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Supplementary Information for Farashishiko *et al.*

Crosslinked Nano-Assembled Capsules (CACs): Potential Molecular Imaging Agents for MRI Capable of Delivering Large Payloads of Very High Relaxivity Gadolinium

Annah Farashishiko,[a] Sally E. Plush,[b] Karley B. Maier,[a] A. Dean Sherry[c,d] and Mark Woods[a,e]*

[a] Department of Chemistry, Portland State University, 1719 SW 10th Ave, Portland, OR 97201, USA.

[b] Sansom Institute for Health Research, University of South Australia, GPO Box 2471. Adelaide, South Australia 5001, Australia

[c] Advanced Imaging Research Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390, USA.

[d] Department of Chemistry, University of Texas at Dallas, 800 W Campbell Road, Richardson, TX 75080, USA

[e] Advanced Imaging Research Center, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA

E-mail: mark.woods@pdx.edu & woodsmar@ohsu.edu

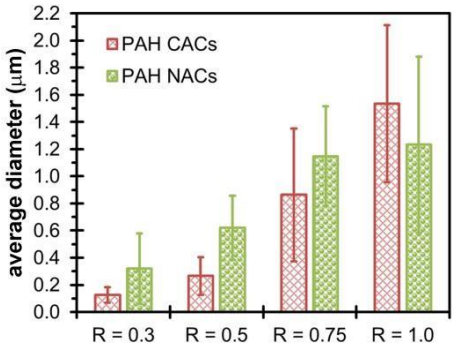

Fig S1. A comparison of the average hydrodynamic diameters of CACs (red) and NACs (green) prepared using PAH and $GdDOTP^{5-}$. Each value is the mean of six nano-capsule preparations, error bars show the 1st standard deviation of the size distribution.

Table S1. The average longitudinal relaxivity (r_1 ($s^{-1}mmolal^{-1}$), 20 MHz and 298 K) of $GdDOTP^{5-}$ containing nano capsules (CACs and NACs) prepared with PAH in 3:2 MeCN/H_2O as a function of the *R* value used in their preparation. The errors values are the 1st standard deviation of values determined from six separate preparations.

	CACs	NACs[a]
R = 0.3	70.7 ± 0.4	46.4 ± 0.3
R = 0.5	46.3 ± 0.4	19.8 ± 0.1
R = 0.75	16.0 ± 0.2	15.8 ± 0.1
R = 1.0	9.17 ± 0.1	7.1 ± 0.1

[a] Data from reference [1]

CAC Stability Investigations

A number of endogenous metal ions are capable of interfering with the action of $GdDOTP^{5-}$ as a contrast agent. In particular metal ions, such as Ca^{2+}, that have a high affinity for phosphonates and silence the relaxivity of $GdDOTP^{5-}$ are of particular concern.[2] However, the list of metal ions capable of interfering with a contrast agent is somewhat shorter than that commonly given. Only metal ions that are not protein bound and unchaperoned in extra- cellular fluids are of relevance and this list is comparatively short. It eliminates from consideration many common endogenous metal ions such as $Cu^{1/2+}$, $Fe^{2/3+}$ and Zn^{2+}. Zn^{2+} in particular is often identified as playing a major role in the demetallation of Gd^{3+}-based contrast agents;[3] however, Zn^{2+} is very tightly controlled *in vivo* and by far the vast majority of endogenous Zn^{2+} is intra-cellular[4] and therefore not accessible to contrast agents (since they are strictly extra-cellular). The extra-cellular Zn^{2+} concentration is very low (μM)[5] and, because Zn^{2+} can be toxic to cells at even nM concentrations,[6] the majority of this is protein bound.[7] The total "free" (that is rapidly exchangeable) extra-cellular concentration of Zn^{2+} is likely to be as low as nanomolar.[7, 8] For these reasons Zn^{2+} is unlikely to play a significant role in affecting the performance or stability of an extra-cellular contrast agent and was not studied. Metals such as $Cu^{1/2+}$ and $Fe^{2/3+}$ are redox active and consequently tightly chaperoned in all extra-cellular fluids. These metals ions also have no significant role to play in the *in vivo* performance or degradation of extra-cellular contrast agents. The metal ions of relevance are those found in solution at appreciable concentrations in extra-cellular fluids: in particular Ca^{2+}, Mg^{2+} and Na^{+}. The cross-linked peptic coating of CACs must therefore be capable of tolerating the presence of these metal ions as well as changes in pH relevant to tissue pathologies *in vivo*.

