RGD-functionalized melanin nanoparticles for intraoperative photoacoustic imaging-guided breast cancer surgery

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Abstract

**Purpose:** Obtaining tumour-free margins is critical for avoiding re-excision and reducing local recurrence following breast-conserving surgery (BCS); however, it remains challenging. Imaging-guided surgery provides precise detection of residual lesions and assists surgical resection. Herein, we describe water-soluble melanin nanoparticles (MNPs) conjugated with cyclic Arg-Gly-Asp (cRGD) peptides for breast cancer photoacoustic imaging (PAI) and surgery navigation.

**Methods:** cRGD-MNPs was synthesized and characterized for morphology, photoacoustic characteristics and stability. Tumour targeting and toxicity were determined by cells and tumour-bearing mice. PAI was used to locate the tumour and guide surgical resection in MDA-MB-231 tumour-bearing mice.

**Results:** The cRGD-MNPs exhibited excellent tumour-targeting *in vitro* and *in vivo*, with low toxicity. Intravenous administration of cRGD-MNPs to MDA-MB-231 tumour-bearing mice showed an approximately 2.1-fold enhancement in photoacoustic (PA) intensity at 2 h, and the ratio of the PA intensity at the tumour site compared to that in the surrounding normal tissue was 3.2 ± 0.1, which was much higher than that using MNPs alone (1.7 ± 0.3). Similarly, the PA signal in the mammary glands containing spontaneous breast cancer was enhanced (2.5 ± 0.3-fold) in MMTV-PyVT transgenic murine model. Preoperative screening by PAI could assess tumour volume and offer a three-dimensional (3D) reconstruction image for accurate surgical planning. Surgical resection following real-time PAI on the tumour bed showed high consistency with histopathological analysis.

**Conclusion:** These results highlight that cRGD-MNPs combined with PAI provide a powerful tool for breast cancer imaging and precise tumour resection. cRGD-MNPs with good PA properties have great potential for clinical translation.

**Keywords:** melanin, bioimaging, photoacoustic imaging, breast cancer, tumour detection, imaging-guided surgery
Introduction

Nearly half of patients with early breast cancer undergo breast-conserving surgery (BCS) with adjuvant radiation therapy [1]. BCS involves the removal of the primary tumour with a margin of surrounding normal breast tissue. Although BCS is the standard of care treatment for early breast cancer patients [2], a potential downside is the failure to achieve clear or negative margins with lumpectomy. In particular, 20–40% of patients require additional operative intervention by re-excision or even mastectomy [3]. Re-excision surgery increases the risk of complications (e.g., infection and unsatisfactory aesthetic results) and potentially postpones systemic treatment. It also increases health care costs and burden and harms patients both physically and psychologically.

During BCS, surgeons typically use visual inspection and tactile feedback to determine the tumour location and set resection margins. However, it is difficult to discriminate tumour margins from surrounding normal tissue macroscopically. Frozen section and imprint cytology are applied clinically for intraoperative margin assessment. These methods have the potential to lower the rates of positive margins, but they are labour-intensive and time-consuming and have low sensitivity due to sampling rate limitations [4, 5]. Currently, there have been multiple emerging intraoperative imaging tools for real-time margin assessment, including wire-guided localization [6], intraoperative ultrasound [7] and postoperative techniques (intraoperative specimen mammography/micro-computed tomography) [8]. Due to their anatomical imaging modalities and limited tumour specificity, these methods have not been widely used for margin assessment. Fluorescence-guided surgical navigation is a high sensitivity technique using the injection of a fluorescent contrast agent that is a promising intraoperative tool for precise tumour resection [9]. However, this technique is hampered by a limited penetration depth because of light scattering and signal attenuation [10].

Recently, photoacoustic imaging (PAI) has been developed as a novel imaging technology for biomedical applications. PAI detects optical absorption contrast acoustically via the photoacoustic (PA) effect, a physical phenomenon that converts
absorbed optical energy into acoustic energy [11]. Based on endogenous contrast molecules (e.g., oxyhaemoglobin, deoxyhaemoglobin, lipid, or DNA-RNA), PAI has been used in the clinic trial to demonstrate its highly desirable capabilities for breast cancer imaging in vivo and ex vivo, particularly for assessing tumour margins macroscopically and microscopically [12-14]. However, intrinsic chromophores provide access to only a limited range of biological processes but low tumour-imaging contrast. Hence, molecular PAI for breast cancer still requires a targeted contrast agent that can selectively bind to surface receptors on cancer cells or respond to the tumour microenvironment [11, 15].

Natural melanin is a group of biopigments with multifunctionality (i.e., ultraviolet protection, radical scavenging, and photothermal conversion) [16]. Due to its good intrinsic biocompatibility, natural melanin or synthetic melanin-like nanomaterials have been successfully developed as novel nano-bioplatforms in bioimaging, therapy, theranostics, and biosensing [17, 18]. As an endogenous PA contrasting agent, melanin was used to detect the metastatic status of ex vivo human melanoma sentinel lymph nodes by multispectral optoacoustic imaging[19]. The results showed an excellent correlation with the histological assessment of melanoma cell infiltration with 100% sensitivity and 62% specificity [19].

