Supplementary Information

Controlled release of carnosine from poly(lactic-co-glycolic acid) beads using nanomechanical magnetic trigger towards the treatment of Glioblastoma

Kinana Habra, [a] Robert H. Morris, [a] Stéphanie E. B. McArdle[b] and Gareth W. V. Cave [a,] *

[a] School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, UK; kinana.habra2017@ntu.ac.uk (K.H.); rob.morris@ntu.ac.uk (R.H.M.)

[b] John van Geest Cancer Research Centre, School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, UK; stephanie.mcardle@ntu.ac.uk (S.E.B.M)

* Correspondence: gareth.cave@ntu.ac.uk; Tel.: +44-115-848-3242

Experimental Section

All reagents were purchased from Sigma-Aldrich (Dorset, UK), were of analytical grade and used without further purification.

Generation of spheroids

Each well of a green coded 96-well round base plate for suspension (Sarstedt, Nümbrecht, Germany) was rinsed by a volume of 50 µL anti-adherence rinsing solution (STEMCELL Technologies, Cambridge, UK). After 15 minutes all wells were washed with 50 µL of serum-free media. U87 MG monolayer cells were detached with Trypsin (Sigma-Aldrich, St. Louis, Missouri, USA) to generate a single-cell suspension. The cells were calculated and seeded (400 cells/well) in 100 µL full media, then spheroid formation was initiated by centrifuging the plates at 3700 RPM for 10 min. The plate was incubated at 37° C, 5% CO_2 in humidified incubators. The full media had been replaced each other day.

Synthesis of superparamagnetic iron oxide nano-rods (IONRs) and Surface Coating

The method was described previously. In summary, aqueous polyethyleneimine (PEI) was dissolved in distilled water and Iron (II) sulfate heptahydrate and anhydrous iron (III) chloride were then added to the cyclohexane. The reaction was gently stirred at room temperature until the iron salts were fully dissolved, with the cyclohexane layer helping to prevent unwanted oxidation. The reaction was then headed (120 °C) with stirring (900 rpm) for 20 h. On cooling and pressure equalisations, the magnetite product was separated using a strong hand magnet and washed with dry dimethyl formamide. The suspended iron oxide nano-rods a double necked round bottom flask before the addition of branched polyethyleneimine (BPEI). The final product was separated using a strong magnet. The coated nano-rods were then separated via ultra-centrifugation. The synthesis produced IONRs of (85.9 ± 17.1) nm length.

PLGA beads synthesis

The flow focusing method was applied for multiple emulsifications using Dolomite microfluidic system (Mitos Fluika Pumps, Cambridge, UK). Carnosine was dissolved in 1% Polyvinyl alcohol (PVA) solution and emulsified with 1% PLGA dissolved in Dichloromethane (DCM). By using a micromixer hydrophobic

microfluidic chip and flow rate (1:1), the seeds of approximately 2µm diameter were produced. This primary emulsion was then transferred to a second junction on another hydrophilic micromixer chip where a second emulsification with 1% PVA solution was proceeded. PLGA beads were dried by gentle rotating for 2 hours. The PLGA beads were purified by applying three cycles of washing with sterilised water and centrifugation (10,000 rpm for 5 mins), then lyophilised. A yield of over 90% was obtained by recycling the top layer of carnosine from the collected emulsion during the first emulsification stage. The bottom layer (seeds) was used for the second emulsification.

Rotating magnet station

The rotating magnetic field station consisted of NdFeB Halbach Array magnet (Bunting Magnetics Europe Ltd., Hertfordshire, UK), which produces a uniform 1 Tesla magnetic field diametrically across the central air gap. The magnet was mounted on a motor and the sample was placed inside the central air gap. Measurement of temperature was conducted with a digital thermocouple using thermometer probe type K (Maplin 500 VGC, Taiwan) with a temperature accuracy of ± 0.3% + 1 °C and a reading rate of 2.5 times per second.

Magnetic Resonance imaging (MRI)

MRI measurements were performed at room temperature and used to measure T_2 time. To investigate the correlation between the relaxivity and sedimentation of the IONRs. Images were collected using a 1.5 T MRI scanner (Avanto, Siemens, Munich, Germany) with transverse slices. Images were acquired using a proton density weighted (TR = 7.14 s and TE = 34 ms) Turbo Spin Echo sequence with a 512 × 160 acquisition matrix and an isotropic resolution of 350 µm. An initial image was taken as a reference and was subtracted from the collected images.

Scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM-EDS)

The morphology of beads was characterised by scanning electron microscopy/energy dispersive X-ray spectroscopy (SEM-EDS, JSM-7100f, JEOL Ltd., Tokyo, Japan) integrated with Aztec software (ver. 2.4, Oxford Instruments, Oxford, UK). SEM-EDS was used to identify the location of iron after mounting the dry sample on a carbon tape stuck to an aluminum stub (13 mm), then sputter coated with 5 nm conductive material (gold).

Fourier transform infrared (FT-IR)

Fourier transform infrared spectra were recorded using an Infrared (FT-IR) spectrometer (PerkinElmer. Ltd, Waltham, Massachusetts, USA) using the dry components of the beads after checking a blank background.

Dynamic light scattering (DLS)

NanoPlus (Particulate systems, Norcross, GA, USA) was used to measure zeta potential for the surface charge of the beads. The suspension was prepared in neutral distilled water. The zeta quartz cell was filled with the diluted suspension and, after checking the optimum density for starting the test, the zeta potential was obtained in triplicate and then was averaged to minimise the error.

Inductively coupled plasma mass spectrometry (ICP-MS)

The levels of iron were determined by inductively coupled plasma mass spectrometry (ICP-MS) using a PerkinElmer NexION 1000 ICP-MS instrument (Waltham, MA, USA). Analysis was performed with standard calibration from Certipur® ICP (Merck, Watford, UK) Single-Element standards of iron. The sample was added to concentrated nitric acid and chloric acid and left overnight. The samples were filtered with a 0.2µm membrane syringe filter before being diluted with Deionised water ready for ICP-MS analysis. Standards were prepared in dilute ultrapure nitric acid (1%) ranging between 10, 20, 50, 100, 250, 500, 750 and 1000 ppb. Data was obtained as ppb.

Liquid Chromatography Mass Spectrum (LC-MS)

The carnosine assay was performed using a SCIEX TripleTOF 5600+ (AB Sciex LLC, Framingham, MA, USA). Positive ion mass spectra were acquired with direct infusion for optimisation and hyphenated LC system. The LC conditions were as follows: The separation was performed on an Eksigent ekspert nanoLC 425 system ACE AQ column (0.5 × 150mm, 3 µm) using ACE chromatography. The mobile phase was composed of MS grade water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), Merck, UK. For quantification, we prepared a standard curve of carnosine at a range of concentrations (1.0, 2.0, 5.0, 10.0, 15.0 µM). The standards and samples, mixed with water, were introduced into the source at a flow rate of 5 µL min^{-1}. The standardised gradient elution program in LC separation was: 0 min, 1% B; 1 min, 1% B; 3 min, 50% B; 6 min, 90% B; 9 min, 90% B; 10 min 1% B re-equilibrate for 3 min. To identify the transitions of carnosine, full Scan MS/MS was performed by fragmenting the precursor ion (m/z 227.11) of carnosine at a fixed collision energy of 25 V and a declustering potential of 80. The transitions were acquired in the mass range of 100 to 300 m/z. The column temperature was controlled at 45 °C and the sample chamber temperature was 8 °C. The injection volume was 2.0 µL. The ion spray voltage was fixed at 5.5 kV, Gas 1 12, Gas 2 0, using a Duospray source and 50 µm electrode. Data were analysed using MultiQuant 3.0.3 (SCIEX) (AB Sciex LLC, Framingham, MA, USA).

Image J analysis

To determine the area and the perimeter of the IONRs within the agarose gel, the images were acquired and analysed on ImageJ software. The IONRs have negative contrast in the field of view and were easily distinguishable from the transparent gel. The images were converted to a black and white mask and the total area of the IONRs was measured. The pixel of the IONRs was determined by the average grey area and perimeter.

