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BIOENGINEERED EXTRACELLULAR VESICLES AS NEXT-GENERATION BIOINSPIRED OPTOACOUSTIC CONTRAST AGENTS

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Abstract

Near-infrared (NIR) optoacoustic (OptA) contrast agents are still dominated by synthetic organic and inorganic materials. Although these agents can generate strong signals, their broader preclinical and clinical use is often limited by concerns over toxicity, poor biocompatibility, and high manufacturing costs. Extracellular vesicles (EVs) offer a promising alternative because they are naturally derived, biocompatible, can be produced in bulk, and exhibit low immunogenicity. Most reported EVs-based contrast agents, however, are developed by loading EVs with synthetic dyes or nanoparticles, which may compromise vesicle integrity, reduce targeting performance, and introduce batch-to-batch variability. In this study, we present a fully biological strategy for optoacoustic imaging by developing genetically modified EVs (GMEVs) from bioengineered mammalian cells. These vesicles are simple to produce and purify, cost-effective, and show strong optoacoustic performance from deep tissue. We further incorporated RVG peptides to support blood-brain barrier crossing after intravenous administration. In a murine brain tumor model, GMEVs enabled clear tumor

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visualisation following systemic delivery, likely due to enhanced tumor accumulation. Importantly, the vesicles were well tolerated in vivo, supporting their potential as bioinspired, bioengineered, and biocompatible NIR optoacoustic contrast agents for in vivo imaging.

Keywords: *Biocompatible, Contrast agents, Extracellular vesicles, Optoacoustics, Photoacoustics*

1. Introduction

Current NIR OptA contrast agents are primarily composed of synthetic materials, including organic and inorganic compounds such as small-molecule dyes (e.g. Cyanine and BODIPY), gold, silver, and quantum dots [1],[2],[3]. Although effective as contrast enhancers, these agents often raise concerns regarding bulk cost-effective synthesis, toxicity, and limited biocompatibility, which hinder their clinical translation. In contrast, bioengineering approaches offer naturally biocompatible, low-immunogenic platforms, such as EVs, which can be produced at scale in a cost-effective manner [4],[5],[6]. Over the past decade, researchers have shown keen interest in developing bioengineered cell-derived vesicles as contrast agents and carrier systems [7],[8],[9]. Bioengineering technologies now provide robust and scalable manufacturing platforms for engineered EVs, helping to address the growing demands of both basic research and commercial applications.

Various strategies have been explored for developing EV-based NIR contrast agents, including labelling EVs with organic dyes or inorganic nanoparticles, conjugation, electroporation, sonication, and extrusion to label or load EVs with synthetic agents [5],[10],[11],[12],[13]. However, these approaches are often constrained by issues that can compromise EV integrity, reduce targeting efficiency, and lead to variability in product consistency. Alternatively, engineering EVs to express imaging proteins offers a fully biological approach for synthetic or hybrid NIR contrast agents. However, this approach remains largely underexplored in NIR optoacoustic imaging applications.

We hypothesized that GMEVs can package NIR imaging proteins and a biological barrier-penetrating peptide to create a bioinspired, biocompatible contrast agent (GMEVs) for effective deep-tissue OptA imaging. For the first time, we demonstrate that genetically modified GMEVs derived from bioengineered mammalian cells can serve as bioinspired NIR OptA contrast agents. These GMEVs can be produced rapidly and cost-effectively, are easy to purify, and exhibit strong OptA performance, making them a promising platform for deep-tissue OptA imaging applications. We have developed GMEVs that express NIR imaging protein- miRFP for deep-tissue visualization and RVG peptides to enable blood-brain barrier (BBB) penetration upon intravenous administration. Leveraging the enhanced permeability and retention (EPR) effect, our GMEVs enabled precise tumor visualization in murine brains. These GMEVs, derived from mammalian cells, are biocompatible and well tolerated after systemic administration. In the future, it will be possible to express additional NIR proteins alongside therapeutic proteins for specific biological applications.

2. Materials and Methods:

Cells: HEK293 cells used for transfection and EV isolation were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma #D5796) supplemented with 10% exosome-depleted fetal bovine serum (FBS) (Thermo Scientific #2720801). The 4T1 cells (ATCC, order nr. CRL2539) used for orthotopic brain tumor implantation in mice were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, Massachusetts, USA). All cells were grown in a 37°C incubator with 5% CO2. All culture media were supplemented with antibiotics (penicillin (100 U/ml) and streptomycin (100 mg/ml)).

