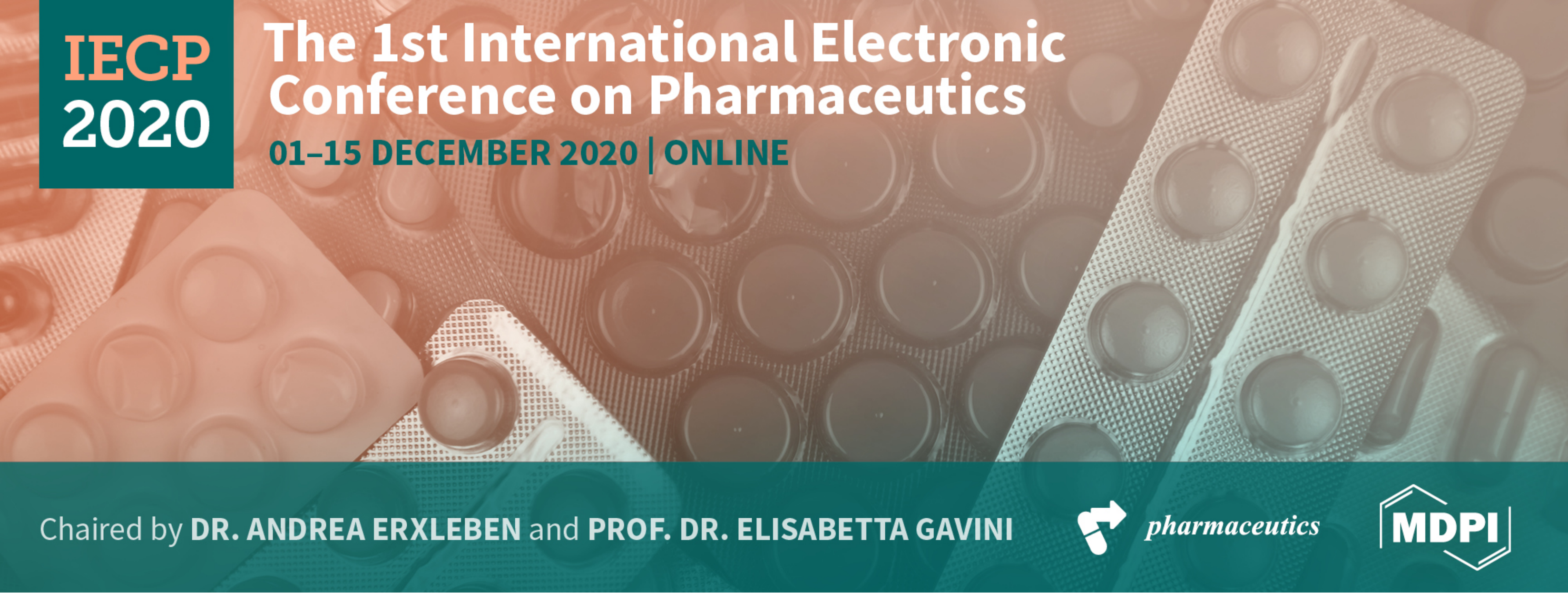

Development of a Nanodroplet formulation for triggered release of BIO for bone fracture healing

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Abstract

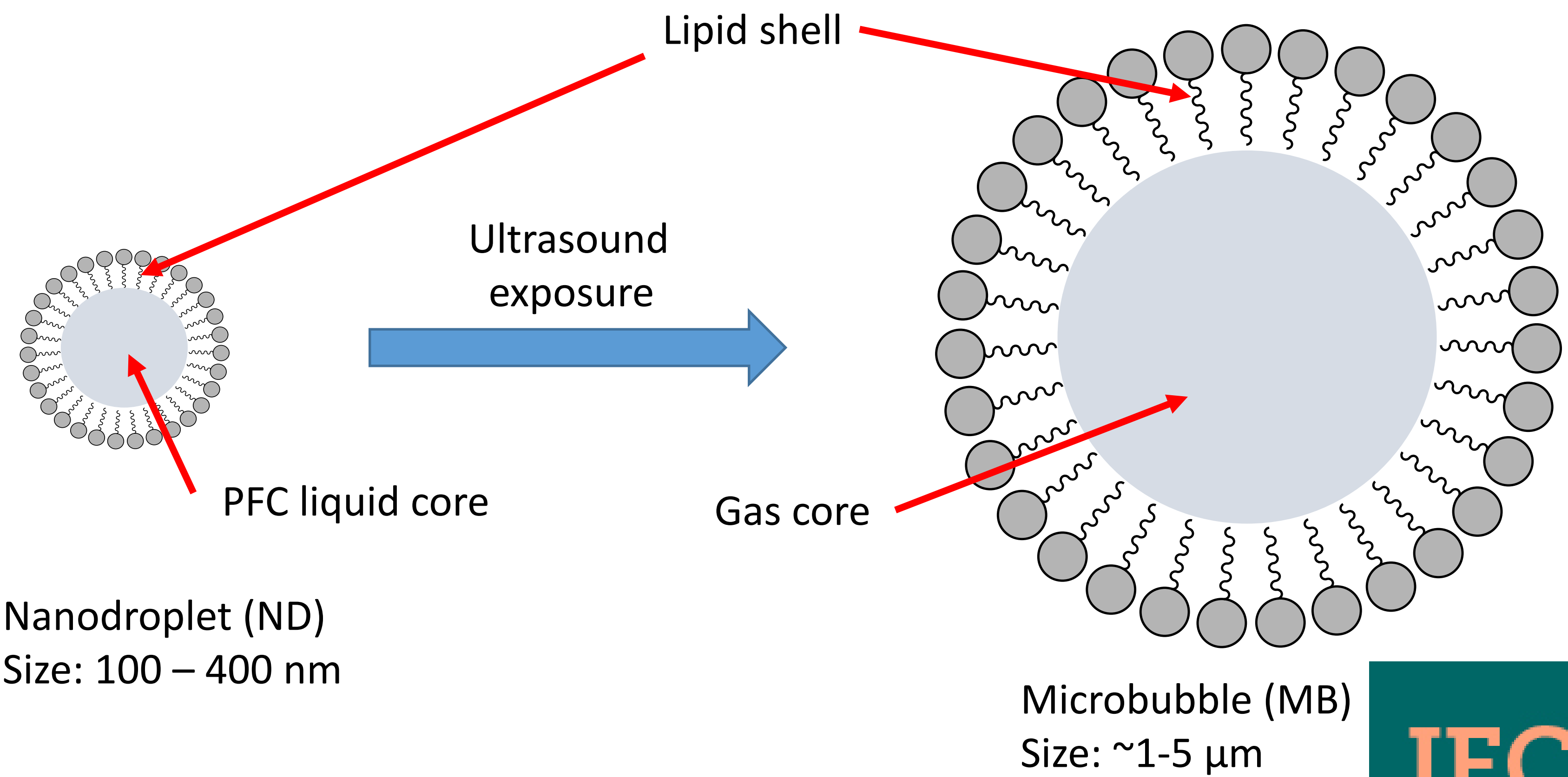
Impaired fracture healing impacts patients' quality of life and imposes a financial burden on healthcare services. Up to 10% of bone fractures result in delayed/non-union fractures, for which new treatments are urgently required. However, systemic delivery of bone anabolic molecules is often sub-optimal and can lead to significant side effects. In this study, we developed ultrasound responsive nano-sized vehicles in the form of perfluorocarbon nanodroplets (NDs), as a means of targeting delivery of drugs to localised tissues.