In this study, we conducted a comprehensive study of the feasibility of the PAI method for improving the detection and accurate removal of breast cancer using targeted melanin nanoparticles (MNPs) conjugated with cyclic Arg-Gly-Asp (cRGD) (Scheme 1). Our data support the potential clinical application of cRGD-MNPs as a novel tumour-specific PA contrast agent for imaging-guided BCS.
Materials and methods

Reagents

Melanin was obtained from Sigma-Aldrich. Sodium hydroxide, hydrochloric acid and NH₄OH solution (28 wt%) were purchased from Sinopharm Chemical Reagent Co., Ltd. Amine-PEG₅₀₀₀-amine (NH₂-PEG₅₀₀₀-NH₂, 5 kDa) was obtained from Shanghai Aladdin Bio-Chem Technology Co., LTD. CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega, phosphate-buffered saline (PBS) was purchased from Solarbio and c(RGDfC) was from GL Biochem. Melanin staining kit was purchased from Servicebio. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco. Mammary Epithelial Cell Growth Medium (MEGM) BulletKit was purchased from Lonza.

Cell culture

The human breast cancer cell line MDA-MB-231 and non-cancerous mammary epithelial cell line MCF-10A were purchased from Procell Life Science & Technology Co., Ltd., and cells with passage number < 25 were used in the experiment. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. MCF-10A cells were cultured in MEGM supplemented with BPE, hEGF, insulin, hydrocortisone, and GA-1000. The cells were grown as sub-confluent monolayers at 37 °C under the water-saturated environment with 5% CO₂.

Animals

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Research Animals established by the Xiamen University Animal Studies Committee. Female BALB/c nude mice and BALB/c mice (4–6 weeks, approximately 20 g) were purchased from the Experimental Animal Center of Xiamen University and kept under sterile conditions. FVB/N-Tg(MMTV-PyVT)634Mul/J transgenic mice were purchased from the Jackson Laboratory and raised under specific-pathogen-free conditions.
Preparation of cRGD-MNPs

The cRGD-MNPs were obtained as previously described [20]. Briefly, melanin (2 mg/mL) was dissolved in NaOH aqueous solution (0.1 N) followed by the rapid addition of HCl aqueous solution (0.1 N) to a pH of 7.0 under sonication. The neutralised solution was centrifuged and washed with deionized water several times. The black solid of MNPs was obtained by freeze-drying. The MNPs aqueous solution (1 mg/mL, pH = 9) was added dropwise into an NH$_2$-PEG$_{5000}$-NH$_2$ aqueous solution. After vigorous stirring for 12 h, the PEG-modified MNPs were retrieved by centrifugation and washed several times to remove the unreacted NH$_2$-PEG$_{5000}$-NH$_2$. The PEGylated MNPs (1 mg/mL, pH = 7.2) were incubated with 4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-SMCC) solution (1.2 mg in 36 μL of dimethyl sulfoxide) for 2 h at room temperature. The complexes were purified using a PD-10 column. The c(RGDfC) solution (120 μL of 5 mM in degassed water) was added to the purified MNPs with stirring for 24 h at 4°C. The excessive RGD peptide was removed using a PD-10 column. The final cRGD-MNPs were filtered through a 0.22 μm filter.

Characterisation of MNPs

Transmission electron microscopy images were recorded using a Talos F200s transmission electron microscope (FEI, USA) at an accelerating voltage of 100 kV. The MNPs or cRGD-MNPs aqueous solution was dropped onto a carbon-coated copper grid and air-dried. The $^1$H NMR spectra were recorded at 20°C on a 400 MHz NMR spectrometer (Bruker) using D$_2$O as the solvent. Zeta potentials were measured using a laser particle size analyser system (Malvern, Zetasizer Nano ZS90). The absorption spectra were obtained using a Multiskan Spectrum Microplate Spectrophotometer (Thermo, USA).

PAI system

Both PA and US images were recorded using the Vevo LAZR-X photoacoustic imaging system (VisualSonics, FujiFilm, Japan). A tunable laser (680–970 nm) with a
repetition rate of 20 Hz and a 256-element linear array with the main frequency of 40 MHz was used to obtain single-plane, full-spectrum and 3D PA images. The 3D scanning was controlled using an electric motor with a step length of 0.14 mm. The quantified PA signal intensities within the region of interest (ROI) of each image were analysed using the Vevo LAB tool (VisualSonics, FujiFilm, Japan).

