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Crosslinked shells for nano-assembled capsules: A new encapsulation method for smaller Gd³⁺-loaded capsules with exceedingly high relaxivities†

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Abstract

Nano-assembled capsules can incorporate large payloads of high relaxivity Gd³⁺, permitting the development of highly detectable molecular imaging agents for MRI. A new encapsulating shell, based upon cross-linked peptides, is found to afford smaller capsules (127 nm average diameter) with exceptionally high per-Gd³⁺ relaxivities (70.7 s⁻¹mmol⁻¹).

Graphical abstract

Nano-capsules with robust, covalently bound, crosslinked shells can be used to encapsulate large payloads of MRI contrast agent, such as GdDOTP⁵⁻, that exhibit very high per-Gd³⁺ relaxivities.

†Electronic Supplementary Information (ESI) available: Experimental details, additional graphics and details of stability testing are provided. See DOI: 10.1039/x0xx00000x

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Low molecular weight Gd^{3+} chelates are widely used as contrast agents in anatomical and physiological magnetic resonance imaging (MRI). Dosing requirements for these agents are high (0.1 mmol kg^{-1}), reflecting the high detection limits of these agents.¹ The emerging field of molecular imaging envisions probing the molecular architecture of diseased tissue through the use of imaging agents that specifically highlight biomarkers of disease.²⁻⁴ Relevant biomarkers are most commonly present in concentrations several orders of magnitude lower than the detection limit of clinical contrast agents. If the advantages of MRI as an imaging modality – exquisite spatial resolution and superior safety profile – are to be exploited for molecular imaging applications then the detectability of contrast agents must be improved. Although a 15 to 20-fold improvement in relaxivity (effectiveness) may be possible for a single Gd^{3+} ion,⁵⁻⁸ increasing relaxivity alone cannot afford detection limits low enough for molecular imaging applications. Improving detection limits by several orders of magnitude will require incorporation of many high relaxivity Gd^{3+} ions into a single imaging agent.

Nano-assembled capsules (NACs)^{9, 10} are a robust and versatile method of achieving this goal.^{11, 12} NACs are formed by encapsulating an aggregate of poly-anionic Gd^{3+} chelate, such as GdDOTP^{5-} , and cationic polymer, such as PAH, within a silica nanoparticle (SNP) shell (Fig 1). NACs have substantial advantages over other methods of incorporating large payloads of Gd^{3+} chelate into a single structure. NACs can be prepared using cheaper, “off-the-shelf” chelates, it is not necessary to undertake synthetically challenging and expensive modifications to the chelate structure.¹³⁻¹⁶ NAC preparation is synthetically straightforward, robust and wastes little or no chelate. Because the molecular motion of the Gd^{3+} ion is strongly coupled to that of the NAC structure, there is no effective separation of local and global rotation, this lifts the limiting effect of rotation on the per- Gd^{3+} relaxivity that can be achieved.^{11, 12}

The NAC platform has proven to be extremely versatile, tolerant of variations in the structure and charge of the contrast agent, the nature of the cationic polymer, the composition of the solvent, and the charge ratio R (eqn 1).^{11, 12} The size of the NACs produced depends on each of these synthetic variables, but most particularly on the charge ratio R . Decreasing the value of R consistently afforded smaller NACs and smaller NACs were found to have higher per-Gd³⁺ relaxivities. Of the systems investigated thus far the smallest NACs and those with the highest per-Gd³⁺ relaxivity were produced by combining the penta-anionic chelate GdDOTP⁵⁻ and the cationic polymer polyallylamine hydrochloride (PAH), at $R = 0.3$ in 3:2 MeCN/H₂O.¹² These NACs had an average hydrodynamic diameter of 320 nm and a per-Gd³⁺ relaxivity of 46.4 s⁻¹mmolal⁻¹ (20 MHz and 298 K). Although the preparation of Gd³⁺-containing NACs is robust to many synthetic parameters, the SNP encapsulating shell has previously always been preserved.^{11, 12} This despite the fact that a variety of other encapsulating shells have been employed in other nano-assembled capsule applications.¹⁷⁻¹⁹

$$R = \frac{[\text{anion}] \times |z-|}{[\text{polymer}] \times |z+|} \quad \text{Eqn 1}$$