We tested the hypothesis that NDs could stably encapsulate BIO (GSK-3β inhibitor), which could be released upon ultrasound (US) stimulation to activate Wnt signaling and induce bone formation.

Keywords: Nanoparticle; Phase-change Nanodroplet; Ultrasound; Externally stimulated triggered release, Bone fracture healing;

Nanodroplets

- Nano-sized particles with a lipid shell and a liquid perfluorocarbon core
- Upon ultrasound exposure the liquid core undergoes a phase-change to form a bubble



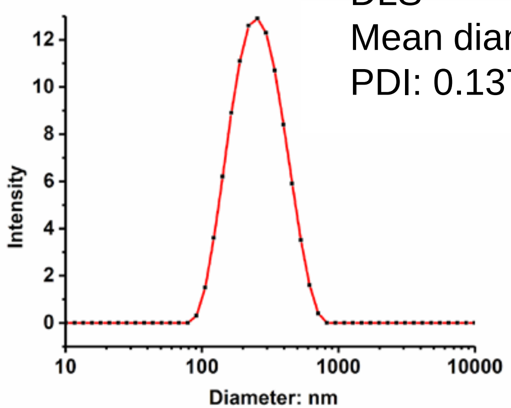
Lipid Shell: DSPC/Cholesterol/DSPE-PEG$_{2000}$ (molar ratio 72:20:8)

Liquid Core: PFC (perfluorobutane)

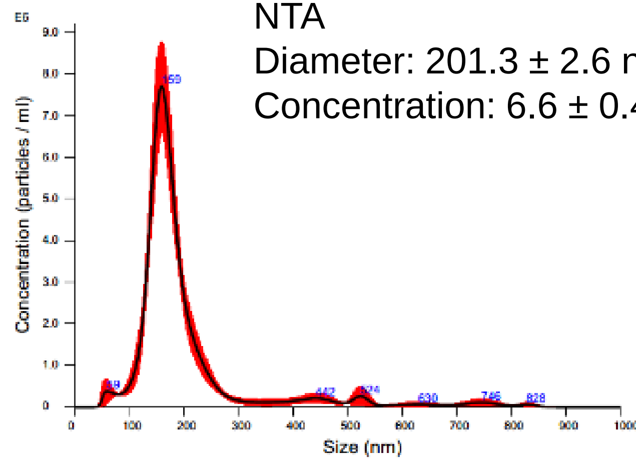
Nanodroplets – characterisation: Size

- The size, distribution and concentration of NDs was analysed by dynamic light scattering (DLS), nano tracking analysis (NTA) and cryo-EM

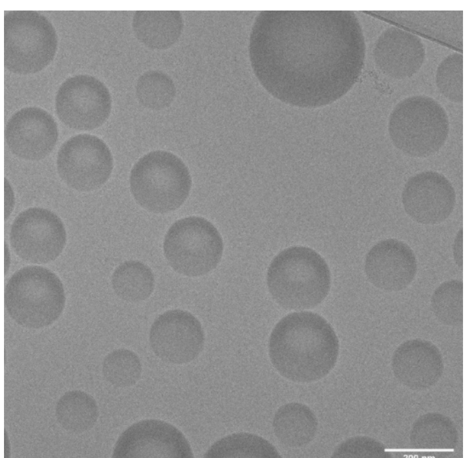
DLS
Mean diameter: 237 ± 16 nm
PDI: 0.137

