Angiogenesis Imaging of Tumor Xenografts by $\alpha_v\beta_3$-Targeted, Dual-Modality Micellar Nanoprobes

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Statement of Purpose: One essential requirement for solid tumor growth is the ability to acquire an adequate blood supply; therefore, tumor vasculature is in a perpetual state of angiogenesis. Integrin $\alpha_v\beta_3$ is a well-established biomarker for angiogenesis with overexpression in tumor vasculature and low to no expression in resting endothelial cells.[1] Non-invasive imaging of tumor angiogenesis is important for early diagnosis of cancer as well as post-therapy assessment.[2,3] Here we describe the development of a dual modality, $\alpha_v\beta_3$ integrin specific fluorescent superparamagnetic polymeric micelle (FSPPM, Figure 1A) that incorporates magnetic resonance imaging (MRI) ultrasensitive contrast and fluorescence into one nanoprobe platform for the in vivo imaging of tumor angiogenesis.

Methods: Methoxy poly(ethylene glycol)-b-poly(D,L lactic acid) (MeO-PEG-PLA) and maleimide-terminated PEG-PLA copolymers were synthesized via ring opening polymerization and used in combination with MeO-PEG-PLA copolymers conjugated with a fluorescent probe, tetramethylrhodamine (TMR, red fluorescence), PEG-PLA-TMR, for micelle formation. Superparamagnetic iron oxide (SPIO) nanoparticles were utilized, and micelles were formed by the solvent evaporation method. The FSPPM were conjugated with cRGD, a targeting ligand for $\alpha_v\beta_3$, or cRAD, non-targeted ligand, by maleimide chemistry. Cellular uptake and targeting specificity was tested in SLK cells, which overexpress $\alpha_v\beta_3$ integrin and facilitate receptor-mediated endocytosis.[4] For in vivo studies, A549 non-small cell lung cancer cells, were injected subcutaneously into the flank of athymic nude mice. MR images were collected using a 3D gradient echo sequence, on a 7 Tesla Varian small animal MRI, including pre- and post-injection (4 hr) images of targeted (cRGD-), or non-targeted (cRAD-) FSPPM (6mg/kg Fe). ImageJ and ITK-Snap were used to display and generate 3D images, respectively.

Results: PEG-PLA-TMR blended with non-fluorescent polymers at 20% (w/w%) was found to have the highest fluorescence on a per micelle basis, and was used for the remainder of the study. Loading of SPIO (9.9 ± 0.4 nm) at a density of 20% yielded FSPPM with mean diameters of 50-60 nm. In vitro studies of the endocytosis pathways used by the FSPPM showed that Genistein, a small molecule inhibitor for caveolae-mediated endocytosis, blocked the uptake of FSPPM by 50%, whereas, Chlorpromazine, an inhibitor for clathrin-mediated endocytosis, did not block uptake of FSPPM (Figure1B). Specific targeting of $\alpha_v\beta_3$ and cellular uptake was analyzed by confocal laser scanning microscopy (CLSM) and showed a significant increase in cellular uptake in cells treated with cRGD-FSPPM compared to cells treated with non-targeted, cRAD-FSPPM. A competitive control of free cRGD peptide co-incubated with cRGD-FSPPM was also analyzed. Prussian blue staining of the cells confirmed the presence of SPIO. Wheat germ agglutinin

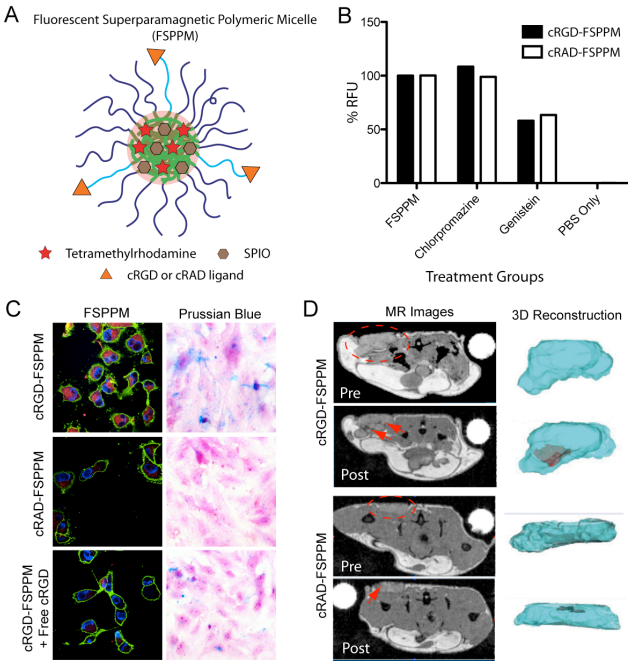

Figure 1. (A) Scheme of a FSPPM nanoparticle. (B) FSPPM uptake in the presence of endocytosis inhibitors chlorpromazine and genistein. (C) In vitro cellular uptake of cRGD-FSPPM using CLSM: red – FSPPM, green –WGA, blue- DAPI. (D) T_2*-weighted MR images of mice pre and post-injection of cRGD- (top pair) or cRAD-FSPPM (bottom pair). Dashed circle delineates tumor and red arrows show darkened areas due to FSPPM accumulation. 3D reconstruction of the MRI data sets show the tumor volume (teal) and areas of darkening (red) overlaid.

(WGA, green) was used to delineate the cell boundary and verify cellular internalization (Figure 1C). We also evaluated the in vivo MR imaging specificity by intravenous injection of cRGD- and cRAD-FSPPM in mice bearing A549 tumor xenograft. Pre- and post-injection images show a greater darkening in the tumor tissue in cRGD-FSPPM treated animals. 3D reconstruction of the tumor tissue was overlaid with the areas of darkening due to FSPPM accumulation. The distribution of the cRGD-FSPPM showed a branched network of darkening throughout the tumor, whereas cRAD-FSPPM treated tumors showed accumulation in the periphery of the tumor (Figure 1D). Immunofluorescence and Prussian blue staining showed targeting of $\alpha_v\beta_3$ integrin by co-localization with cRGD-FSPPM.

Conclusions: This study describes the development of $\alpha_v\beta_3$ specific dual modality FSPPM that are sensitive to a wide spectrum of fluorescent imaging modalities and MRI. cRGD-FSPPM showed specific targeting in vitro and in vivo, and allowed for non-invasive imaging of tumor angiogenesis.

References: 1. Brooks PC, et al. Science 1994. 2. Weissleder R, et al. Nature 2008. 3. Cai W, et al. J Nucl Med 2008. 4. Nasongkla N, et al. Nano Lett 2006.