Mice and brain tumor model: All procedures involving animal experiments were approved by the Government of Upper Bavaria and were performed in accordance with the institutional guidelines and in compliance with the ARRIVE guidelines. In this study female Crl:NU-Foxn1nu mice were used as an orthotopic brain tumor model for optoacoustic MSOT imaging. Orthotopic brain tumor models were prepared by implanting 2.5 × 10 ^5 4T1 cells in 3 µl PBS into the mouse striatum (bregma +1.0 mm, left lateral 2.0 mm, and depth 3.0 mm) (N. Liu et. al., 2021) [14]. Brain imaging was conducted ten days after implantation.

Plasmid constructs: A customized DNA construct (Fig 1A) containing the signal peptide (SP), RVG (Rabies Virus Glycoprotein) peptide, LAMP2b (EV marker protein) [15] and miRFP720 (fluorescence tag) was codon optimized and commercially purchased from Life Technologies GmbH Thermofisher Scientific (GeneArt Strings (TM) DNA Fragment). The

commercial DNA constructs were PCR amplified with forward primers (5' AAAAAGCTTGCCACCATGGTCTG 3') and reverse primer (5' GGATATCTGCAGAATTCTCAGTGGTGGTGGT GGTGGTGTTCTTCCATCAC 3') containing His-tag at the 3' end. The PCR product was gel purified and cloned downstream of the CMV promoter into a pcDNA3.0(+) vector linearized by restriction digestion with HindIII and EcoRI via Gibson Assembly Master Mix (New England Biolabs). Negative control plasmid clone lacking the RVG peptide sequence were generated by the same cloning strategy using the forward primers (5' GCCACCTGTCTGTACGGCAGCGGCGCTAAAT GGCAG 3') and the reverse primer (5' GGATATCTGCAGAATTCTCAGTGGTGGTGGT GGTGGTGTTCTTCCATCAC 3') containing His-tag at the 3' end. All the plasmid constructs were confirmed by DNA sequencing.

HEK293 cells transfection and EV isolation from the conditioned medium: HEK293 cells were cultured in DMEM supplemented with 10% exosome depleted FBS and antibiotics in a T175 flask until 75-80% confluency. The confluent HEK 293 cells were washed with Dulbecco's Phosphate Buffer Saline (D-PBS) (Sigma #D8537) and transfected with plasmid constructs using Lipofectamine 2000 reagent using the manufacturer protocol (Invitrogen #11668027). 4 to 5 hrs after the transfection the cells were washed again with D-PBS and DMEM supplemented with 10% exosome depleted FBS was added to the flask. After 72 hrs, the cell-conditioned medium was collected for EVs isolation using the differential centrifugation method as described in Annamaria et al. 2021 [16]. Briefly, the collected cell-conditioned medium was spun at 300xg at 4°C to remove the cell debris. The supernatant was then centrifuged for 30 min at 10,000xg at 4°C to remove other large vesicles and remaining cell debris. The supernatant was ultracentrifuged for 1,10,000xg at 4°C to collect the EVs. The collected EVs were resuspended in 200 µl PBS, and all the downstream analyses were performed on freshly isolated EVs. The yield of EVs was approximately 1.0-1.5 µg/ul (based on Pierce™ BCA Protein Assay Kits) per T175 flask.

Multispectral optoacoustic tomography (MSOT) imaging: Optoacoustic property of the isolated GMEVs was characterised by MSOT, ((MSOT256-TF, iThera Medical GmbH, Munich, Germany) using a tissue mimicking agar phantom as described in N. Liu et. al., 2021 [14]. The GMEVs and transfected HEK 293 cells were filled in a thin tube and inserted into the tissue-mimicking phantoms for optoacoustic spectral measurement using an MSOT. The MSOT scanning was performed from 680 to 760 nm with a step size of 5 nm and 10 signal averages. For *in vivo* imaging, mice bearing brain tumors were anesthetized by 1% isoflurane delivered via a nose cone, and 150 µL of RVG-miRFP EVs or No RVG-miRFP EVs were injected via the tail vein. *In vivo* optoacoustic scanning images were acquired before EVs injection and 48 hrs after EVs injection. Image reconstruction, unmixing, and analysis were done as described in N. Liu et. al., 2021 [14].