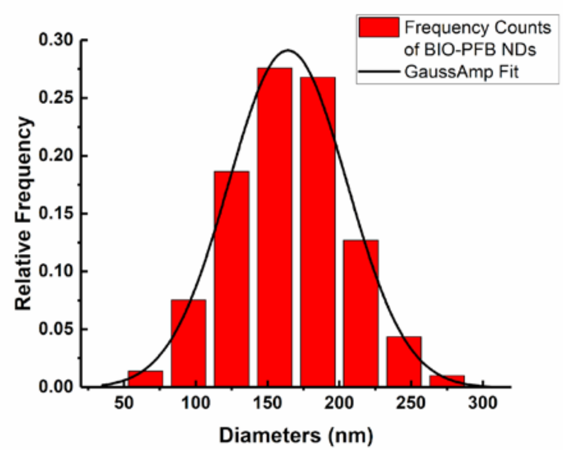


NTA
Diameter: 201.3 ± 2.6 nm
Concentration: 6.6 ± 0.4 x10^{11} mL^{-1}



Cryo-EM image of BIO-PFB NDs





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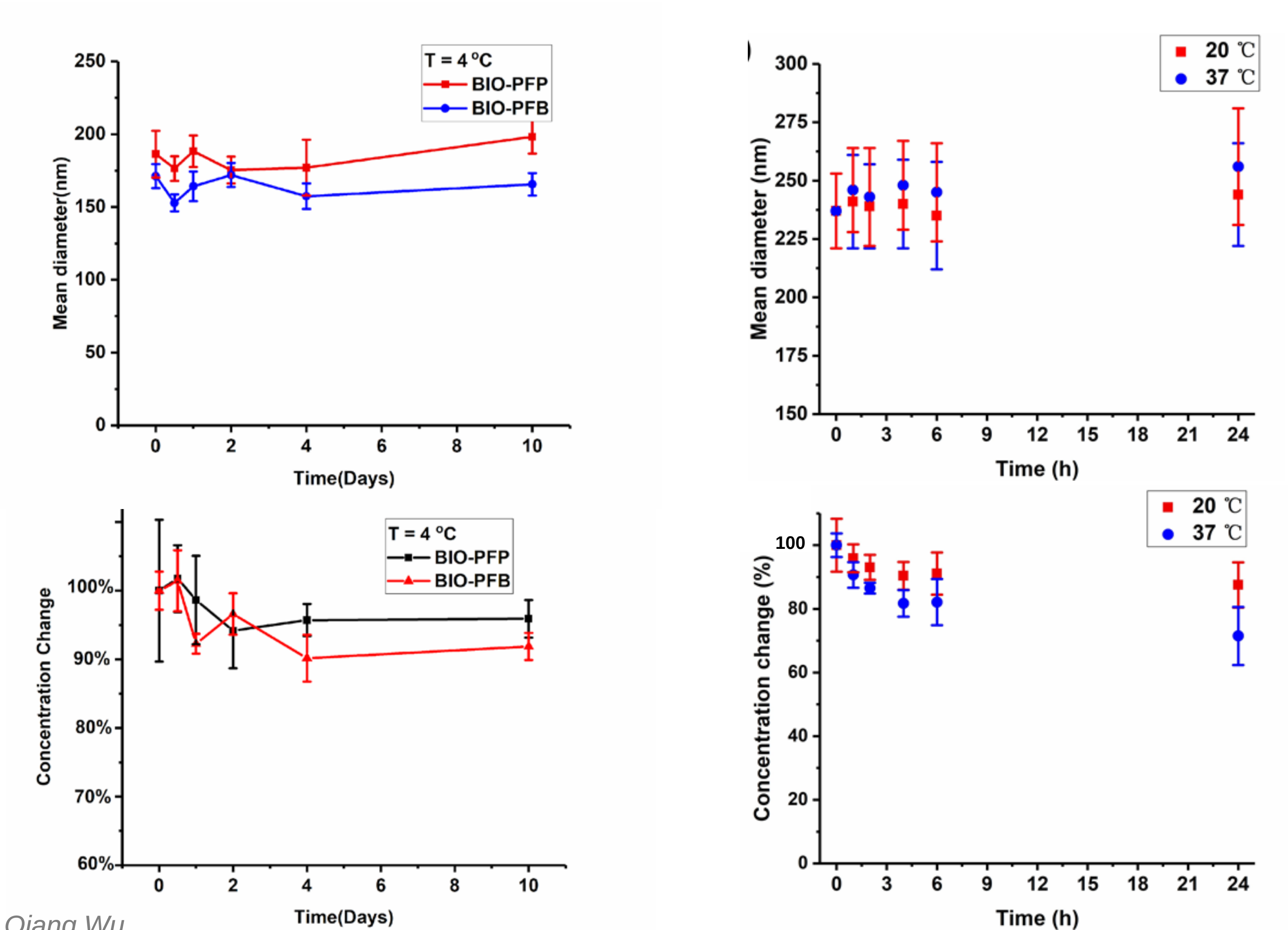
Nanodroplets – characterisation: Stability

- Size and concentration were used to assess ND stability at 4, 20 and 37°C using DLS and NTA



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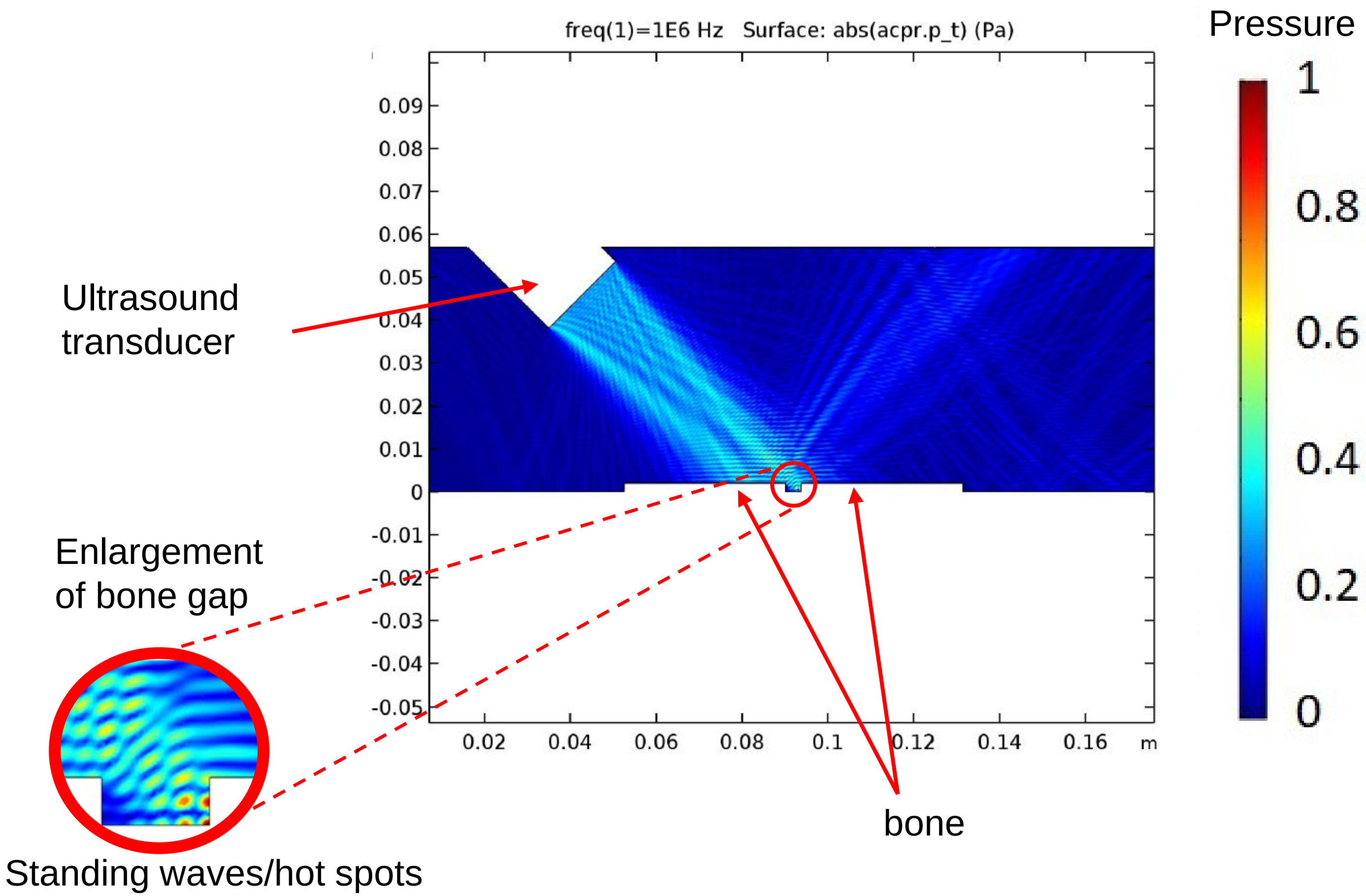
Nanodroplets – acoustic characterisation