Supplementary Figures

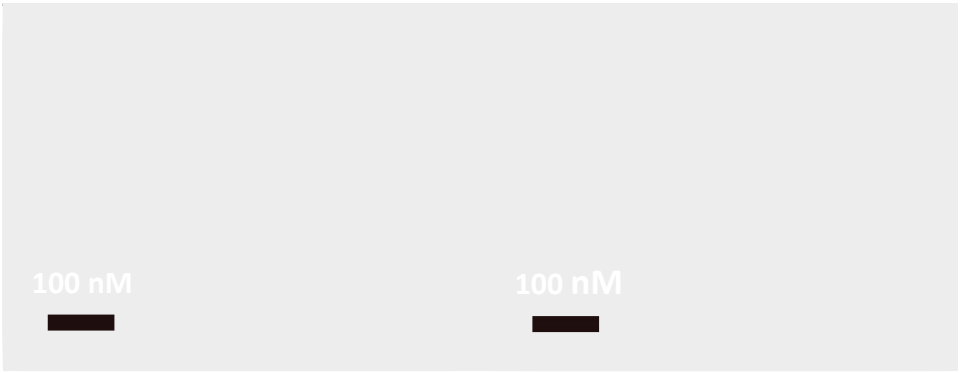


Fig S1. Transmission electron microscopic (TEM) images of mono disperse BPEI-coated IONRs with a tetragonal prism-dipyramid morphology. Scale bar 100 nM.

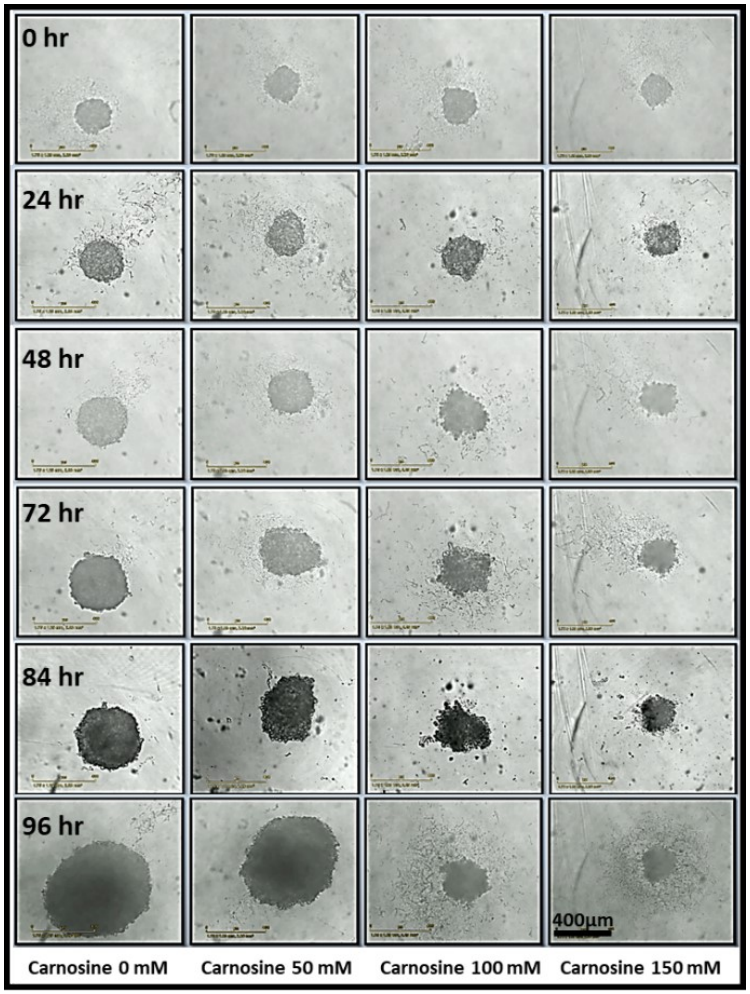


Fig S2. IncuCyte images show the effect of the sustained treatment using carnosine on U87 single spheroids. Carnosine was added every other day, and the images were collected over 4 days. The spheroid tightness was affected with carnosine > 100 mM. Scale bar 400 µM.