Preliminary stability tests were performed by incubating CACs (R = 0.3) against Ca^{2+}, Mg^{2+} and Na^{+} (at concentrations substantially higher than those found in extra-cellular fluids) and over the pH range 3 – 11 (wider than the physiologically relevant range) for 4 weeks.

Capsules were assessed in terms of their robustness and performance. Every 7 days changes in capsule size (DLS) and relaxivity were monitored; capsule morphology was assessed at the end of the 4 week incubation period.

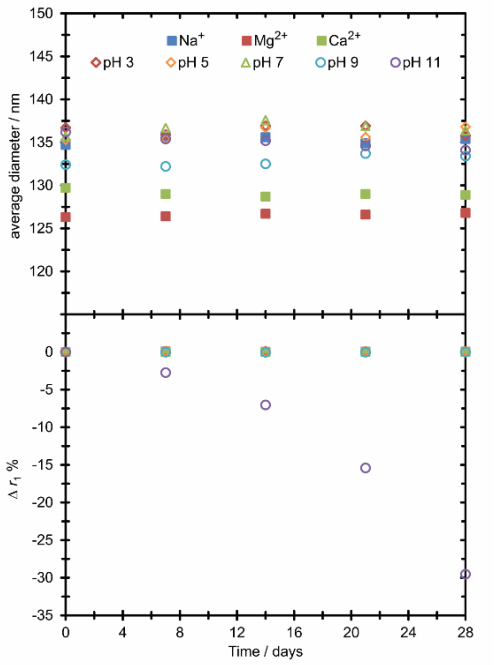

Fig S2. The effect of several biologically relevant factors on the size and effectiveness of CACs. The change in hydrodynamic diameter (top) and per-Gd^{3+} relaxivity (bottom) for samples of PAH and $GdDOTP^{5-}$ based CACs (*R* = 0.3) during incubation against relevant endogenous metals (filled squares) and changes in pH that extend beyond the physiologically relevant range (open circles). Both the size and relaxivity of CACs are unaffected by these conditions with the exception of high pH (pH 11).

Suspensions of $GdDOTP^{5-}$ containing CACs (*R* = 0.3) were placed in a dialysis membrane (30 kD MWCO) and then incubated under sink conditions (that is to say that the volume of the dialysis bath medium was 3× more than that required to form a saturated solution) in solutions of $CaCl_2$ (5 mM), $MgCl_2$ (5 mM) and NaCl (200 mM) and at pH 3, 5, 7, 9 and 11. For CACs incubated against the three metal ions and pHs between 3 and 9 no change in size, morphology or per-Gd^{3+} relaxivity of the CACs was observed over the 28 day incubation period (Figs S2 & S3). This seems to indicate that the electrostatic interactions between the phosphonate groups of $GdDOTP^{5-}$ and the polymer do not appear to be disrupted by any of

these metal cations or pH conditions. It seems likely that the encapsulating shell of CACs is sufficiently robust for in vivo applications.

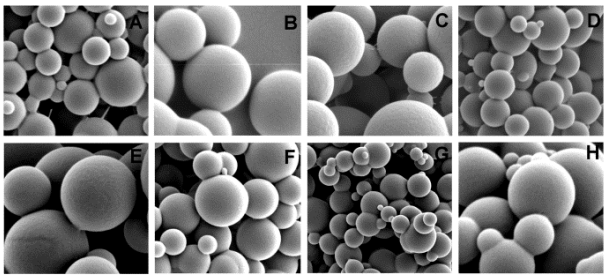

Fig S3. SEM images of PAH/$GdDOTP^{5-}$ CACs (R = 0.3) after incubation against A) 5 mM $CaCl_2$, B) 5 mM $MgCl_2$, C) 200 mM NaCl, D) pH 3, E) pH 5, F) pH 7, G) pH 9 and H) pH 11 for 4 weeks.

The picture at pH 11 is quite different. Although there is no discernable change to the overall capsule morphology after incubation at pH 11 for 28 days, DLS indicates a small (~ 5 nm), but consistent change in the hydrodynamic diameter of the CACs over the 4 week incubation period. However, there is quite a marked change in relaxivity over this period, eventually leading to a 35% decrease after 28 days. One possible explanation for this decrease is that the high pH of the solution hydrolyzes the metal from of the chelate, which would reduce its relaxivity. To examine this possibility a sample (0.3 mM) of $GdDOTP^{5-}$ alone was incubated at pH 11 for 4 weeks and the relaxivity monitored every week for 4 weeks. Changes in relaxivity, consistent with a tiny amount of metal hydrolysis, were observed in this experiment but the extent of dechelation is much lower than would be required to give rise to the drop in relaxivity seen for CACs in Fig S2. We suggest therefore that either the capsule interior (and in particular the protonated amines of the polymer) assist the hydroxide in the hydrolysis of the metal ion. Or, as seems more likely, the chelate remains intact and that the elevated pH (above the pK_a of the polymer amines) disrupts hydrogen bonding interactions between the chelate phosphonates and the protonated polymer amines. These interactions are critical for high CAC relaxivity since they must hold the chelate rigid within the capsule,

populate the chelate's "second-sphere" and help modulate rapid proton transfer with water in the capsule exterior.