Objective: Design a setup to perform the acoustic characterization of NDs in a fracture gap

Initially this was studied in a model to check ultrasound interference and reflection



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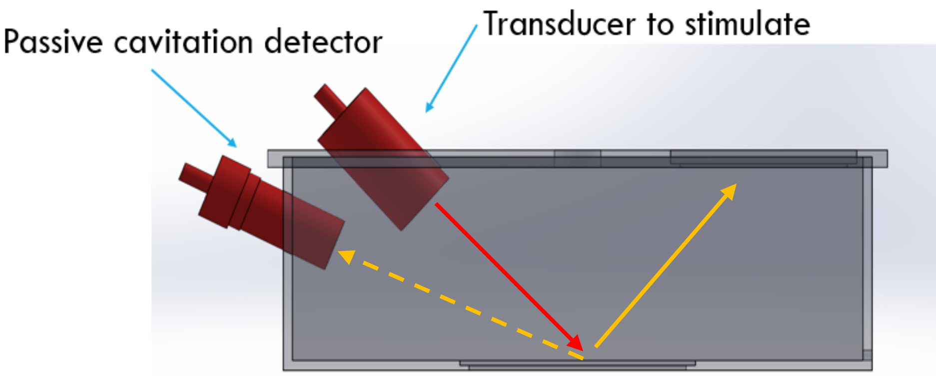
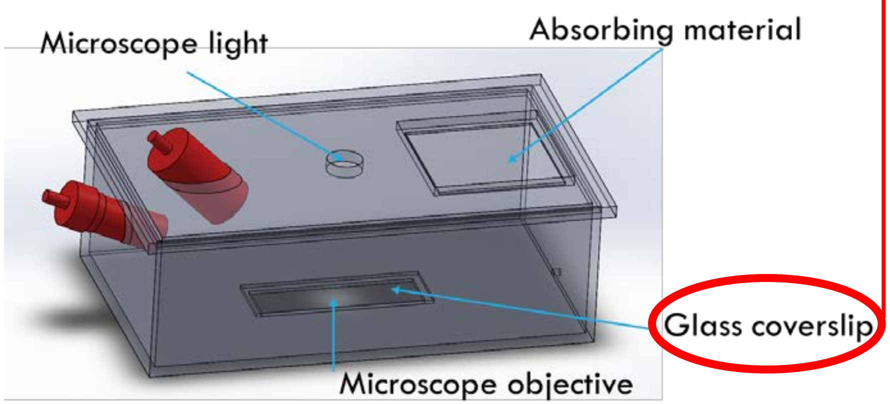
Nanodroplets – acoustic characterisation

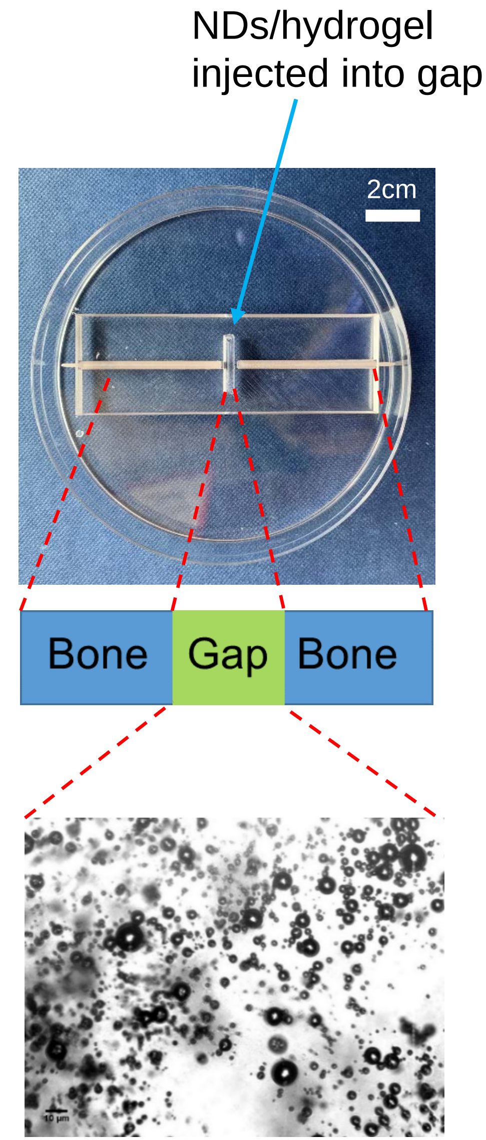
A hydrogel containing NDs was injected into a bespoke bone fracture model and stimulated by ultrasound allowing simultaneous capture of optical images and acoustic emissions



1 MHz transducer to stimulate; 7.5 MHz transducer to record



Microscope-compatible water tank (190x110x60 mm)



Hydrogel containing NDs/MBs

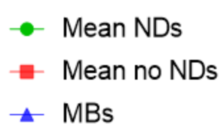
NB. Bone impedance: 5.41 x 10^6 kg/(m^2s)