**PA signal of cRGD-MNPs**

For evaluating the PA performance of nanoparticles, MNPs and cRGD-MNPs (120 μM) were scanned for PAI at different wavelengths ranging from 680 to 970 nm (interval = 5 nm) to detect the optimum excitation wavelength. Different concentrations (3.75–120 μM) of MNPs and cRGD-MNPs were dispersed in PBS and triggered by the optimal excitation wavelength to acquire the corresponding PA images. For photostability analysis, PA images of the MNPs and cRGD-MNPs solutions (120 μM) were obtained at different time points (0, 2, 4, 6 and 7 days) using a 680 nm excitation.

**Cellular uptake of cRGD-MNPs**

MDA-MB-231 cells (3 × 10^5 per well) were seeded in 6-well plates and cultured for 24 h. The old medium was replaced with medium containing 0.125 μM cRGD-MNPs. After incubation for various times (1, 2, 4 or 8 h), the medium was removed, and the cells were washed three times with PBS. The cells were harvested, fixed with 4% paraformaldehyde solution for 20 min, resuspended in PBS and counted. The same amount of cell suspension (20 μL) was placed in a microcentrifuge tube and then the tube was fixed on the imaging table. PAI was performed on the MDA-MB-231 cell samples at a wavelength of 680 nm using the Vevo LAZR-X system. For the concentration gradient experiment, MDA-MB-231 cells were incubated in medium containing the cRGD-MNPs (0.125, 0.25, 0.5 or 1 μM) for 4 h. The cells were collected and imaged as above. To investigate the effect of RGD on MNPs uptake, MDA-MB-231 cells were treated for 4 h with medium containing 0.5 μM MNPs, PEG-MNPs or cRGD-MNPs and then subjected to PAI analysis. For the RGD blocking experiment, MDA-MB-231 and MCF-10A cells were seeded in 6-well culture plates and incubated
with 0.5 μM cRGD-MNPs for 4 h. For the αvβ3 integrin-blocking group, MDA-MB-231 cells were co-incubated with free RGD (100 μM). The samples were assessed by PAI.

**Preparation of Rho-MNPs and Rho-cRGD-MNPs**

Rhodamine (30 μL, 5 mg/mL) was added to the MNPs or cRGD-MNPs solutions (1 mL, 2 mg/mL). After stirring at room temperature for 1 h, the solution was transferred to an ultrafiltration centrifuge tube (Millipore Amicon Ultra, 30 kDa) and centrifuged several times (4000 rpm, 15 min) to remove the free or unstable rhodamine, yielding the rhodamine-labelled MNPs (Rho-MNPs) and cRGD-MNPs (Rho-cRGD-MNP).

**Location of cRGD-MNPs by confocal microscopy**

MDA-MB-231 (1 × 10^5 cells) were seeded in confocal dishes and allowed to adhere for 24 h. The Rho-cRGD-MNPs were added to the culture medium with or without free RGD (100 μM) at the times and concentrations described above. The cells were fixed with 4% paraformaldehyde and counterstained with DAPI to visualise nuclei. The fluorescence images of the cells were collected using a Nikon confocal laser scanning microscope.

**In vitro cytotoxicity assay**

MNPs cytotoxicity was determined in MDA-MB-231 cells using the MTS assay. MDA-MB-231 cells (5 × 10^3/well) were incubated in 96-well plates for 24 h. Cells were then cultured in medium supplemented with the different MNPs (0.625, 1.25, 5 and 10 μM) for 72 h. After the incubation, MTS (10 μL, 0.5 mg/mL) was added to each well, and the cells were incubated for 2 h at 37°C. The absorbance was measured at 490 nm using a microplate reader. The absorbance of the untreated cells was used as the reference value for calculating 100% cell viability. Five replicates were conducted for each group.

**MDA-MB-231 breast cancer xenograft mouse model**

MDA-MB-231 cells (1×10^6 in 100 μL PBS) were inoculated subcutaneously in a
single flanks of nude mice. When tumour volume reached 100 mm$^3$, the tumour-bearing
mice were subjected to in vivo PAI and biodistribution studies.

In vivo tumour PAI in MDA-MB-231 tumour-bearing mice

To determine the optimal concentration range of the MNPs, mice were randomly
allocated into groups and injected with different concentrations of RGD-PEG-MNPs
(50 μM, 100 μM or 200 μM, 200 μL) via the tail vein. PA images were collected at
different time points (pre, 1, 2, 4 or 12 h) using the Vevo LAZR-X at 680 nm, and the
average PA intensities in the tumour regions were measured. To detect the tumour
targeting capability of the cRGD-MNPs, MDA-MB-231 tumour-bearing mice were
randomly divided into MNPs and cRGD-MNPs groups (n = 3 per group). The mice
were intravenously injected with the MNPs or cRGD-MNPs (100 μM, 200 μL),
respectively. PA images were collected, and the intensities measured.

The biodistribution of MNPs and cRGD-MNPs in MDA-MB-231 tumour-bearing
mice

To determine the biodistribution of the nanoprobes in vivo, MDA-MB-231 tumour-
bearing mice (n = 3 per group) were intravenously injected with Rho-MNPs or Rho-
cRGD-MNPs (100 μM, 200 μL). The major organs and tumours of mice were harvested
2 h post-injection for ex vivo fluorescence imaging using the IVIS Lumina imaging
system (PerkinElmer, USA). The relative fluorescence intensities were measured.