The encapsulation step in NAC preparation is achieved through the electrostatic attraction of SNPs to the aggregate surface. This SNP shell has proven quite permeable to water proton transport,¹¹ which is crucial if the encapsulated Gd³⁺ chelates are to operate as effective T_1 relaxation contrast agents. However, the electrostatic nature of this encapsulation strategy raises questions about the potential robustness of NACs *in vivo*. A new, three step, encapsulation method is reported herein that employs peptide coupling chemistry to build up a covalently linked capsule shell. The first step of the preparation of these Crosslinked nano-Assembled Capsules (CACs) is the same as for NACs – preparation of a charge driven aggregate (Fig. 1). Initial investigations were undertaken using simple diamagnetic polyanions. Aggregates were prepared by mixing solutions of PAH and pyrophosphate in water (pH 9). The encapsulating shell is then constructed by reaction of a diacidic acid with primary amines of the polymer on the aggregate surface. L-Glutamic acid was added to the aggregate in order to coat the surface, after ageing for 3 minutes the surface morphology

of the aggregate was found to change markedly as seen by comparison of the TEM images before and after coating (Figs 2A & B). The coated aggregates were then treated with the peptide coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 30 minutes in order to crosslink the aggregate and glutamate coating. The capsules were then purified by centrifugation and resuspension. TEM images of the final CACs (Fig 2C) clearly show the presence of an outer shell surrounding the inner aggregate.

CACs were produced using R values from 2.5 to 12.0 with pyrophosphate and PAH, and encapsulated using L-glutamate and EDC. This generated large capsules with average diameters of several microns. The robustness of this new synthetic methodology was verified by preparing CACs using poly-L-lysine and citrate, followed by coating with succinic acid and crosslinking with EDC. This showed that CAC formation was tolerant of variations in the poly-anion, polymer and dicarboxylic acid cross-linking reagent. Varying the nature of the various synthetic constituents did not appear to have a consistent effect on the size of the CACs produced.

This encapsulation strategy was then applied to the preparation of Gd^{3+} -containing CACs. The procedure followed as closely as possible that employed for the preparation of the smallest and most successful Gd^{3+} -containing NACs.¹² The polyanion (GdDOTP^{5-}) and cationic polymer (PAH) were stirred together at R ratios from 0.3 to 1.0 in 3:2 MeCN/ H_2O (pH 9). Treatment with succinic acid followed by EDC accomplished crosslinking and encapsulation. After purification by centrifugation through a 10kD MWCO centrifuge filter Gd^{3+} -containing CACs were obtained (Fig. 2D & E).

The hydrodynamic size and polydispersity of CAC samples were determined by dynamic light scattering (DLS) (Table 1). As with NACs, CAC size depends strongly upon the charge ratio R : lower R values producing smaller CACs. The dependence of capsule size on the R value was found to be significantly more pronounced for CACs than for NACs.^{11, 12} At $R = 1.0$ CACs are larger than the equivalent NACs by about 20%, but at $R = 0.3$ they are less than half the size of the equivalent NACs. As in the NAC preparations, we found that nearly all (> 98%) of the chelate was incorporated into the CAC, meaning that there is very little wasted chelate in the preparation of these CACs.

The CACs prepared with the lowest R values afforded the highest per- Gd^{3+} relaxivities, the same trend was observed for NACs.¹¹ This result was expected based on the size of the capsule alone. For an encapsulated chelate to be effective at shortening the water proton T_1 it is necessary that protons be able to rapidly diffuse between solvent water and chelates in the interior of the CAC. Since the rate of this diffusion is inversely proportional to the diameter of the spheroid, the per- Gd^{3+} relaxivity of larger CACs will tend to be limited by slow diffusion. The per- Gd^{3+} relaxivity of the largest CACs ($R = 1.0$) is comparable to that of NACs and only slightly higher than that of GdDOTP^{5-} itself.¹¹ When Gd^{3+} chelates are attached to the surface of macromolecular structures the coupling between global rotation (that of the macromolecule) and local rotation (the remaining freedom of motion of the chelate) can be weak.^{20, 21} This is thought to limit the gain in relaxivity because tumbling is not as slow as that of the macromolecule.²²⁻²⁵ GdDOTP^{5-} is capable of a very large number of “second-sphere” interactions,^{12, 26} which leads to a high second sphere contribution to

