In Vivo Photoacoustic Lifetime Based Oxygen Imaging with Tumor Targeted G2 Polyacrylamide Nanosonophores

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Supporting Information

G2-PAA NP characterization

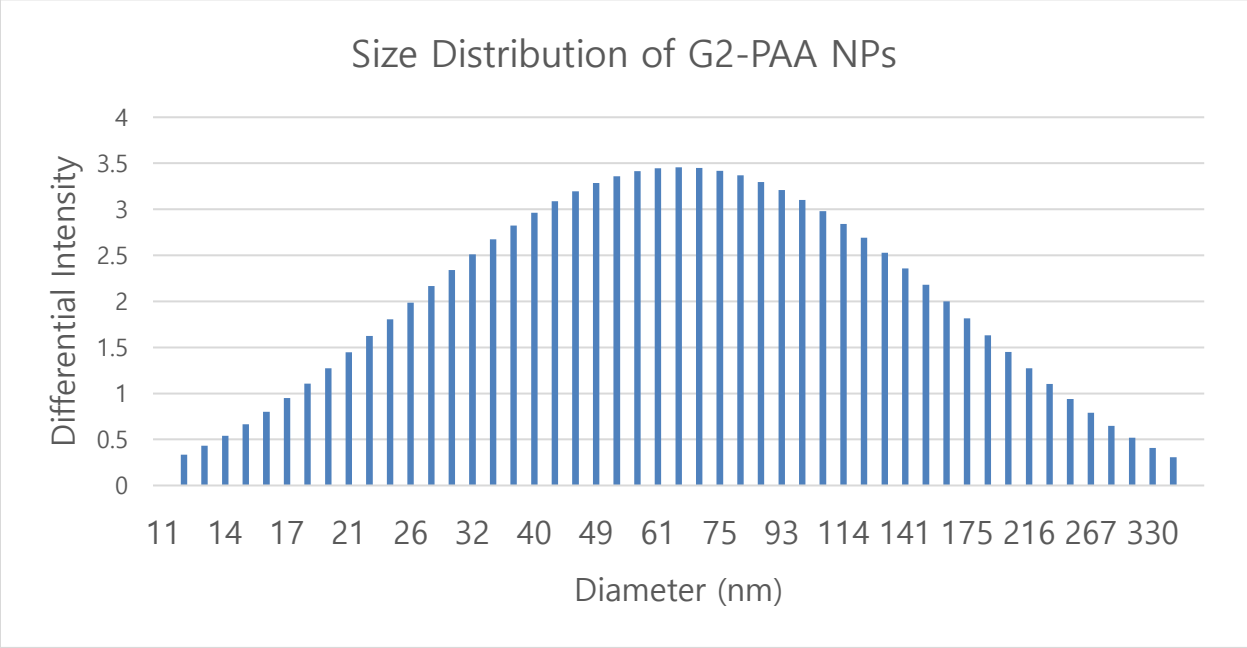

Figure S1. Hydrodynamic size measurements of G2-PAA NP using Dynamic Light Scattering (DLS). The average NP diameter was ~60nm, and the polydispersity index was 0.28.

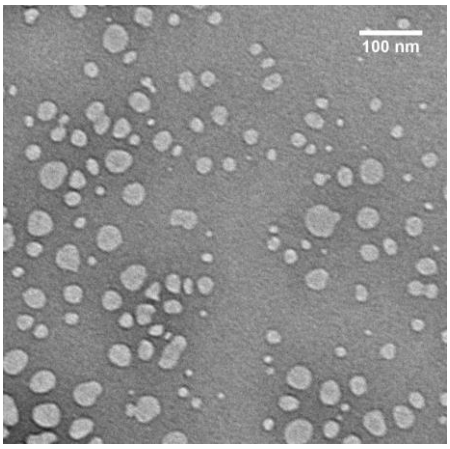

Figure S2. TEM image of G2-PAA NPs.

