a natural drug carrier because of its inherent biocompatibility, abundance, and versatile roles in drug delivery [45]. ICG is a registered and FDA-approved fluorescent dye for optical imaging in clinical settings, which has low toxicity, good light stability and excellent biocompatibility [46]. ICG is recognized as a safe and inexpensive, water-soluble amphiphilic molecule with a molecular weight of 775 Daltons and a hydrodynamic diameter of 1.2 nm, rendering it an excellent vascular contrast agent if injected intravenously [47]. Therefore, the nanoprobe prepared by encapsulating ICG will have a good prospect for clinical application. Moreover, we can also use ICG as a photosensitizer in our further study for photothermal and photodynamic therapy of atherosclerotic plaque. In this study, we combined ICG with human serum albumin (HSA) to improve its preclinical performance.

As a critical requirement for plaque vulnerability, angiogenesis is regulated by many proteins [48]. Among angiogenesis factors, integrins are responsible for cellular adhesion to extracellular matrix proteins in intercellular spaces and basement membranes and regulate cellular entry and withdrawal from the cell cycle [49]. Integrins are made up of alpha and beta subunits which have both adhesion and signaling functions. It has been shown that cyclization of RGD peptides recognizes the \( \alpha v \beta 3 \)-integrin via linkers, often leading to increased receptor binding affinity and selectivity [50]. The cyclic peptide (RGDK), originally designed and synthesized by Kessler’s group, is a cyclic RGD peptide with good affinity and selective inhibition against integrin \( \alpha v \beta 3 \) [51]. Among the members of the integrin family, integrin \( \alpha v \beta 3 \) is extensively expressed in endothelia with statistically significant differences between tumor and normal tissue [32]. In a recent study, tumor-targeting theranostic nanoprobes were constructed by assembly of both HSA-Ce6 and HSA-RGD simultaneously, which could target \( \alpha v \beta 3 \)-integrin, as evidenced by both in vitro and in vivo experiments [52]. Therefore, our designed HSA nanoparticles containing ICG and RGDK could be used as a photoacoustic probe targeting new blood vessels in vulnerable plaques. The metabolism of molecular probes in vivo is still unclear and needs to be further clarified in follow-up work to further evaluate the opportunity for imaging after the probe is injected, optimize the examination procedure and improve the feasibility of clinical application.

A hybrid IVPA/IVUS imaging system that can simultaneously perform photoacoustic and ultrasonic imaging was used to acquire functional information of multiple components in atherosclerotic plaques (lipid core and neovascularization), as well as arterial structural information. The relevant light excitation and ultrasound generation in photoacoustic imaging precludes harmful radiations to the experimental system platform. Meanwhile, the lipid content was also detected through 1720 nm wavelength by IVPA, which also were confirmed by pathological staining.

The obtained comprehensive information is valuable in assessing the severity and vulnerability of atherosclerotic plaques and potentially enabling the development of improved interventional treatment for atherosclerosis [54]. Although the imaging system was successfully applied in lipid and neovascularization detection, the in vivo identification of lipids was not achieved in this study due to the high absorption (brown staining, Fig. 9B).
of blood and water at 1720 nm. In the ex vivo experiment, we filled the space between the imaging catheter and artery with heavy water, which had low light scattering and absorption at 1720 nm, to reduce the absorption and ensure excitation in the artery wall. However, blood flushing with heavy water is highly difficult during in vivo imaging. Due to the relatively high cost and the complications to the experimental subject, such as higher concentrations (usually >20 % of body weight) of heavy water can be toxic to human and animal cells. Effects on the nervous system and formation of different blood cells have been concerned [55]. Thus, a more efficient blood flushing operation is still needed, as blood scattering causes signal attenuation and further reduces the imaging contrast, which we are pursuing in follow-up work.

Not only was the presence of lipids detected using hybrid IVUS/IVPA imaging, but the presence of neovascularization was also detected directly for the first time with intracavitary imaging technology. In this study, under excitation at 780 nm combined with targeted nanoparticle probes, an obvious photoacoustic signal was detected inside the abdominal aorta atherosclerosis in the HFD group, suggesting the possibility of neovascularization. At the same time, according to the results of subsequent pathological and immunohistochemical staining, neovascularization formation was found within the blood vessels, which agreed with IVUS/IVPA imaging. The above results indicate that the photoacoustic nanoprobe developed in this study can successfully position the activation of endothelial cells or angiogenesis in plaques and may provide strong evidence for assessing plaque vulnerability and guidance on treatment strategies. In addition, the hybrid imaging technique can provide information on the overall structure and components of the plaque in a single imaging process. The technology has potential in preclinical scientific research and clinical translation in the future.

Despite these encouraging results, there are still some limitations in our research. First, the probe of IHR-NPs can only be utilized in the diagnosis of plaques instead of therapy integration, and further discovery of portable antiatherosclerotic drugs or siRNAs that can be used for probe encapsulation is needed. Second, we have demonstrated the ability of IVUS/IVPA imaging technology to offer comprehensive imaging information, but further application of the technology for in vivo imaging or clinical purposes is still need further study. Third, we cannot provide the lipid data and biomarker data in vivo simultaneously. Further IVPA application in RGD detection for in vivo imaging or clinical purposes is still hindered by the limited imaging speed. During operation in the intravascular environment, high-speed imaging is important to reduce the risk of ischemia.

5. Conclusion

We successfully constructed an imaging probe for RGD-targeted HSA nanoparticles that can specifically visualize angiogenesis formation within progressed plaques. The dual-probe designed hybrid intravascular imaging probe can provide both structural information and inflammatory component information in rabbit abdominal atherosclerotic plaques simultaneously in vivo with the hybrid intravascular imaging system. Our results suggested that hybrid intravascular molecular imaging technology may provide further strategic guidance for the interventional treatment of coronary heart disease.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pacs.2021.100262.

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