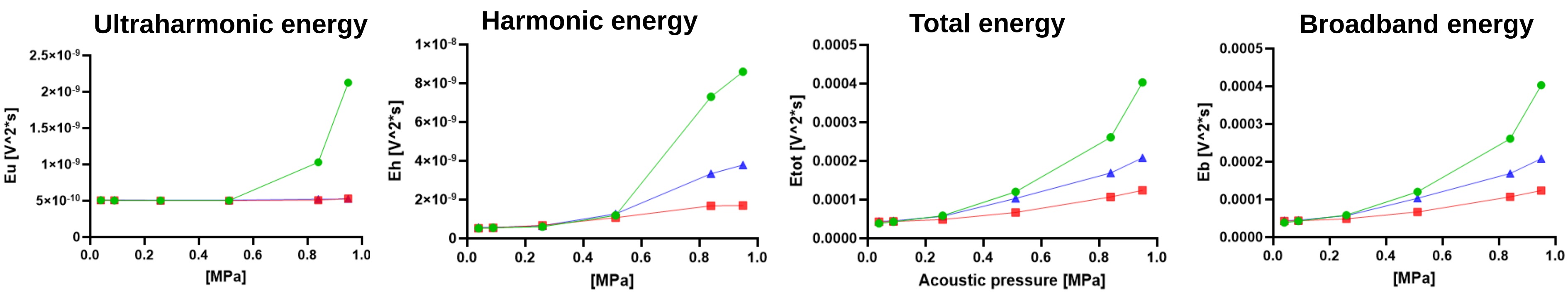
Nanodroplets – acoustic characterisation

Experiments were performed to investigate the ND vaporisation within the fracture model

The Pwelch method was used to study the strength of the signal as a function of frequency

Harmonic (1, 2, 3… MHz), ultraharmonic (1.5, 2.5, 3.5… MHz) and broadband energies of the recorded signals were calculated for a range of applied pressures



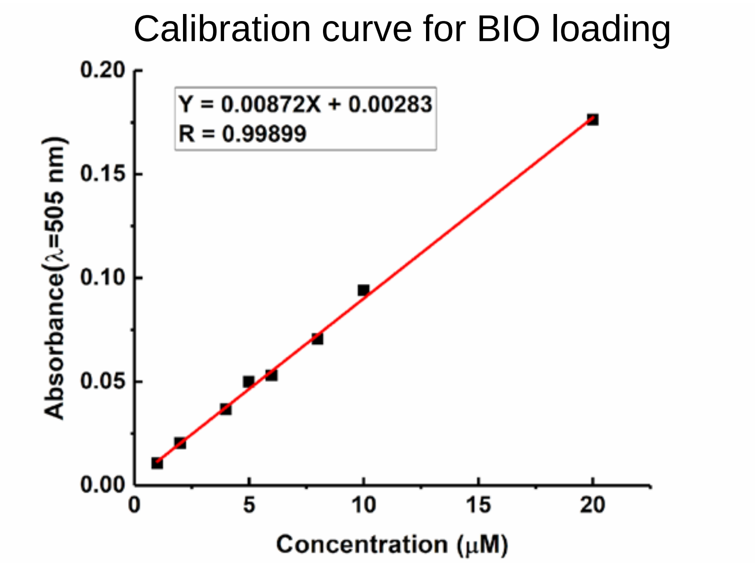


- All energies increased when the acoustic pressure exceeded 0.5 MPa
- This increased response is likely due to ND vaporisation
- This is encouraging for the potential use of NDs as therapeutic agents

ND: PFP DSPC/PEG40s; Hydrogel: 2% Low Melting Point Agarose gel
Acoustic parameters: f: 1 MHz, PRF: 1 Hz, Duty cycle: 1-5%, T: 30s, P: 0.04 – 0.95 MPa

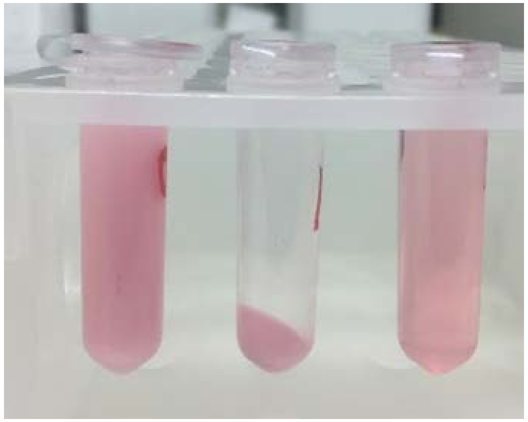
Nanodroplets - BIO loading

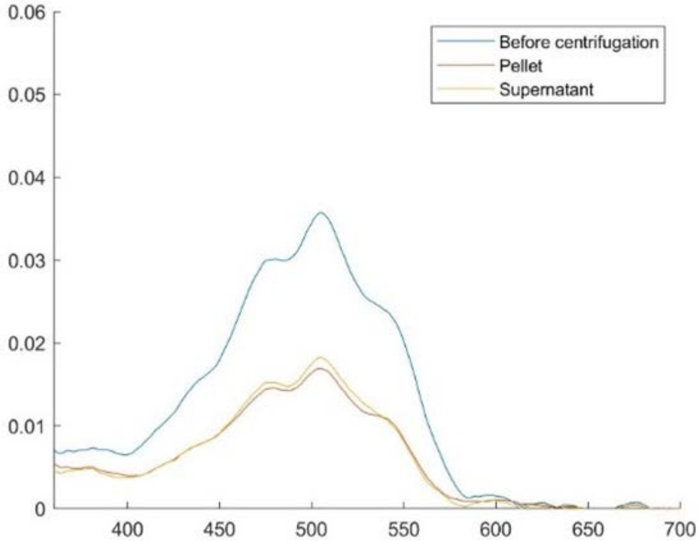
NDs were loaded with BIO during preparation
BIO loading was assessed by HPLC (Abs_{max}: 505 nm)



- Initial loading was found to be: 48.7%
- Following centrifugation and filtration: 21.9%