relaxivity that may be enhanced by causing the chelate to tumble more slowly.^{27, 28} These second-sphere interactions are responsible for anchoring the chelate within the capsule interior. Presumably these interactions hold the chelate quite rigidly in 3-dimensions which will tend to couple the molecular motion of GdDOTP⁵⁻ to that of the nano-capsule more effectively. The result is a chelate that has slow molecular rotation with many proximate protons that have comparatively long residence lifetimes in the second-sphere. These features will tend to render the encapsulated chelate a highly effective relaxation agent. The limiting factor in relaxivity is likely to be the rate at which protons diffuse between chelate and bulk water. Thus large CACs exhibit only modest gains in relaxivity owing to slow diffusion kinetics. In contrast, the smallest CACs ($R = 0.3$) have remarkably high per-Gd³⁺ relaxivities ($70.7 \text{ s}^{-1} \text{ mmol}^{-1} - 20 \text{ MHz}$ and 298 K). This compares favorably with some of the best relaxivities obtained for discrete octadentate Gd³⁺ chelates.²⁹ These CACs (diameter = 127 nm) are less than half the size of NACs produced at the same R value¹¹ and have a per-Gd³⁺ relaxivity that is nearly twice as high. It appears that the crosslinked encapsulating shell has good permeability to water (or water proton) exchange. Nonetheless, the encapsulating shell does seem to limit the per-Gd³⁺ relaxivity of CACs. GdDOTP⁵⁻ in aggregates with hydrogels of a similar size without an encapsulating shell has a slightly higher relaxivity.³⁰ However, aggregates without a robust encapsulation shell are unlikely to be suitable for *in vivo* use. CACs incubated in solutions of physiologically relevant metals and in solutions in the pH range 3 to 9 exhibited no change in per-Gd³⁺ relaxivity, size or CAC morphology (for more details see ESI). This suggests that CACs are resistant to degradation or loss of performance in physiological environments and are suitable for *in vivo* applications.

However, if CACs are to be incorporated into molecular imaging agents then it will be necessary to conjugate the capsule to a targeting biomolecule. Bioconjugation technology is now quite advanced and many technologies exist to facilitate this type of coupling.¹³ One of the most popular is the use of isothiocyanates to selectively couple primary amino groups under mild conditions.²² To establish whether free primary amines remain on the capsule surface after encapsulation CACs prepared with $R = 0.5$ were treated with the isothiocyanate-containing dye FITC. After washing thoroughly to remove unreacted dye, the CACs were imaged using confocal microscopy (Fig. 4). The intense green emission from the capsules indicates that FITC was able to access and react with primary amino groups on, or close to, the surface of capsules. It seems reasonable to suppose that the CAC surface can be readily functionalized using established bioconjugation techniques to produce super high relaxivity molecular imaging agents.

CACs are a new, robust and general method for encapsulating large payloads of Gd³⁺ chelate. These new CACs can be produced to be significantly smaller than the previously reported NAC system. In consequence it is possible to generate exceptionally high relaxivities for the Gd³⁺ chelates encapsulated within CACs. The encapsulating shell is robust under conditions relevant to physiological use, but also readily modified to permit bioconjugation and development of molecular imaging agents based on this system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

A schematic representation of the preparation of both NACs (encapsulation with SNPs) and CACs (encapsulation through peptidic cross-linking).

Fig. 2.

Electron micrographs of different stages of CAC preparation. A) TEM image of a single pyrophosphate/PAH aggregate ($R = 5$) showing the loose morphology of the aggregate; B) TEM image of a pyrophosphate/PAH aggregate with a glutamate coating showing the tighter surface morphology of the coated aggregate; C) TEM image of a pyrophosphate/PAH CAC after treatment with EDC showing the crosslinked capsule shell; and SEM images of GdDOTP⁵⁻/PAH CACs after treatment with succinic acid and EDC D) $R=0.5$ and E) $R = 1.0$.

Fig. 3.

A comparison of the the per-Gd³⁺ longitudinal relaxivities (20 MHz and 298 K) of CACs (red) and NACs (green) prepared using PAH and GdDOTP⁵⁻. Each value is the mean of six nano-capsule preparations, error bars (black) show the 1st standard deviation.

Fig. 4. Optical microscopy image of $R = 0.5$ CACs prepared with GdDOTP^{5-} and treated with FITC after. 63 $\times$ magnification; excitation laser line 488 nm; excitation wavelength – 500 nm; emission wavelength – 555 nm; scan format 512 $\times$ 512 pixels.

Table 1

The average hydrodynamic diameter, D (with 1st standard deviation of the size over 6 preparations) and the polydispersity index (PDI) determined by dynamic light scattering, and the percentage incorporation of GdDOTP⁵⁻ into nano-capsules (CACs and NACs) prepared with PAH in 3:2 MeCN/H₂O as a function of the R value used in their preparation. Data are the average of six separate preparations.

	$R = 0.3$	$R = 0.5$	$R = 0.75$	$R = 1.0$
<i>CACs</i>				
D (nm)	127 (2.3)	265 (2.5)	863 (10.9)	1534 (15.8)
PDI	0.20	0.27	0.32	0.14
% Gd	99.2	98.7	98.6	99.5
<i>NACs</i> ^[a]				
D (nm)	320	620	1147	1232
PDI	0.66	0.14	0.10	0.28
% Gd	99.9	99.1	98.9	98.6

^[a]Data from reference 12