PAI and pathological analysis of spontaneous breast cancer in transgenic mice

The FVB/N-Tg(MMTV-PyVT)634Mul/J transgenic mice model spontaneously
develops invasive breast cancer in each mammary gland between 6 and 12 weeks of
age. To further confirm the tumour targeting specificity of the cRGD-MNPs, mice with
breast cancer (age 6–8 weeks, n = 6) were intravenously injected with the cRGD-MNPs
(100 μM, 200 μL). US and PA images were obtained before injection and 2 h post-
injection with excitation at 680 nm. In addition, the 4th and 5th mammary glands of the
transgenic mice (age 8 weeks, n = 3) with breast cancer were divided into four sections
(P1-4) for PAI in vivo and in vitro 2 h after nanoprobe injection. After imaging, the
mammary tissue was fixed with formalin and embedded in paraffin. Tissue sections (4 μm) were stained with haematoxylin and eosin (H&E), according to standard methods.

**Melanin staining**

The transgenic mice with breast cancer were divided into PBS and cRGD-MNPs groups (age 8 weeks, n = 3 per group). Two hours post-intravenous injection, the tumour tissue was removed and subjected to Fontana–Masson staining. The slides were immersed in the Fontana–Masson solution, placed in the dark at 4°C for 12-18 h, and then rinsed with distilled water. Next, the slides was put into VG staining solution and stained for 1 minute. Finally, the slides were washed, sealed with neutral balsam and observed under the microscope.

**PAI-guided breast cancer surgery on MDA-MB-231 xenograft mice**

For surgical resection, cRGD-MNPs (100 μM, 200 μL) were intravenously injected into MDA-MB-231 tumour-bearing mice via the tail vein. Each mouse was anesthetised and placed on a mouse table for position adjustment. The mice remained anesthetised during the entire imaging acquisition process. Tumour profiles were detected using 3D PA/US imaging two hours after nanoprobe injection. For simulated tumour surgery resection, the surgery procedure was performed in four steps: PAI-guided tumour detection, partial tumour resection (P1-3), PAI detection of the residual tumour, and re-resection of the tumour bed (P4). PA images were acquired both before and after each sequential removal of tumour. Each resection was about a 5-6 mm long and 2-3 mm wide piece of tissue. After each resection, we irrigate the surgical area with saline to resolve the bleeding issues. Surgically excised tissue was stained with haematoxylin and eosin (H&E), according to the standard protocol. To detect the depth of PAI, tumours covering chicken breast with various thickness (1, 2, 3, 5, 7 mm) were performed PAI using the PAI system.

**cRGD-MNPs biosafety evaluation**

BALB/c mice (4–6 weeks, approximately 20 g) were randomly divided into four groups: control, 1-d cRGD-MNPs, 7-d cRGD-MNPs, and 30-d cRGD-MNPs (n = 5 per
The mice were intravenously injected with saline (control) or cRGD-MNPs (100 μM, 200 μL). Body weight was monitored throughout the experiment. Blood samples and major organs (heart, liver, spleen, lung and kidney) were collected after 1 (1-d group), 7 (7-d group) or 30 days (control and 30-d groups) post-injection. The blood samples were analysed for serum biochemistry parameters. These parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (CR). H&E staining was performed on sections of the major organs for histological analysis.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, CA). The two-tailed paired t-test was used to compare the changes in photoacoustic signals. Data are presented as the mean ± standard error of the mean. Significant differences between or among the groups are indicated as follows: ns for no significant difference, * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$. 


Results

Synthesis and characterisation of cRGD-MNPs

The water-soluble MNPs were approximately 4 nm in size based on transmission electron microscopy (Fig. 1a). Polyethylene glycol (PEG) chains were introduced into the MNPs for further biomodification, which was confirmed by $^1$H NMR spectra (Fig. S1a). This can reduce the liver accumulation because of the enhanced water solubility. Lastly, the PEG-MNPs were further modified with cyclic Arg-Gly-Asp-d-Phe-Cys [c(RGDfC)] peptide (cRGD), which targets tumour $\alpha_v\beta_3$ integrin [21]. The diameter of the RGD-functionalized PEG-MNPs (cRGD-MNPs) increased to about 9 nm (Fig. 1a).

With the modifications of PEG and RGD (i.e., cRGD-MNPs), the surface potential of the MNPs increased from -48 to -8.6 mV (Fig. 1b). MNPs have strong and broad optical absorbance and PA spectra in the wavelength range of visible light to the near-infrared region. The similar absorption and PA spectra between cRGD-MNPs and MNPs indicated that the biomodifications did not influence the absorption properties and intrinsic PA properties of melanin (Fig. 1c, d). Moreover, both the cRGD-MNPs and MNPs showed a linear relationship between concentration and absorbance intensity ($R^2 = 1$ and 0.9999 for MNPs and cRGD-MNPs, respectively) (Fig. S1b, c, d) and PA intensity ($R^2 = 0.9637$ and 0.9513 for MNPs and cRGD-MNPs, respectively) (Fig. S1e, f, Fig. 1e) at 680 nm. In addition, the cRGD-MNPs exhibited excellent stability, with no attenuation of the PA signal intensity after storage for a week at 4°C (Fig. 1f).

