Title: Dynamic Biomolecular Reporters for Ultrasound Imaging of Protease Activity

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Background, motivation and objective:

A major goal of biomolecular ultrasound is to develop contrast agents that can dynamically sense physiologically relevant molecules or their activity within living cells. Gas vesicles (GVs) – genetically encoded gas-filled protein nanostructures – are a promising candidate for the development of such contrast agents due to their ability to be engineered at the genetic level and expressed heterologously as genetic reporters (Bourdeau et al. *Nature*, 2018; Lakshmanan et al., *ACS Nano*, 2016). Building on these capabilities, we set out to engineer 'dynamic GVs' (dGVs) that change their non-linear ultrasound contrast dynamically in response to the activity of proteases: an important class of enzymes underlying cellular homeostasis and disease processes, and a target of drug discovery.

Statement of Contribution/Methods:

We engineered dGVs that produce enhanced non-linear acoustic signals upon sensing the activity of three types of proteases **(Fig. 1A)**. Genetic modification of the GV shell protein 'GvpC', by incorporation of a protease recognition motif or degradation tag, enables its subsequent cleavage or degradation by the protease in reconstituted cell-free systems *in vitro*, in probiotic *E. coli* Nissle cells and *in vivo* (**Fig 1A-C**). This dynamic weakening or removal of GvpC causes the GV shell to become less stiff, undergo non-linear buckling in response to ultrasound, and produce enhanced contrast under amplitude modulation imaging. Ultrasound imaging of purified dGVs, cells expressing dGVs and mice injected with dGVs was performed at 15.625 MHz using a cross-amplitude modulation (xAM) pulse sequence.

Result/Discussion:

dGVs showed significant enhancement in non-linear contrast upon sensing protease activity *in vitro* and inside bacteria **(Fig. 1D, E)**. Furthermore, intracellular non-linear contrast could be 'tuned-down' dynamically by expression of non-degradable GvpC upon addition of a chemical inducer **(Fig. 1F)**. Harmonic dGV-expressing bacteria could also be acoustically distinguished from bacteria expressing normal, non-degradable GVs inside the colon of live mice **(Fig. 1G)**. These results show that GVs can be engineered as dynamic biomolecular sensors for ultrasound, extending the capabilities of this modality into an important area of biological imaging.

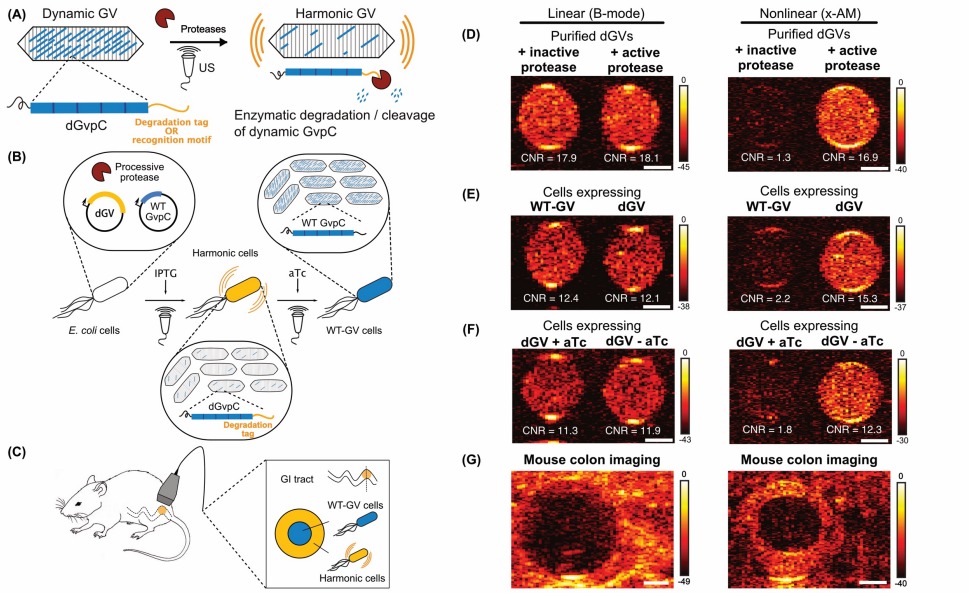

Fig.1 (A) Schematic illustration of dynamic gas vesicles (dGVs) respond to proteases and produce enhanced nonlinear ultrasound signal in reconstituted cell-free systems. **(B)** Schematic illustration of nonlinear contrast produced by dGV expressing probiotics *E. Coli* Nissle cells and eliminated by expression of non-degradable GvpC upon addition of anhydrotetracycline (aTc). **(C)** Schematic illustration of mouse colon imaging with dGV expressing cells and wild type GV expressing cells. Ultrasound image acquired at 15.625 MHz using B-mode and a custom cross-amplitude modulation pulse sequence (xAM) for **(D)** dGVs in reconstituted cell-free system *in vitro*, **(E)** dGV expressing cells and WT-GV expressing cells, **(F)** dGV expressing cells with and without aTc, **(G)** mouse colon imaging *in vivo*. Scale bars: 1 mm.