Biosafety assessment of genetically modified extracellular vesicles (GMEVs): To study the effect of GMEVs on blood biochemistry and haematology parameters, we used C57BL/6 mice. Mice were randomly divided into 5 groups with 3 mice in each group. The control group was intravenously injected with 100 µl of PBS buffer, and the other 4 group of mice were intravenously injected with 300 µg of GMEVs in 100 µl PBS. After injection, blood was collected from each group (PBS, RVG GMEVs, No-RVG GMEVs) on day 1 after the injection. Blood was sampled from groups 3 and 4 on day 7 after injection, respectively. Whole blood samples were collected in EDTA tubes for full blood count analysis. To assess clinical chemistry parameters, whole blood was collected in Li-heparin tubes and centrifuged at 5000 g for 10 mins at room temperature to collect plasma. The analyses were conducted using an AU480 analyzer from Beckman Coulter.

3. Results and Discussion:

To obtain genetically modified EVs (GMEVs), we designed a plasmid construct. This plasmid construct was designed to expresses a fusion protein containing a signal peptide (SP), Lamp2B as a unique protein marker present in EVs, miRFP720, a fluorescence protein which generates a strong optoacoustic (OptA) signal spectrum in the near-infrared (NIR) window, RVG peptide for a biological application in brain tumor imaging, and a His-tag to confirm that this fusion protein is loaded in the GMEVs **(Fig 1A)**. Another plasmid construct was designed to express all the

above-mentioned peptides and proteins except for RVG peptide, which served as a negative control in the *in vivo* tumor imaging using MSOT. This plasmid construct was transfected into the HEK293 cells and the GMEVs were isolated using differential ultracentrifugation method. The isolated GMEVs were further characterized using WB, TEM, and DLS **(Fig. 1B, C)**. The WB from the GMEVs with and without RVG peptide showed nice bands when probed with anti-HA-tag antibody which suggests that the isolated EVs are successfully loaded with the fusion protein construct. Other EVs marker proteins, such asLAMP2b, TSG101, and CD81, were detected using the respective antibodies.

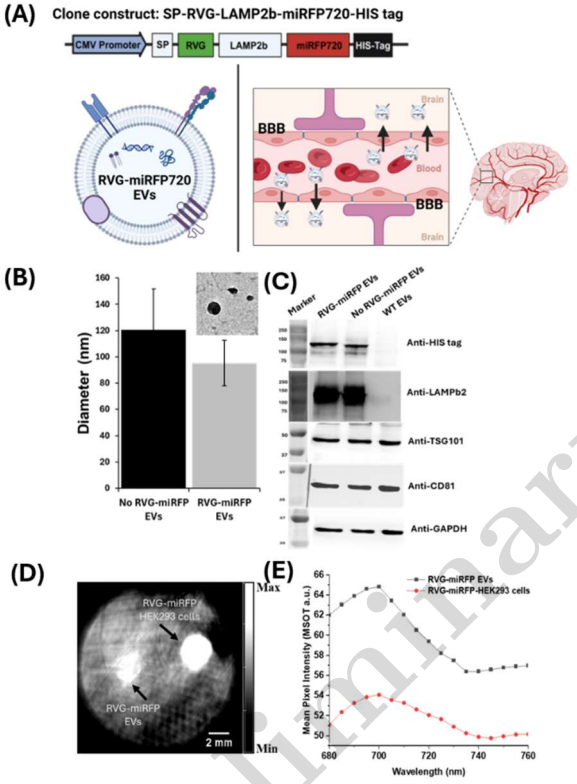

Figure 1. Schematic and characterization of EVs. (A) Schematic diagrams illustrating cloning strategy, (B) Dynamic light scattering (DLS) determined the diameters of EVs and Representative Transmission Electron micrographs (TEM), (C) Western blot (WB) characterization of EVs, (D) Optoacoustic imaging of GMEVs and transfected HEK293 cells in a tissue-mimicking agar phantom using MSOT, (E) Optoacoustic spectra of modified EVs and cells.

The typical structure of the EVs was seen using TEM **(Fig 1B)**. The DLS analyses showed the diameter of the isolated EVs was in range of 100 nm to 120 nm **(Fig 1B)**, this diameter of the GMEVs is optimal for intravenous injections and enhanced permeability via leaky blood vessels in tumors. After the biochemical and structural characterization of the GMEVs, we performed MSOT analysis using tissue-mimicking agar cylindrical phantoms to study the optoacoustic properties of the GMEVs **(Fig 1D)**. For this purpose, we used the HEK293 cells transfected with RVG-Lamp2b-miRFP720-His tag fusion plasmid construct as a positive control. The OptA imaging of the HEK293 cells and the GMEVs carrying the RVG-Lamp2b-miRFP720-His tag fusion protein using MSOT showed a high optical absorbance with a pronounced signal maximum at approximately 700 nm in both, the transfected cells and in the GMEVs **(Fig 1D, E)**. This finding confirmed the presence of the miRFP720 fluorescence tag in the GMEVs and suggests that this EVs could be used for the *in vivo* MSOT OptA imaging. Wild-type or control-non-transfected HEK 293 cells or EVs isolated from the conditioned medium of these cells did not show any absorption at 700 nm.