ND centrifugation:
Before Pellet Supernatant





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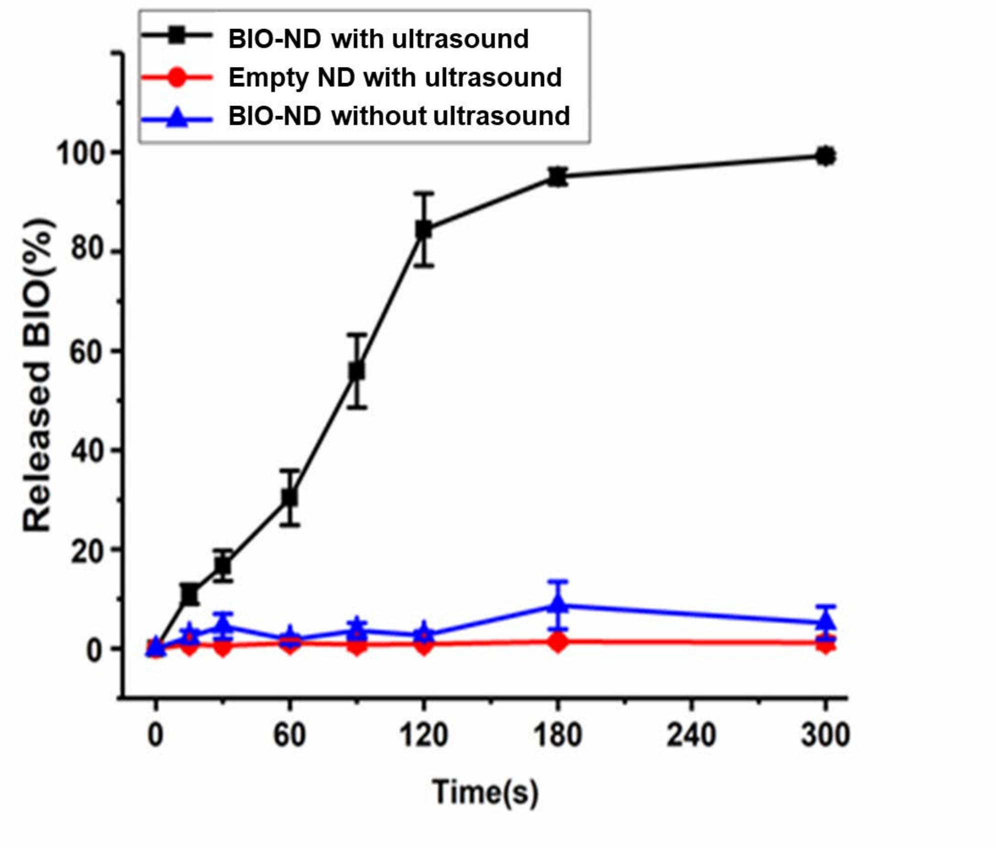
Nanodroplets - BIO release

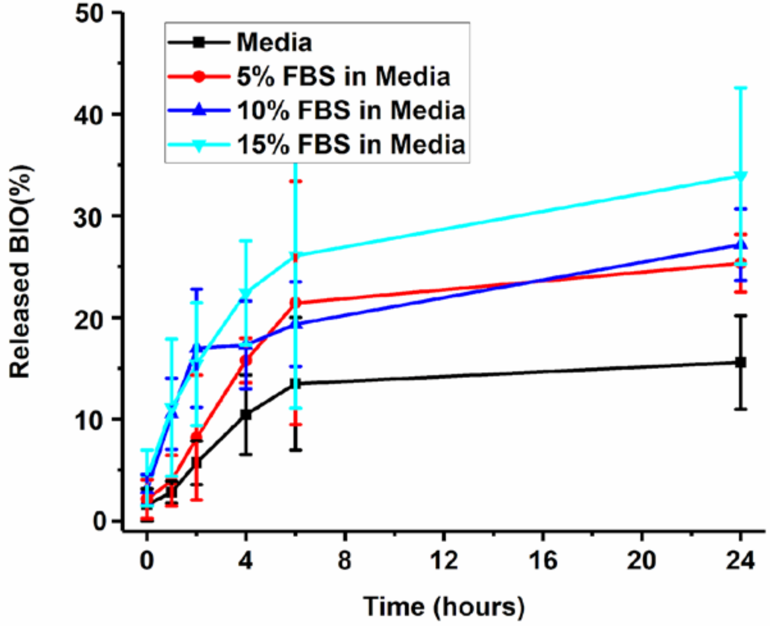


- BIO loaded NDs in PBS remain stable with no BIO release
- Ultrasound activation causes near quantitative BIO release

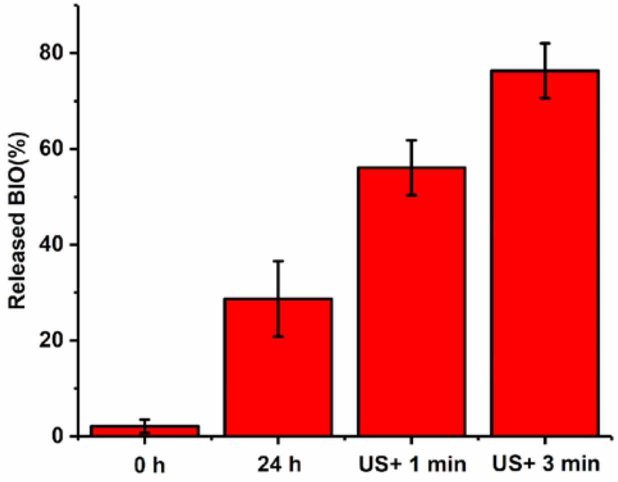
ND: BIO loaded DSPC/Chol/DSPE-PEG/PFB in PBS buffer
Acoustic parameters: f: 1 MHz, Duty cycle: 5%, P: 1.1 MPa

Nanodroplets - BIO release

BIO release from NDs at 37°C
in FBS containing media



BIO release from NDs
when stimulated with US



- Some BIO leakage was observed in serum containing buffers at 37°C
- Remaining BIO was released using ultrasound (US)

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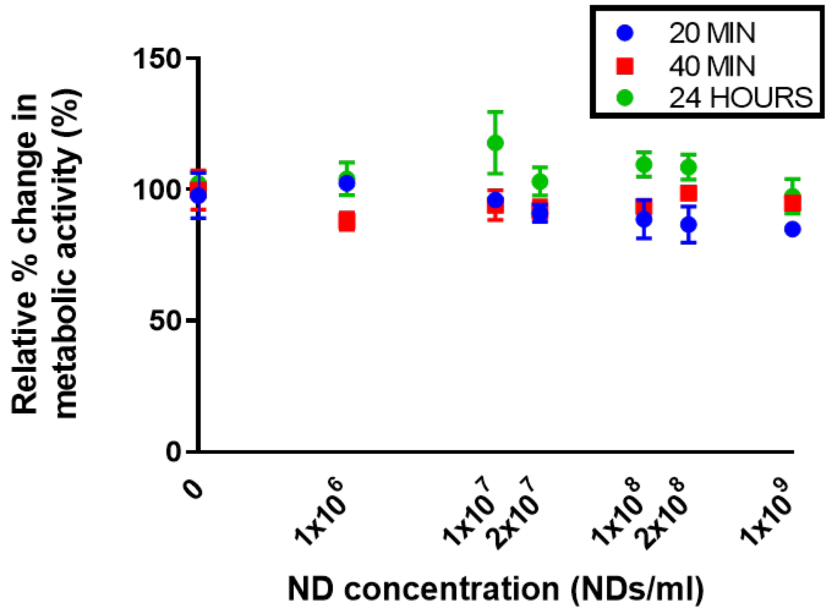
Nanodroplets cell toxicity