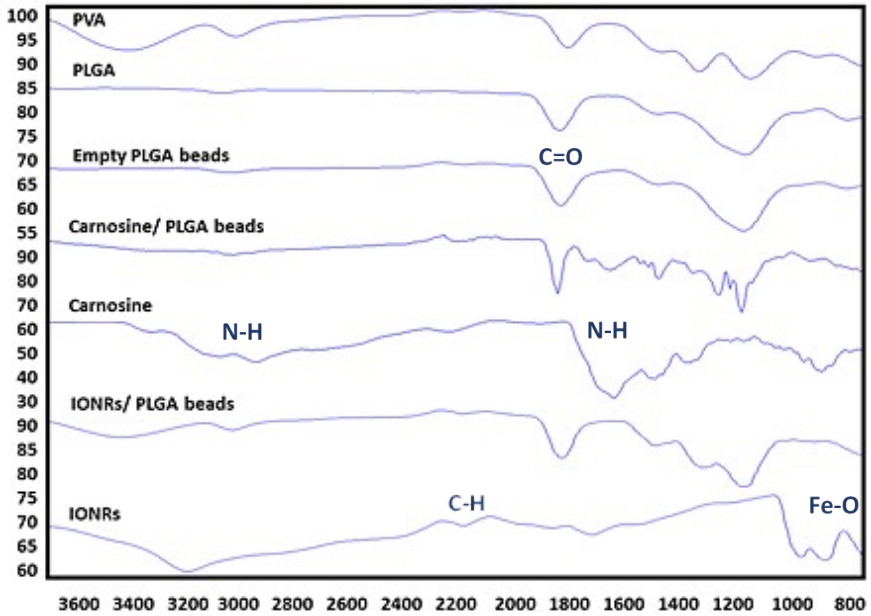


Fig S3. FT-IR spectra for PVA, PLGA, empty beads, L-carnosine (with/without PLGA), and IONRs (with/without PLGA).

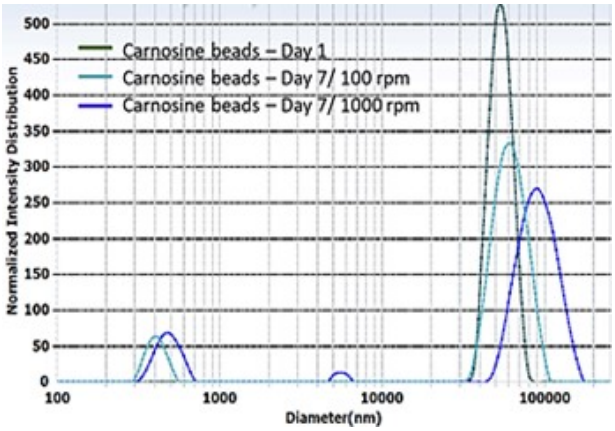


Fig S4. The DLS results of normalised intensity distribution of the beads over a week with different stirring speeds.

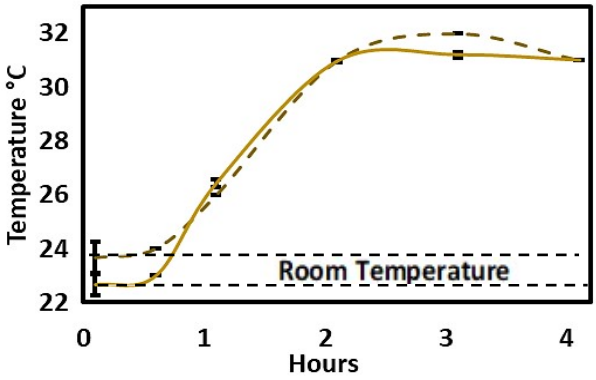


Fig S5. The temperature fluctuations for applying the rotating MF on __ water vs. _ _ PLGA beads.