Targeting ability and cytotoxicity of cRGD-MNPs in vitro

As seen from the overlay images and quantitative results, the PA signal intensity of MDA-MB-231 cells increased with incubation time from 1 h to 8 h (Fig. 2a). The cells produced significant PA signal intensities after incubation for 4 h, and there was no statistical difference between the 4 h and 8 h time points. As the concentration of cRGD-MNPs increased, the uptake of the probe by the MDA-MB-231 cells also increased (Fig. 2b). The concentration of 0.5 μM was selected for subsequent experiments because there was no statistical difference between 0.5 μM and 1 μM in PA signal intensity.
Compared to the same concentrations of MNPs and PEG-MNPs, MDA-MB-231 cells incubated with RGD-MNPs showed stronger PA signal intensity (Fig. 2c), which confirmed that more cRGD-MNPs could be internalised by MDA-MB-231 cells.

To prove the ability of the cRGD-MNPs to target the integrin αvβ3 receptor, we determined their binding affinity using MDA-MB-231 and MCF-10A (a non-neoplastic breast cell line with low expression of αvβ3) [22] cells. The PA intensity was markedly enhanced for the MDA-MB-231 cells compared to that of the MCF-10A cells after a 4-h incubation (Fig. 2d). Additionally, when the cells were co-incubated with excess free RGD molecules, the PA signal intensity of the MDA-MB-231 cells became weaker. These data indicated that indeed RGD enhanced the internalisation of the nanoparticles by cancer cells overexpressing the integrin αvβ3 receptor. Similarly, strong red fluorescence was observed in MDA-MB-231 cells incubated with Rho-cRGD-MNPs, while MCF-10A cells showed very weak red fluorescence under the same conditions. This phenomenon could be blocked by excessive unlabelled RGD peptide (Fig. 2e).

Finally, analysis of cell viability suggested low cytotoxicity for the MNPs, even at a high concentration up to 10 μM (Fig. 2f).

**In vivo cRGD-MNPs tumour uptake and the biodistribution of fluorescence-labelled cRGD-MNPs**

Based on the PAI performance of the cRGD-MNPs in vitro, we performed tumour PAI in vivo. The in vivo PA signal intensity at the tumour site of MDA-MB-231 tumour-bearing mice at various concentration of cRGD-MNPs (50, 100, and 200 μM) increased gradually and reached a peak two hours post-injection (Fig. S2a). Moreover, the 100 μM and 200 μM groups exhibited similar PA intensities, which were higher than that observed for the 50 μM group 2 h post-injection. The tumour targeting capability of the cRGD-MNPs in living mice was further investigated by intravenous injection cRGD-MNPs into tumour-bearing mice using MNPs as a control) (Fig. 3a, b). We showed that tumour sites exhibited a higher PA signal 1 to 2 h after intravenous administration of cRGD-MNPs than MNPs in vivo. Moreover, the cRGD-MNPs group had a significantly higher signal-to-background ratio (tumour versus surrounding normal tissue) than the
MNPs group two hours post-injection (3.2 ± 0.1 versus 1.7 ± 0.3, P < 0.05) (Fig. 3c).

In addition, the PA signal increased approximately 2.1-fold two hours post-injection compared to pre-injection (Fig. S2b). These results indicated that cRGD-MNPs accumulated more in tumours than normal tissue and provided clearer tumour contrast.

We also used fluorescence imaging to track the biodistribution of Rho-cRGD-MNPs and Rho-MNPs two hours post-injection (Fig. 3d). The ex vivo fluorescence images and intensities of the major organs and tumours suggested more effective accumulation of Rho-cRGD-MNPs into tumour tissue than Rho-MNPs (Fig. 3d, e).

**In vivo cRGD-MNPs PAI in MMTV-PyVT transgenic mice**

To evaluate the PAI performance of cRGD-MNPs, we used the FVB/N-Tg(MMTV-PyVT)634Mul/J spontaneous breast cancer model that closely recapitulate human disease [23]. The transgenic mice underwent PAI before or two hours post-injection (Fig. 4a). Compared to the imaging signal before administration, the PA signal intensities of the mammary glands containing tumour increased 2.5 ± 0.3-fold (Fig. 4b). In contrast, the PA signal intensities of the normal mammary glands did not increase post-injection (0.9 ± 0.1-fold, Fig. 4b). The pathological status of the mammary glands was confirmed by H&E staining (Fig. 4c). Furthermore, melanin staining confirmed the presence of the MNPs in the tumour tissue after injection of the probe (Fig. 4d). These results indicate that cRGD-MNPs provide a high signal intensity at the tumour site and could distinguish between normal mammary glands and breast tumours.