The dried G2-PAA NPs can be stored at -20℃ for several months. Aqueous G2-PAA NPs are always freshly prepared prior to in vivo experiments. Our G2-PAA NPs are cross-linked by ester

bonds (AHM), which are expected to be cleaved by esterase presented in body in the long term. Polyacrylamide NPs are prepared via free-radical polymerization of monomers and a cross-linker within reverse micelles (aqueous droplets in hexane). G2-modified polyacrylamide NPs are composed of three major monomers (acrylamide (AA), 3-(Aminopropyl) methacrylamide hydrochloride salt (APMA), and 3-(acryloyloxy)-2-hydroxypropyl methacrylate (AHM)) and acrylated G2 dyes. AA is the base unit of polyacrylamide NPs, and APMA (~3% molar ratio) is added to grant primary amine groups in the polyacrylamide NPs. AHM is the cross-linker, which cross-links linear polyacrylamide chains randomly within the micelles. Acrylated G2 dyes are prepared in situ through carbodiimide chemistry (NHS/EDC) with APMA prior to radical polymerization. Each oxyphor G2 dye has 16 carboxyl acid groups, which can readily be conjugated to primary amine groups of co-monomer APMA.[1]

Free-radical polymerization is induced via addition of ammonium persulfate (APS, radical source) and tetramethylethylenediamine (TEMED, radical polymerization catalyst) under constant stirring. The size of the NPs is dictated by the size of the reverse micelles formed prior to polymerizations. Any unreacted monomers and surfactants are thoroughly washed using Amicon filtration system (size exclusion, 300 kDa cutoff) both with ethanol and water (unreacted monomers, dyes, and surfactants are much smaller than 300 kDa). As the NPs are prepared within the reverse micelles, they typically form spherical shapes. Previous studies have demonstrated characterization of blank polyacrylamide NPs through NMR showing simply monomer peak disappearance during purification steps.[2] We note that any un-polymerized or under-polymerized substances are secreted during the size exclusion purification step (cutoff 300 kDa). Any over-sized NPs are excluded by using conventional syringe filters (0.45 μm).

To the bare G2-PAA NPs, we first modify the surface with bifunctional polyethylene glycol (NHS-PEG-MAL, 2 kDa). Primary amine groups on the bare G2-PAA NPs from APMA content conjugates to NHS-PEG end under neutral pH at room temperature. PEG-MAL (maleimide) end undergoes chemical conjugation with thiol groups presented in cysteine end in the F3-peptides under neutral pH at room temperature.

Although the exact morphology of our polyacrylamide NPs is undetermined, we believe it to have a sponge-like morphology, as polyacrylamide is a hydrogel (its size differs under dry and wet conditions). The overall size of the NPs is dictated by the size of the reverse micelles formed prior to polymerizations. The sizes of NPs are ~60 nm and relatively narrow with polydispersity index (PDI), 0.28 for G2-PAA NPs, when measured by DLS (Figure S1). TEM images show average size of ~27 nm (Figure S2). We note that the TEM images are taken under dried out conditions. The G2-PAA NPs are composed of polyacrylamide, which is a type of hydrogels; thus, it is known to swell under aqueous conditions (i.e. ~27 nm measured by TEM and ~60 nm measured by DLS).

NP toxicity was assessed with an MTT assay (Figure S3). HeLa cells were plated in 24 well plates at a density of 25,000 cells/well. After 1 day of growth, different concentrations of G2-PAA NP

were added to the wells and the cells were incubated for another 24 hours. Following the incubation, cell media was replaced with MTT reagent and incubated for 4 hours. Formazan crystals were then dissolved in DMSO and their absorption measured and compared to a control that received no N P. The G2-PAA NP showed minimal toxicity up to 5 mg/ml NP concentrations.

We hypothesized that the NP accumulation rate would be similar to our previous report as the NP composition is almost identical except for the dye (Oxyphor G2 in this work and SNARF 5F for our earlier work).[3] The NP accumulated peaked at ~75 min after tail vein injection then decayed over time. As the temporal resolution of the current PALT imaging system is slow (typically on the order of 20 min), we started taking PALT images 60 min after tail vein injection.