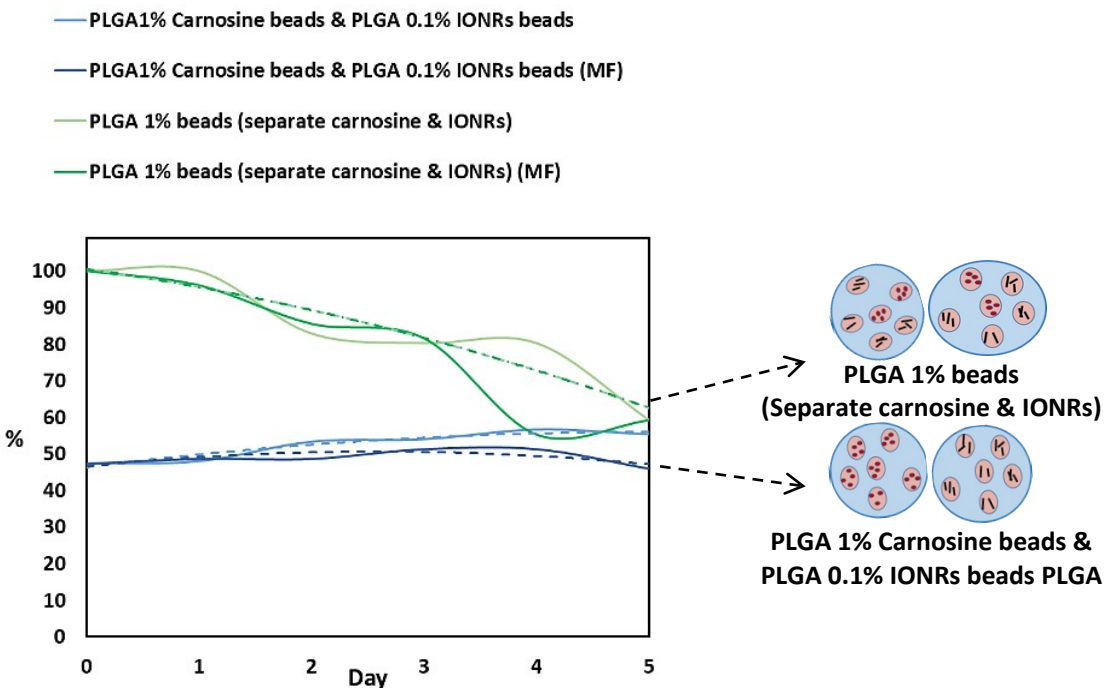


Fig S6. ICP assay for IONRs normalised concentrations shows the comparison between adding the encapsulated IONRs to the carnosine as seeds vs. beads. The decrease in concentration because of the attachment with the released carnosine over time. The dotted lines are trend lines fitted to the normalised concentration of the released IONRs.

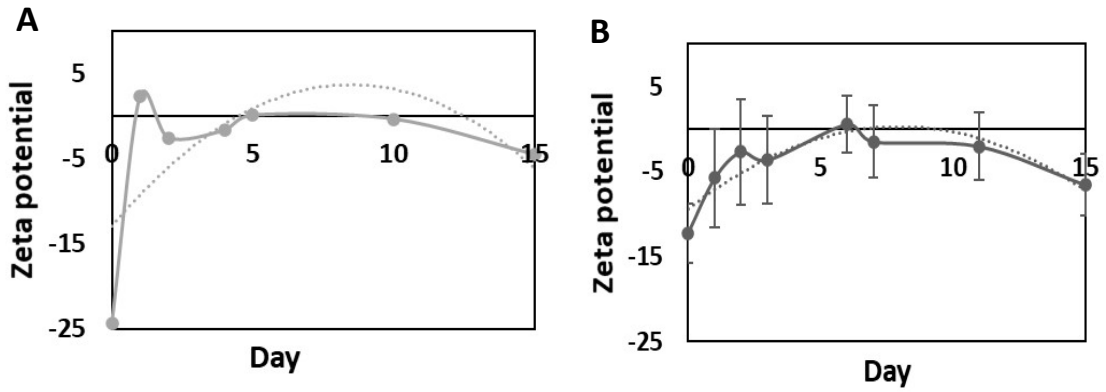


Fig S7. Zeta potential measurements for 15 days to compare the freeze frying effect on the stability profile. **(A)** Freeze drying the sample induced the initial release for carnosine, while **(B)** shows multiple waves for the released carnosine because of using gentle vacuum drying. The dotted lines are trend lines fitted to the surface charge of the carnosine loaded PLGA beads.