In order to further assess the feasibility of using the cRGD-MNPs to detect a tumour, segmented tumour PAI was performed in MMTV-PyVT transgenic mice with breast cancer (Fig. 4e). The fourth and fifth mammary glands of the transgenic mice were divided into four segments (P1–4), and PA imaging showed complete and intense enhancement of each part of the tumour. Ex vivo tissues were imaged using the PAI system, and the tissue signal distribution was consistent with that observed in vivo and correlated with the pathological examination (Fig. S3a). The PA images demonstrated
an improved contrast profile for breast cancer detection with the cRGD-MNPs.

**PAI-guided resection of breast cancer using cRGD-MNPs**

We evaluated cRGD-MNPs-based PAI for tumour detection, delineation, and imaging-guided resection. First, the tumours of MDA-MB-231 tumour-bearing mice were examined by PAI preoperatively. The reconstructed three-dimensional (3D) image was used to show the spatial distribution of the cRGD-MNPs at the tumour site, which provided the general profile of a tumour for the development of a surgical plan (Fig. 5a). From the PA signals of the cRGD-MNPs, the size and position of the MDA-MB-231-tumour could be defined.

Next, we investigated whether the nanoprobes could guide intraoperative tumour resection. In this experiment, MDA-MB-231 tumour-bearing mice underwent consecutive tumour resections two hours post-injection of cRGD-MNPs. Ultrasound (US) images depicted representative subcutaneous transplanted tumours (Fig. 5b). A strong PA signal showed the tumour location before the resection. We then performed partial resection under PAI guidance. The resected tissues for each step are shown in Figure 5b (P1-4). After removal of most of the tumour, the remaining tumour (about 2 mm wide) was still visible by PAI. Finally, the remaining tumour was resected until no obvious PA signal was detected. For comparison, tissues with negative PA signals on the tumour bed (P4) were excised again for subsequent pathology examination. The ex vivo tissue PA signal distribution was consistent with that obtained in vivo (Fig. S3b).

Pathological examination showed that the tumour had been completely removed, and the tissue with a negative PA signal on the tumour bed was muscle tissue (Fig. 5c). To detect the depth of PAI, we covered the tumours with chicken breasts of various thicknesses and found that the maximum imaging depth was up to 5 mm (Fig. 5d). Taken together, PAI can effectively detect tumours, identify residual masses, and guide surgical resection.

**In vivo cRGD-MNPs biosafety**

The in vivo biocompatibility was further evaluated in mice for prolonged durations
(1, 7 or 30 days) after intravenous administration of cRGD-MNPs. Saline served as a negative control. No considerable body weight loss was observed for the cRGD-MNPs and control groups over 30 days, indicating that cRGD-MNPs had no significant side effects in mice (Fig. 6a). The serum biochemistry analysis, which included liver function (ALT, AST) and kidney function (BUN, CR) markers, showed negligible variations between the different groups, indicating no detectable toxicity shortly after or a relatively long time after exposure (Fig. 6b). Although cRGD-MNPs also accumulated in the liver and kidneys, negligible liver and kidney damage was induced. H&E staining of the major organs (heart, liver, spleen, lung and kidney) at different times after intravenous injection showed no significant acute or chronic physiological toxicity compared to the control group, indicating the high histocompatibility of the cRGD-MNPs (Fig. 6c). These results indicate that the cRGD-MNPs have high in vivo biocompatibility.
Discussion

It remains a significant challenge in BCS to acquire clean margins during primary surgery due to the lack of precision in localising tumours and the inaccuracy of tumour excision by visual inspection and tactile feedback. Imaging-guided surgery is gaining increasing importance in the operating room. It can help detect microscopic tumours or residual lesions that are readily missed during surgery and guide intraoperative surgical margin assessment [24]. Thus, this approach could potentially improve patient outcome following oncologic surgery. In the present study, we demonstrated for the first time that the use of cRGD-MNPs as a targeted PA contrast has the potential to locate tumours and offer a 3D reconstruction of the breast cancer for surgical planning. Although the utility of cRGD-MNPs-mediated PAI or photothermal therapy has been previously demonstrated in animals [17], our study further translates these findings to imaging-guided cancer surgery, showing the feasibility of cRGD-MNPs PAI for consecutive imaging-guided resections of the remaining margins of breast cancer.