Of course the situation in physiological fluids is more complicated, with multiple components that could even potentially act in concert to affect the CACs. At the suggestion of a reviewer we attempted to assess how a more complex medium such as serum might affect the stability of CACs by incubating capsules in DMEM (hepes-buffered low-glucose Dulbecco's Modified Eagle Medium) following the method described above. Over the course of the 4 week incubation the mean capsule diameter was found to increase slightly (about 18% over the course of 4 weeks) and since this change is substantially less than the size distribution of the sample it is not obvious that this change is statistically significant. However, the relaxivity of the CACs over the 4 week incubation period was similar to the behavior of the CACs incubated at pH 11 (above), with relaxivity dropping significantly over the course of the experiment. Sodium azide had been added to the DMEM for this experiment in an attempt to maintain the integrity of the DMEM. The manufacturer recommends that DMEM be stored in the dark at 4 – 8 ºC, quite different conditions than the room temperature conditions required for these stability experiments. Despite the presence of the sodium azide we found that the DMEM degraded over the course of the experiment; in particular it was noted that the pH increased to about 11, and the T_1 (in the absence of contrast agent) increased by more than 10%. It is unclear what is happening to the medium to cause these changes but it seems that the changes in relaxivity can readily be attributed to the observed change in pH of the medium. Although the change in CAC size may be attributed to incubation in DMEM, it seems that the known degradation of the medium prevents any conclusive statement that physiological fluids may negatively affect the integrity of CACs from being made.

Experimental Section

General remarks. 'Water' refers to deionized water with a resistivity of 18.2 MΩ. All solvents and reagents were purchased from commercial sources and used as received unless

otherwise stated. Polyallylamine hydrochloride (PAH) (56,000 MW), poly-L-lysine (50,000 MW), L-glutamic acid, succinic acid and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from the Sigma-Aldrich corporation and used as received. H_8DOTP was purchased from Macrocyclics. $GdDOTP^{5-}$ was synthesized by previously reported methods.[REF][9]

General Procedure for the Synthesis of CACs with Simple Diamagnetic Anions.

Stock solutions of polymer and anion were prepared in 100% water at pH 9. Stock solution concentrations were as follows: [polymer] = 89 μM, [citrate] = 4.2 to 19.9 M and [pyrophosphate] = 2.9 to 14.9 M. The polymer (20 μL) and anion (120 μL) stock solutions were then added to water (10 mL) at pH 9. The charge ratio *R* was varied by varying the initial concentration of anion. After mixing the reaction immediately became turbid reflecting the formation of a polymer-anion aggregate. The solution was vortexed at low speed for 10 s and then aged for 3 minutes without agitation. After aging, a solution of dicarboxylic acid (20 μL, 0.089 M) was added. Succinic acid or L-glutamic acid were used at identical concentrations. The solution was gently mixed and then aged for 3 min. A solution of 1-ethyl-3-(3-dimethylaminopropyl carbodiimide, EDC, (20 μL, 0.174 M) in H_2O was then added, gently mixed and aged for 30 min. The reaction was then gently centrifuged, the supernatant removed and the capsules recovered by resuspension in water.

General Procedure for the Preparation of Gd^{3+}-containing CACs.

An 89 μM stock solution of PAH was prepared by dissolving PAH (0.0498 g, 0.89 μmol) in a 3:2 *v/v* mixture of acetonitrile and water (10 mL). A stock solution of $GdDOTP^{5-}$ was prepared by diluting a concentrated solution into a 3:2 *v/v* mixture of acetonitrile and water to afford a 0.757 mM stock solution. The pH of the chelate stock solution was adjusted to 9, by addition of 1M NaOH solution. Stock solutions of EDC (0.174 M) and succinic acid (0.089 M) were prepared in pure water. For CACs R = 0.5, stock solutions of the polymer (20 μL) and