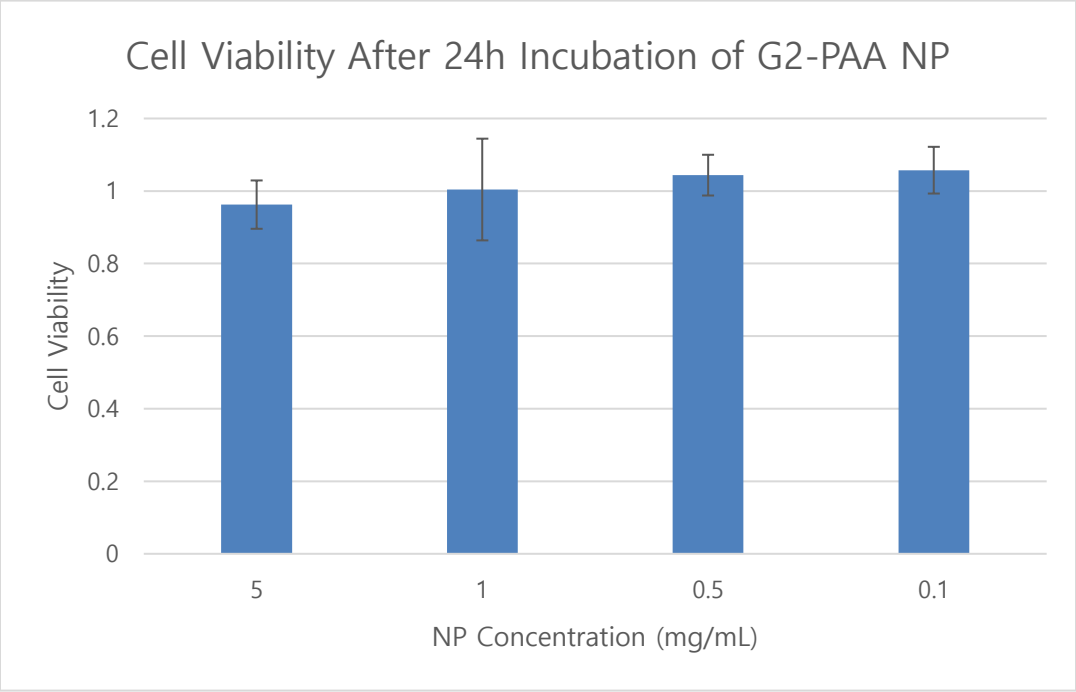

Figure S3. Cell viability, assessed via MTT assay, after 24-hour incubations with different NP concentrations.

To confirm polymerization of the nanoparticles, ^{1}H NMR spectra of monomers (acrylamide, APMA, and AHM in the ratio; 9.7 mol:0.3 mol:2 mol) and blank polyacrylamide nanoparticles were taken (Figure S4). The ^{1}H NMR peaks residing at from around 5 to 7 ppm correspond to vinyl groups (R-C***H***=C***H*****$_2$**) from acrylamide (acrylamide and APMA) and acrylate (AHM) (Figure S4a). During radical polymerization, vinyl groups are polymerized (becoming R-C***H***-C***H***-R). As shown in Figure S4b, vinyl groups' NMR peaks have completely disappeared in the polyacrylamide nanoparticles indicating that all monomers have successfully either polymerized or washed out during purifications.