Next, we evaluated the optoacoustic imaging potential of bioengineered and bioinspired GMEVs *in vivo* using MSOT in a metastatic tumor mouse model. The metastatic tumor model was developed by intracranial implantation of 4T1 tumor cells. 10 days after the tumor implantation, mice were injected intravenously with GMEVs carrying RVG along with the miRFP or with GMEVs carrying no RVG but miRFP as a negative control and imaged 48 hours after injection using MSOT. **Figure 2 A, B** indicate a very strong OptA signal that was recorded in the mouse brain, mainly from the tumor region, due to RVG-miRFPEVs. These results indicate that RVG-miRFPEVs generate strong OptA signals at depth, which can be used for deep-tissue OptA imaging, including brain tumors. The e*x-vivo* fluorescence imaging and histology further confirmed that the OptA signal is specific to the brain tumor and localized GMEVs **(Fig 2C)**. *In vivo* biosafety and biocompatibility of the GMEVs was also evaluated through blood biochemical analyses and hematology. The results from C57BL/6 mice were analyzed on day 1, and 7 days after the GMEVs injection. PBS injection served as control. GMEVs administration in mice caused no significant alterations in blood biochemistry parameters, indicating no detectable systemic toxicity under the tested conditions. Although these preliminary results in mice are encouraging, further

blood chemistry and safety profile analyses in higher animals will be required.

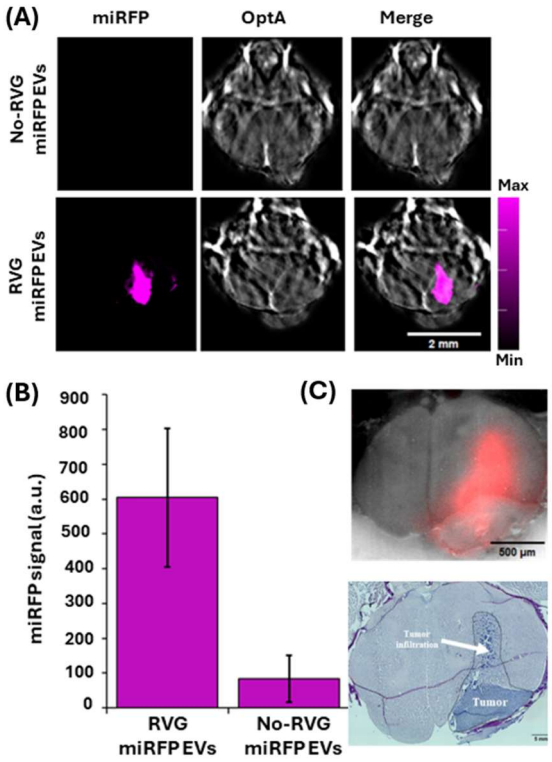

Figure 2. *In vivo* optoacoustic imaging and tissue analysis. (A) MSOT visualisation of GMEVs in 4T1 orthotopic brain tumors, (B) Quantification of miRFP signal within the brain tumor using ImageJ, (C) Cryo-sliced image of the brain indicating fluorescence due to accumulated GMEVs (Top), and H&E staining indicating the tumor region in the brain (white arrow, Bottom).

4. Conclusion

There is a growing need for bioinspired contrast agents suitable for deep-tissue *in vivo* optoacoustic imaging in the NIR range. Our reported bioengineered GMEVs offer a promising strategy to generate biologically derived, scalable NIR imaging agents with optimal optoacoustic properties and favourable *in vivo* safety profiles. These agents may serve as an attractive alternative to synthetic agents, including organic and inorganic contrast enhancers, whose clinical translation is often limited by challenges in scalability, biocompatibility, and long-term safety.

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Disclosures

V.N. is a founder and equity owner of Maurus OY, sThesis GmbH, Spear UG, Biosense Innovations P.C. and I3 Inc.

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