$GdDOTP^{5-}$ (120 μL) were added to a stirred solution of 3:2 *v/v* MeCN/H_2O (1 mL). Upon addition of the chelate stock solution the reaction rapidly became turbid. The reaction was vortexed at a medium speed for 10 seconds. Aggregates were then allowed to age for 3 minutes before addition of the succinic acid stock solution (20 μL). The reaction was vortexed for a further 10 seconds at medium speed and then allowed to age without agitation for 3 minutes. A solution of EDC (20 μL) was added and the reaction was aged for 1 hour. Unreacted starting materials were removed by filter centrifugation using 10 kDa MWCO filter centrifuge tube. The CACs retained in the filter centrifuged tube were washed with water and filtered by centrifugation for 30 minutes at 9,000 rpm a total of six times. The CACs were then taken up into water (1 mL) and recovered into a sample vial. All CAC preparations were repeated six times and data are an average of all of these independent capsule preparations.

Electron Microscopy. Scanning electron microscopy (SEM) was performed on a FEI Sirion FEG electron microscope equipped with an energy dispersive X-ray (EDX) detector. A droplet of NACs suspension was placed on the aluminium stub and dried in air, the sample was then sputter coated with gold for 55 seconds. Secondary electron images were taken at 5kV with a working distance between 5 - 10 mm. Transmission electron microscopy (TEM) imaging was performed using carbon/copper TEM grids on a JEOL 1200 EX system with an accelerating voltage of 40 kV and mounted with a Sis Morada 11 Mpixel CCD camera.

Confocal Microscopy. Confocal images were captured using a Leica TCS-SPE II DM 2500 with 63× magnification and a 488 nm laser line to excite FITC at 500 nm. Emission was detected at 555 nm. Scan format was 512 × 512 pixels. Samples were mounted on conventional glass slides and sealed under cover slip to prevent drying.

Light Scattering. Dynamic light scattering was performed on a Horiba LB-550 dynamic light scattering instrument. For these measurements freshly syringed filtered samples were dispersed in water and measured at four dilutions to ensure size distributions were

independent of concentration effects. Samples were regularly agitated to guard against settling of larger particles.

Relaxometry. Water proton T_1s were measured on a 0.47 T Bruker MiniSpec contrast agent analyzer operating at 19.99 MHz using an inversion recovery pulse sequence. NACs were suspended in water (1 mL) at Gd^{3+} concentrations ranging from 0.22 mM to 2.37 mM. Samples were regularly agitated to guard against settling of larger particles. Relaxivity values were determined by linear regression analysis of the experimentally determined R_1 values as a function of Gd^{3+} concentration in Excel.

ICP-OES Gd^{3+} Concentration Determinations. Concentration determination of gadolinium was performed using a Perkin Elmer Optima 2000 inductively coupled plasma optical emission spectrometer (ICP-OES). Gd^{3+} standards were produced by quantitative serial dilution of a commercial 1000 mgL^{-1} gadolinium in 2 % nitric acid standard (Fluka Analytical) into a 0.1% nitric acid solution. Samples were prepared by digesting the dried samples with 70% HNO_3 (100 µL) and water (900 µL). The resulting analyte was vortexed thoroughly, and then filtered to remove any particulate using a 0.2 µm filter to produce the final sample for ICP-OES analysis. Sample analyte concentrations were calculated to fall in the middle of the constructed ICP-OES Gd^{3+} calibration curve to ensure accurate gadolinium spectral readings. Readings were taken in triplicate and averaged. The highest percent relative standard deviation allowed between these replicates was 1%, to ensure precise Gd^{3+} spectral readings for the samples analyzed in this work. The concentration of Gd^{3+} was determined for each sample and then multiplied by the dilution factor.

Stability Tests. 100 mL of incubation bath solutions were prepared by adding a combination of HCl and NaOH to adjust the pH to 3, 5, 7, 9 and 11, $CaCl_2$ (0.06g, 0.05 mol), $MgCl_2$ (0.048g, 0.05 mol) and NaCl (1.169g, 0.2 mol) to water. Dialysis membranes (50 kDa MWCO) were loaded with suspensions of R = 0.3 $GdDOTP^{5-}$ loaded CACs by centrifuging a stock

suspension of CACs (2 mL, [Gd] = 0.2 mM) to remove the supernatant, the residue was then divided equally between each of the 8 dialysis membranes. Each dialysis membrane was placed in a separate incubation bath and the bath stirred at room temperature for 4 weeks. Immediately after the incubation period had been initiated and at intervals of 7 days a sample was removed from the dialysis membrane and analyzed by ^{1}H relaxometry and DLS (after dilution by about 1 order of magnitude). After 4 weeks the dialysis membrane was removed from the incubation bath and the CACs were analyzed by SEM.

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