Melanin is a naturally occurring biopolymer present in many organisms, particularly in the skin and hair. It has a good intrinsic photoacoustic property. By mimicking natural melanin, recent studies have demonstrated that melanin-based nanoparticles could serve as a multimodality nanoplatform for molecular imaging [16, 25, 26]. As in the report of Fan et al [20], we prepared artificial ultrasmall MNPs and modified them by conjugating them with c(RGDfC) peptides. cRGD-MNPs have a high affinity for integrin αvβ3, which is expressed predominantly on neovascular endothelial cells and tumour cells, including breast cancer cells [27, 28]. Consistent with previous findings, our results demonstrated a higher tumour-targeting capability of cRGD-MNPs compared to MNPs using MDA-MB-231 tumour-bearing mice, indicating that cRGD-MNPs could more specifically target breast cancer xenografts, possibly via the binding to integrin αvβ3. Moreover, mammary glands of FVB/N-Tg(MMTV-PyVT)634Mul/J transgenic mice containing carcinoma showed excellent PA intensity enhancement after the administration of cRGD-MNPs compared to the normal mammary gland tissue. These data further indicated that cRGD-MNPs could conveniently serve as a good contrast for breast cancer-specific PAI.
MNPs have been used in diverse biomedical applications, such as imaging, controlled drug release, bioengineering and bioelectronics, antioxidant applications and theranostics [29]. Based on the dramatic in vivo PAI properties of the cRGD-MNPs in this study, we used these MNPs for PAI-guided surgical navigation. To simulate PAI-guided consecutive resections in a small animal model, we sequentially removed breast tumour tissue using the real-time guidance of PAI, which was validated by subsequent histological analysis. In addition, we found that the tumour, when artificially located in deep tissue, could be clearly imaged and discriminated with a depth of up to 5 mm. A previous study reported the application of PAI with endogenous melanin as contrast for the resection of B16 melanoma liver metastasis [30]. Hepatic melanoma in vivo as small as 400 µm could be detected at a depth of up to 7 mm and precisely resected using PAI guidance, demonstrating the advantages of PA (i.e., high resolution, high sensitivity, deep penetration and early detection of hepatic micrometastasis). In a clinical study, PAI-guided pathological evaluation improved the detection rate of metastases compared to the standard protocol in excising sentinel lymph nodes in patients with melanoma (22.9% versus 14.2%) [19]. Taken together with the results of the present study, PAI using melanin or melanin-based nanoparticles could offer a rapid and effective tool for non-invasive detection of small tumour disease of a certain depth.

In addition to melanin nanoparticles, several exogenous contrast agents have been used for photoacoustic surgical navigation in animals, including gold nanoparticles [31, 32], superparamagnetic iron oxide [33], dye-based agents [34, 35], and carbon-based nanomaterials [36]. All these agents could generate non-invasive PA contrast enhancement when stimulated by laser irradiation at specific wavelengths. However, clinical translation of these agents is prohibitive because of biosafety issues, poor biodegradability, low photostability and unclear biocompatibility [37, 38]. In the present study, the biocompatibility and biosafety of cRGD-MNPs were systematically evaluated both in vitro and in vivo. As reported previously, the strengths of melanin-like nanomaterials include good biocompatibility and long-term photostability [39, 40], prompting us to explore their biomedical applications, particularly for in vivo imaging. Our findings demonstrated that cRGD-MNPs represent a promising contrast agent for
further clinical translation.

However, this study has a few limitations. First, only PAI alone was applied to surgical navigation. As a previous study reported [20], MNPs are an active platform to simplify the assembling of different imaging moieties, such as positron emission tomography and magnetic resonance imaging. Thus, complementary use of multimodality imaging is promising not only for accurate tumour imaging but also for guiding tumour resection. Further research efforts should be devoted to precise, targeted tumour multimodality imaging. Second, the current methodology using MNPs as a contrast agent is unsuitable for deep-tissue imaging in the human body. Therefore, further improvements of the imaging agents are needed to increase the imaging depth.

In this report, we demonstrated the feasibility of PA augmented by the systemic delivery of cRGD-MNPs to detect tumours for preoperative 2D or 3D imaging and guide initial and subsequent resections of breast cancer xenografts. With further development and optimisation, PAI using cRGD-MNPs is expected to be evaluated in breast cancer patients for surgical navigation in the near future.
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Declarations

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Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Contributor Information

Jing-Jing Liu conducted experiments and wrote the manuscript. Zun Wang coordinated and performed the study and drafted the manuscript. Li-Ming Nie directed the experimental methods. Yuan-Yuan Zhu, Ge Li and Lin-Ling Lin cultured cells, raised mice and conducted experiments. Min Chen developed the experimental design and directed the study. Guo-Jun Zhang designed and directed the study and finalised the manuscript.

Ethical approval

Approval was obtained from the ethics committee of Xiamen University. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.
Figure Legends

Scheme 1 Workflow of cRGD-MNPs for intraoperative imaging-guided surgery by photoacoustic imaging.

**Fig. 1 Characterisation of cRGD-MNPs.** a Representative transmission electron microscopy images of MNPs (left) and cRGD-MNPs (right), scale bar: 20 nm. b Zeta potential of MNPs, PEG-MNPs and cRGD-MNPs in aqueous solution. c UV-vis-NIR spectra of MNPs and cRGD-MNPs. d PA spectra of MNPs and cRGD-MNPs. e The photoacoustic signal produced by MNPs and cRGD-MNPs was linear at concentrations from 3.75 to 120 μM. Upper panel: the original PA image. f PA signal intensities of cRGD-MNPs in PBS (pH = 7.4) stored for one week. Data are presented as the mean ± SD (n = 3).