ND cytotoxicity was assessed in patient derived, bone marrow stromal cells (BMSCs) with Alamar Blue (24 h)



No safety concerns were observed for NDs (conc. <10^9 NDs/mL) when incubated with BMSCs for 24 h

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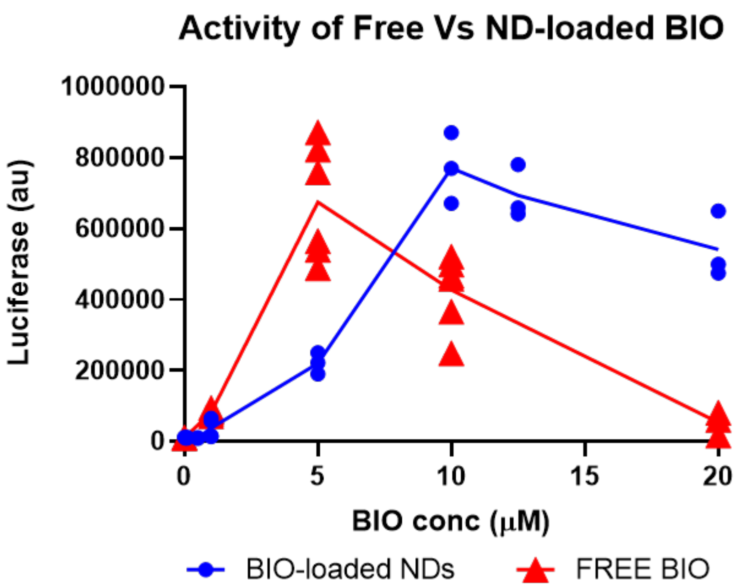
Nanodroplets - BIO release (cell read-out)

In vitro bioactivity of BIO-NDs was evaluated in a 3T3 Wnt-pathway reporter cell line with luciferase readout



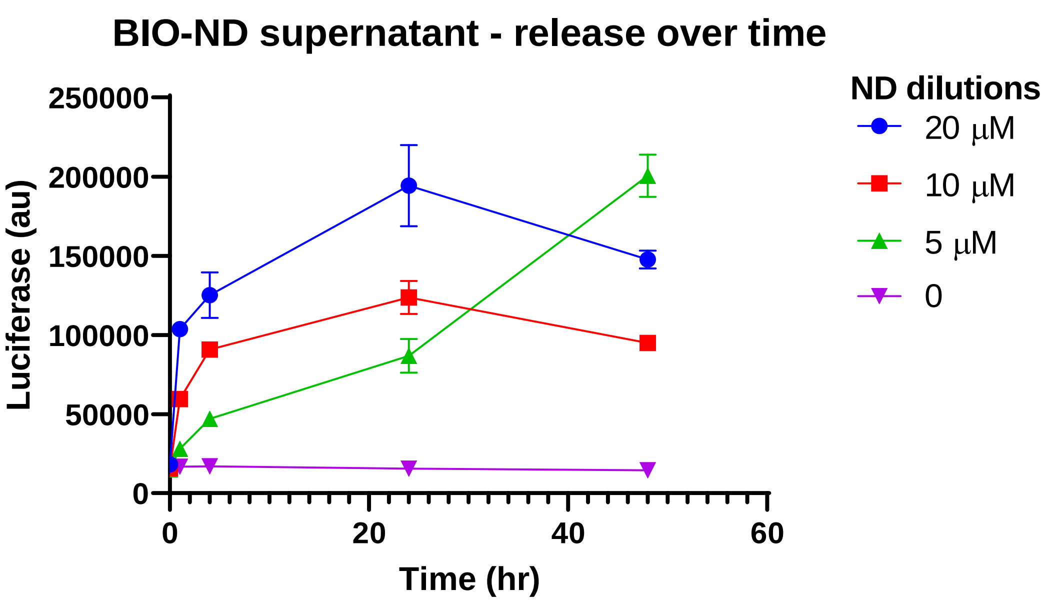
- Free BIO peak activity = 5 μM
- BIO-NDs, induce 70% less activity at 5 μM

Note: BIO becomes toxic to the cells above 5 μM (Free BIO), this value is higher for BIO-NDs (~10 μM) due to the relative amount of BIO available

Anastasia Polydorou

Nanodroplets - BIO release

In vitro bioactivity of BIO-NDs was evaluated in a 3T3 Wnt-pathway reporter cell line with luciferase readout



Incubation of BIO-NDs in cell medium at 37°C results in passive release of BIO

- This suggests that the 30% activity seen in the previous figure is mostly caused by the passive release of BIO from the NDs
- Reduction of bioactivity at 48 h for 10-20 μM is due to BIO cell toxicity