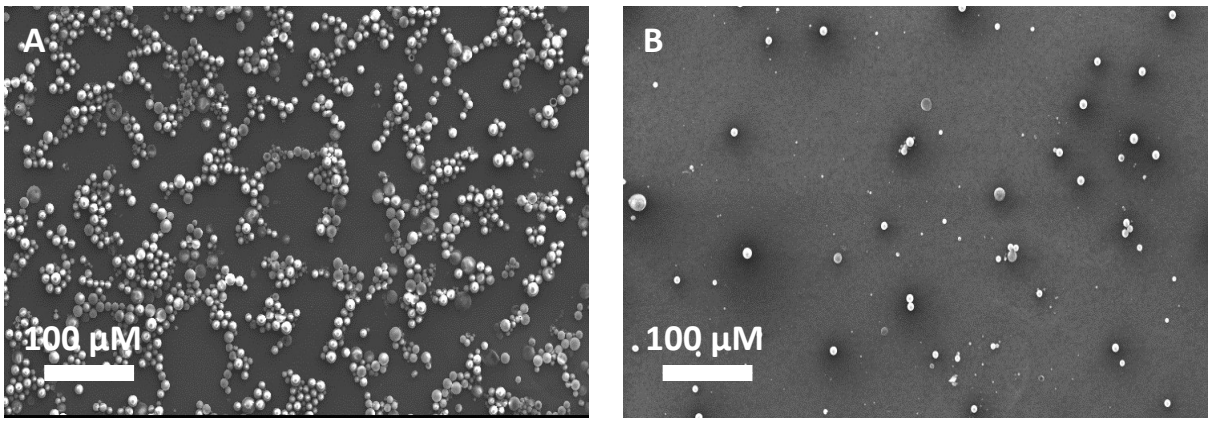
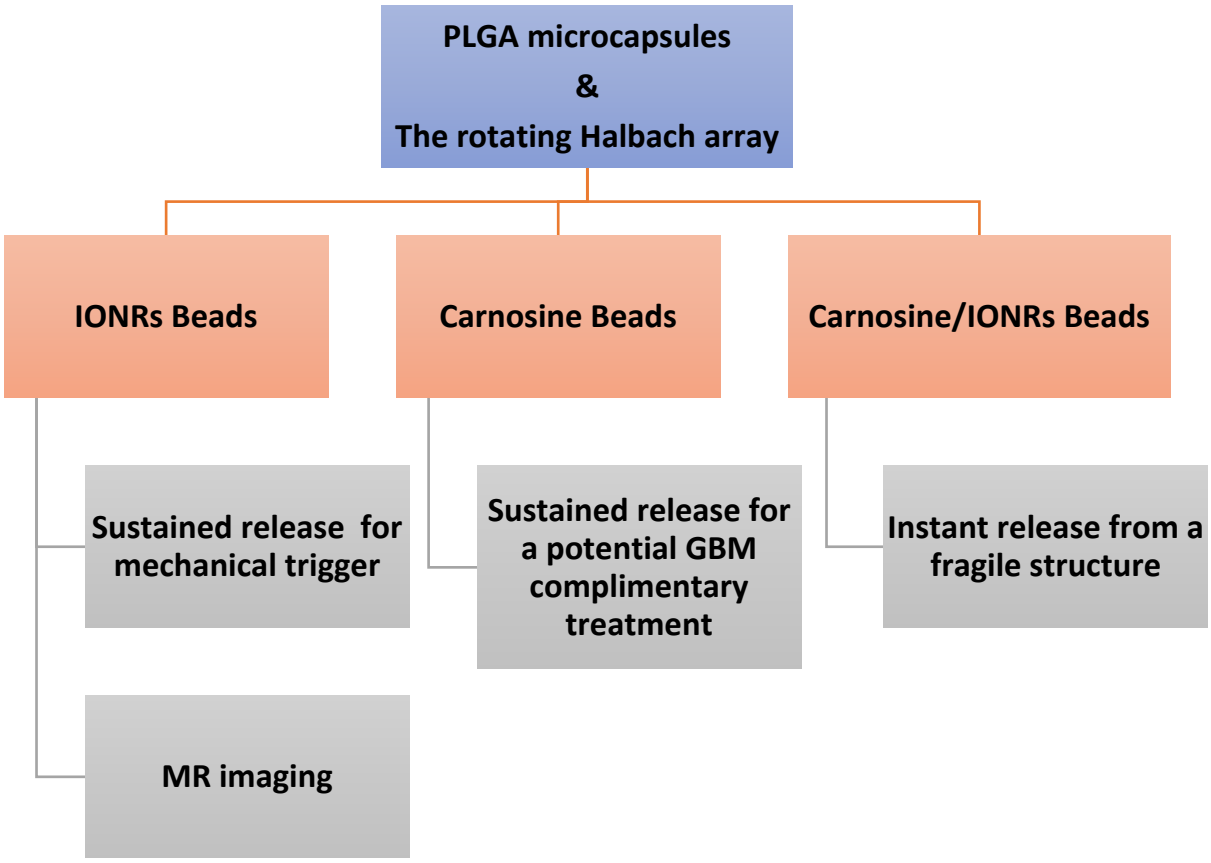


Fig S8. Scanning electron microscopic (SEM) images show the PLGA beads **(A)** The beads cluster in PBS buffer, and **(B)** The beads are stable in the Media with serum. Scale bar 100 nM.



Scheme 1. Flow chart summarizing the work presented.