**Fig. 2 In vitro uptake and cytotoxicity of cRGD-MNPs.** a PA images and signal intensities of MDA-MB-231 cells incubated with cRGD-MNPs for various times (0, 1, 2, 4 and 8 h). b PA images and signal intensities of MDA-MB-231 cells incubated with cRGD-MNPs at various concentrations (0, 0.125, 0.25, 0.5 and 1 μM). c PA images and signal intensities of MDA-MB-231 cells incubated with 0.5 μM MNPs, PEG-MNPs or cRGD-MNPs for 4 h. PBS was used as a negative control. d PA images and signal intensities of MCF-10A and MDA-MB-231 cells after incubation with cRGD-MNPs for 4 h with or without free RGD blocking. e CLSM images of MCF-10A and MDA-MB-231 cells incubated with Rho-cRGD-MNPs for 4 h with or without free RGD blocking. Scale bar: 20 μm. f MDA-MB-231 cell viability after incubation with MNPs, PEG-MNPs or cRGD-MNPs at gradient concentrations using standard MTS assay. Data are presented as the mean ± SD (n = 3), *** P < 0.001.

**Fig. 3 Tumour-targeting and biodistribution of cRGD-MNPs using an MDA-MB-231 xenograft mouse model.** a In vivo merged PA and US images of MDA-MB-231 tumour-bearing mice at various times (pre, 1, 2, 4 and 12 h) after intravenous injection.
of MNPs or cRGD-MNPs (100 μM, 200 μL). Scale bar: 2 mm. b Quantitative analysis
of PA intensities of tumour sites at the different time points in (a). c Signal-to-
background ratio of the tumour region two hours post-injection of MNPs or cRGD-
MNPs. d The biodistribution of rhodamine-labelled MNPs or cRGD-MNPs in the heart,
liver, spleen, lung, kidney and tumour from MDA-MB-231 tumour-bearing mice two
hours post-injection. e Quantitative analysis of the fluorescence intensities of the tissues
in (d). Data are presented as the mean ± SD (n = 3); *** P < 0.001, * P < 0.05.

Fig. 4 PAI for mammary glands containing spontaneous breast cancer in MMTV-
PyVT transgenic mice. a Representative US and PA images of cRGD-MNPs
accumulation in mice with normal mammary glands or breast cancer. Scale bar: 2 mm.
b PA signal intensities of breast tumours and normal mammary glands in transgenic
mice two hours after tail-vein injection of cRGD-MNPs compared to 0 h. c
Histopathological examination of the tissues from (a). Scale bar: 2 mm. d Fontana–
Masson staining of ex vivo tumour tissue. Black particles representing the MNPs were
not observed in the non-injected nanoprobe group (left). Black particles representing
the cRGD-MNPs were observed in the injection probe group (right). The image in the
lower right corner (black square) is an enlarged image of the upper left corner area.
Scale bar: 200 μm. e US (top), PA (middle) and histological (bottom) images of the 4th
and 5th pairs of mammary glands in an 8-week-old MMTV-PyVT mouse. The enlarged
mammary glands and different regional tissue (P1–4) are outlined with dotted orange
and green lines, respectively. The dotted blue line outlines the tissue inferior to the
mammary gland. Scale bar: 2 mm. Data are presented as the mean ± SD (n = 3); * P <
0.05.

Fig. 5 PAI-guided tumour resection in MDA-MB-231 tumour-bearing mice. a
Render, axial, sagittal and coronal images showing the tumour distribution. Scale bar:
2 mm. b Anatomical US (top) and PA (bottom) images showing the tumour region
(dotted orange line) in MDA-MB-231 tumour-bearing mice in vivo. The tissue portion
to be resected (P1-4) is highlighted with a green dashed circle. Scale bar: 2 mm. e
Histological images (top) of resected tissue pieces. Scale bar: 2 mm. Enlarged images of regions marked with black boxes are also shown (bottom). Scale bar: 100 μm. d PA and US images of tumour tissues covered by different thicknesses of chicken breast under 680 nm laser excitation in vitro. Scale bar: 2 mm.

**Fig. 6** The biosafety of cRGD-MNPs. a Body weight curves for mice treated intravenously with saline (control) or cRGD-MNPs. b Serum biochemistry results (liver function and renal function) for BALB/c mice at various time points (1, 7 and 30 days) after intravenous injection of cRGD-MNPs or saline (controls). The units of ALT and AST are U/L; the units of BUN and CR are mmol/L and μmol/L, respectively. c H&E staining of vital organs (heart, liver, spleen, lung and kidney) from each group. Scale bar: 200 μm. Data are presented as the mean ± SD (n = 5).