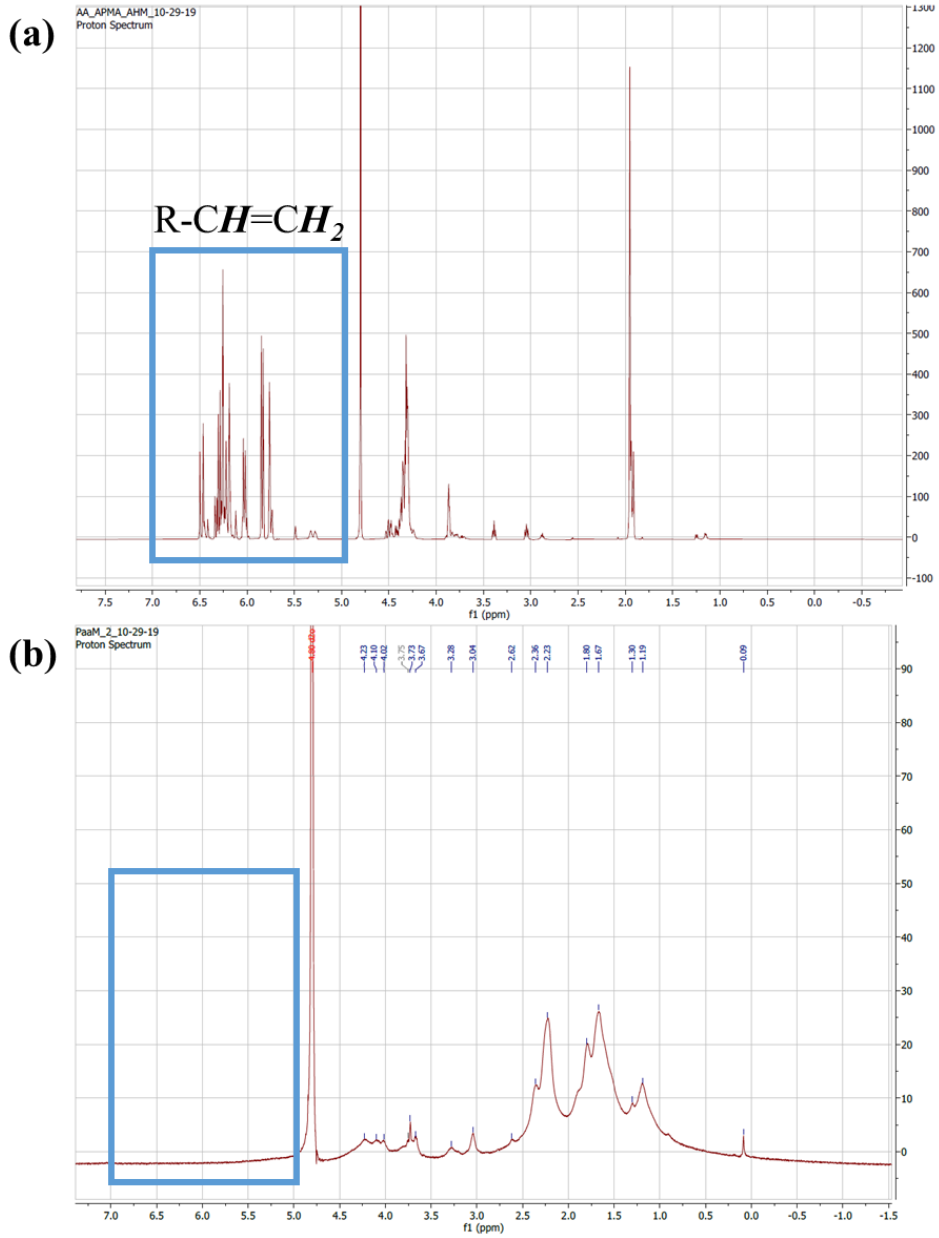

Figure S4. ^{1}H NMR spectra of (a) the monomers (acrylamide, APMA, and AHM in the ratio; 9.7 mol: 0.3 mol:2 mol) and (b) the blank PAA NP dissolved in D_2O.

Table S1 shows the measured zeta-potential of blank PAAm NP after various stages of conjugation to F3 peptides (we note that oxyphor G2 contains 16 carboxylic acid groups, which may interfere the zeta potential measurements.). We expect the blank NP to be positively charged due to the presence of primary amines (21.5 mV). As expected, initial PEGylation of PAAm NP slightly reduced the surface charge as PEG is known to be neutrally charged (14.1 mV). After F3-peptide conjugation, the surface charge slightly increased back to 21.3 mV as F3-peptide contains more positively charged amino acids.

Table S1. Measured zeta-potentials for blank PAAm NPs after each stage of conjugation to F3 peptides.

Particle Formulation	Unmodified PAAm	PAAm + PEG	PAAm + PEG + F3
Zeta-potential (mV)	21.5	14.1	21.3

References

1. Lee, Y.-E. K.; Ulbrich, E. E.; Kim, G.; Hah, H.; Strollo, C.; Fan, W.; Gurjar, R.; Koo, S.; Kopelman, R., Near Infrared Luminescent Oxygen Nanosensors with Nanoparticle Matrix Tailored Sensitivity. *Anal. Chem.* **2010,** *82*, 8446-8455.
2. Gupta, A.; Wang, S.; Marko, A.; Joshi, P.; Ethirajan, M.; Chen, Y.; Yao, R.; Sajjad, M.; Kopelman, R.; Pandey, R. K., Polyacrylamide-Based Biocompatible Nanoplatform Enhances The Tumor Uptake, PET/Fluorescence Imaging and Anticancer Activity of A Chlorophyll Analog. *Theranostics* **2014,** *4*, 614-628.
3. Jo, J.; Lee, C. H.; Kopelman, R.; Wang, X., *In Vivo* Quantitative Imaging of Tumor pH by Nanosonophore Assisted Multispectral Photoacoustic Imaging. *Nat. Commun.* **2017,** *8*, 471.