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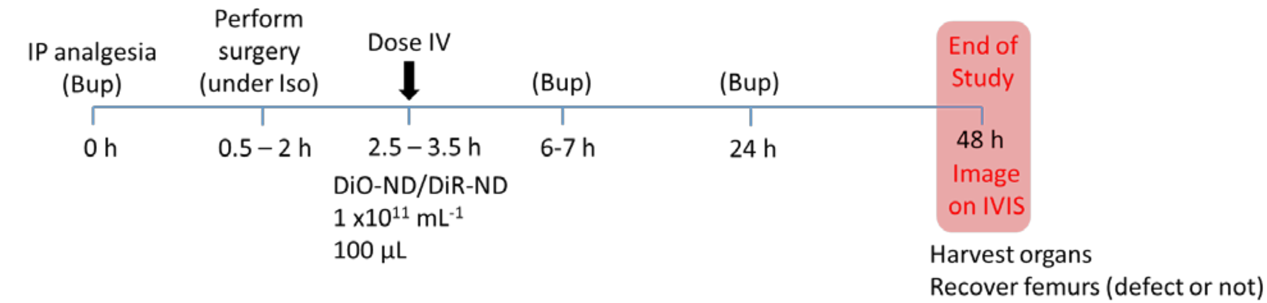
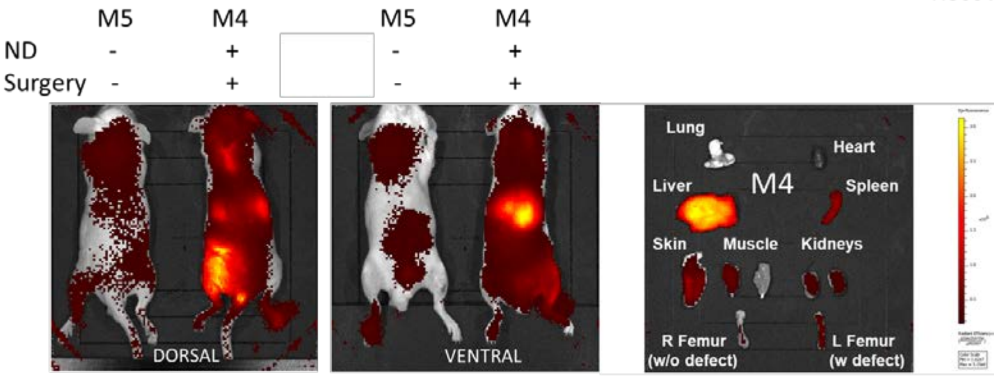
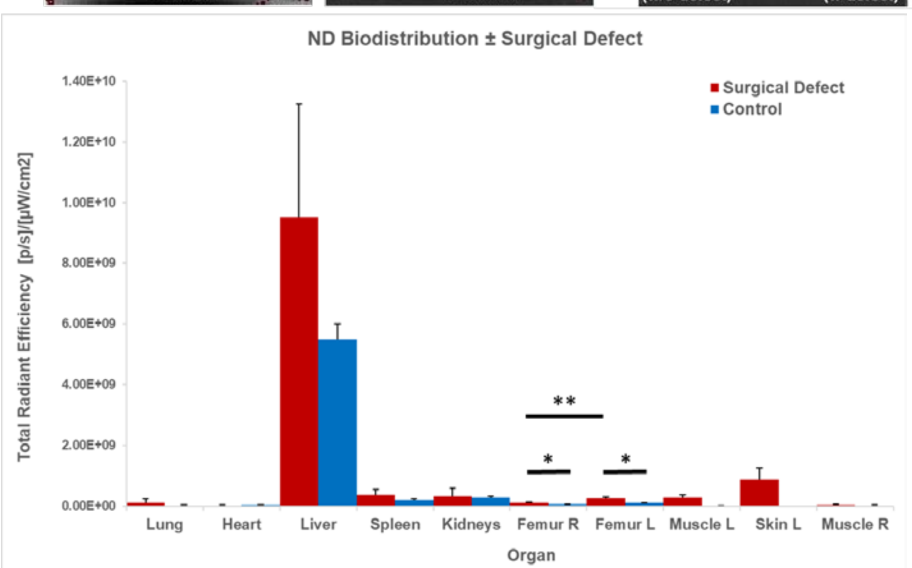
Nanodroplets - in vivo biodistribution







Femoral bone hole defects (1-2mm) were made in WT-MF1 mice (age: 8-12wks) and DiR-labelled NDs (100μL, 10^{11} NDs/mL, i.v.) were injected post-fracture to determine biodistribution by imaging

Groups:	Surgery	ND
1. Injury (4)	+	+
2. Healthy control (2)	-	+
3. Naïve control (1)	-	-

Organs were imaged and the majority of fluorescence (DiR) is observed in the liver

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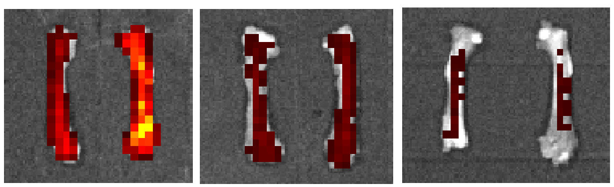
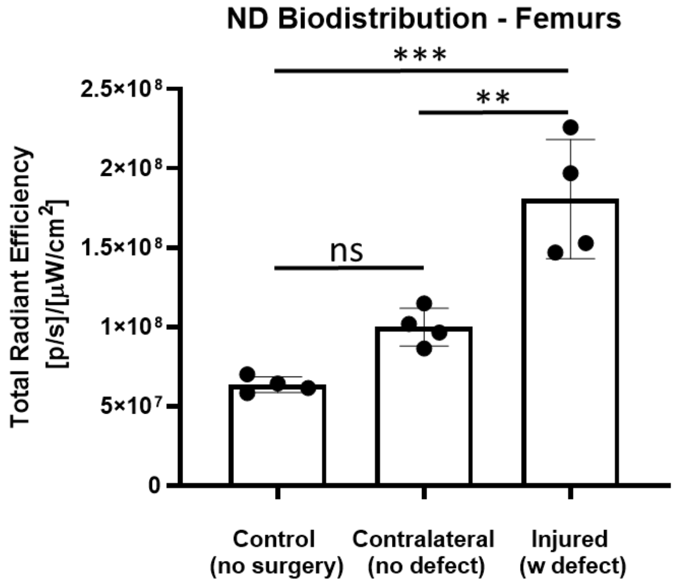
Nanodroplets - in vivo biodistribution



Limb	R	L	R	L	R	L
NDs	+	+	+	+	-	-
BHD	-	+	-	-	-	-



One-way ANOVA w Tukey's post-hoc analysis

Analysis of femurs:

- An increase in fluorescence intensity was observed in the femur with a defect, over the contralateral femur (without a bone hole defect)

- All femurs from a mouse which underwent surgery have higher levels of fluorescence intensity to those from a mouse that did not undergo surgery

Conclusions

ND formulation:
- Nanodroplets were prepared by sonication ~230 nm
- Nanodroplets have been loaded with BIO and were shown to be relatively stable in PBS at 4°C and 37°C
- NDs retained >90% BIO in PBS until US was applied, which caused ~100% release

ND acoustic characterisation:
- NDs responded well to US exposure at biologically compatible parameters

ND In vitro:
- ND exposure up to a concentration of 10^9 NDs/mL showed no cytotoxicity (24 h)
- BIO-loaded NDs induced Wnt pathway activation

ND In vivo:
- NDs were shown to localise at the fracture site in a murine model

Ongoing and future work:
- Improve BIO-ND loading stability
- Investigate ND vaporisation by using ultrahigh speed imaging
- Observe where NDs localise on a cellular scale in bone/liver (histology)
- Study ND release in vivo using ultrasound

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