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Supramolecular protamine/Gd-loaded liposomes adducts as relaxometric protease responsive probes

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ABSTRACT

A new approach to enzyme-responsive MRI agents based on the use of liposomes loaded with a high number of paramagnetic metal complexes (Gd-HPDO3A) is presented. It relies on the disruption of low relaxivity aggregates formed by liposomes and a macromolecular substrate that is selectively cleaved by the enzyme of interest. The interaction of anionic liposomes composed of POPC:CHOL:DPGS and the cationic protein protamine yields a poorly soluble supramolecular assembly endowed with a low relaxivity. The action of the serine protease trypsin causes the digestion of protamine and the consequent deassembly of the supramolecular adduct. The process is accompanied by an overall relaxation enhancement of solvent water protons as consequence of the dissolution of the aggregated liposomes. The observed increase of relaxivity is linearly dependent on the enzyme concentration.

An illustrative example of the possible use of the herein presented responsive agent has been reported. It consists of the entrapment of the supramolecular assembly in alginate microcapsules that have often been used as envelopes for in vivo applications of stem cells and pancreatic islets. The change in the observed longitudinal relaxation rate R_1 (leading to an hyperintense signal in the corresponding MR images) may act as a sensor of the protease activity in the biological environment in which the capsules is located.

1. Introduction

Magnetic resonance imaging (MRI) is a well-established diagnostic imaging technique based on the visualization of the very intense ^{1}H NMR water signal. The contrast in MR images arises mainly from differences in T_1/T_2 of tissue protons and can be improved by the use of paramagnetic contrast agents (CAs), which shorten the longitudinal (T_1) and transverse (T_2) relaxation times of water protons.[1–3] The most widely used class of MRI CAs is represented by paramagnetic chelates of the Gd^{3+} ion.[4,5] Their efficiency in enhancing the water proton relaxation rates ($1/T_1$ and/or $1/T_2$) is usually expressed by the longitudinal and transverse relaxivity values, r_1 and r_2, respectively (in s^{-1} per mM of Gd), measured at a given Larmor frequency and temperature.[1,6]

As far as molecular and cellular imaging applications are concerned, MRI, despite its high spatial resolution (tens of μm), is limited by the low sensitivity of its probes.[7] Due to this limitation, addressing molecular events at the cellular level by MRI requires large local concentrations of the CA in order to achieve a detectable contrast change. This is usually not feasible with targeted CAs containing as imaging reporter one or even a small number of Gd^{3+} chelates.[8] Thus, it is necessary to design amplification procedures that lead to the accumulation of a high number of imaging reporters at the site of interest. Among several possibilities, one route deals with the use of nanovesicular systems, like liposomes, that may be loaded with many Gd-containing complexes.[9–11]

Liposomes are nanocarriers made of naturally-occurring or synthetic phospholipids widely used as biocompatible systems for drug delivery.[4,9,12–14] The high versatility of such systems enables them to carry drugs with different physico-chemical properties, as well as MRI contrast agents.[6] Hydrophilic molecules can be easily encapsulated in the aqueous cavity of the vesicle, whereas hydrophobic compounds can be incorporated in the membrane bilayer. Thus, liposomes offer a solution to the low sensitivity of MRI, by increasing the local payload of Gd^{3+}chelates as reporter groups.[12]

So far, the main applications of the liposome-based agents in MRI have been in the field of tumor targeting either by exploiting the passive targeting of the vesicle (through the enhanced permeability and retention—EPR—effect)[15,16] or by active targeting through the conjugation of a specific vector on the surface of the liposome.[9,14,16–18] Thus, liposomes loaded with paramagnetic metal complexes yield an amplification of the MR response because the solvent water molecules have access to the intraliposomal compartment containing a high number of paramagnetic complexes. Concentrations up to hundreds millimolar of paramagnetic complexes in the aqueous cavities can be obtained, thus providing a powerful relaxation 'sink'

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for the 'bulk' solvent molecules.[4,12,19] The efficiency in transferring the paramagnetic effect to the bulk water is dependent on the permeability of the liposomal membrane to the water molecules. It is well established that the permeability of the membrane of liposomes is dependent on the formulation of the membrane components (i.e., through a proper modulation of the relative amounts of saturated and unsaturated phospholipids and cholesterol).[20]

As far as the design of a responsive agent is concerned, in analogy to what is done with simple molecular systems, the relaxivity of Gd-loaded liposomes has to be modulated by the specific parameter of interest that can affect one or more of its determinants.

In principle, the relaxometric response of a Gd-loaded liposome can be made dependent of processes like association, collapsing, degradation or precipitation. Any of these modifications is expected to have a profound effect on the relaxation enhancement brought by the paramagnetic metal complexes included or encapsulated in the liposomes. Whereas a change in the permeability may affect the accessibility of external water molecules to the paramagnetic centers, a tight association of liposomes may lead to an aggregation and insolubilization of the system with a consequent complete silencing of the paramagnetic effect on the longitudinal relaxation time of water protons. Conversely, the latter process can maintain and even increase the effect on transverse relaxation rates as result of the magnetic susceptibility brought about by compartmentalized systems.[21] On the other hand, the disruption of the liposomes may lead to the complete release of the paramagnetic payload, thus leading to a situation, as far the in vivo MRI application is concerned, ruled only by the biodistribution properties of the paramagnetic chelate.[14,16]

In this work, we propose an enzyme responsive MRI liposomal probe based on the aggregation/de-aggregation process. The aggregation step, promoted by an electrostatic interaction between anionic liposomes and the cationic protein protamine, leads to insoluble micro-sized particles that de-aggregate upon the action of the enzyme trypsin, a representative example of protease (Fig. 1).

2. Experimental

2.1. Preparation of paramagnetic liposomes

The lipids used in the liposomal membranes were purchased from Avanti Polar Lipids. They are 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol (CHOL), 1,2-dipalmitoyl-*sn*-glycero-3-succinate (DPGS) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethyleneglycol) 2000] (DSPE-PEG2000).

Gd-HPDO3A (HPDO3A = 1,4,7-tris(carboxymethyl)-10-(2′-hydroxypropyl)-1,4,7,10-tetraaza-cyclododecane) is commercially available from Bracco Imaging S.p.A under the trade name of ProHance®.

Liposomes have been prepared by the thin lipid film method. Briefly, the proper lipids mixture (POPC:CHOL:DPGS at a 2:2:1 molar ratio or POPC:CHOL:DPGS:DSPE-PEG2000 at a 2:2:0.9:0.1 molar ratio, total lipid amount 20 mg) were dissolved in the volatile solvent chloroform, which was slowly evaporated until a thin film was formed. The lipidic film was left under vacuum until total evaporation of chloroform and then hydrated with 1 mL of a 200 mM aqueous solution of the paramagnetic complex Gd-HPDO3A (ProHance). The suspension was then extruded through polycarbonate membranes with a pore diameter of 200 nm (Northern Lipids, USA) and the final suspension was purified from non-encapsulated metal complex by dialysis carried out against an isotonic HEPES buffer (5 mM HEPES, 0.15 M NaCl, pH 7.4). The mean hydrodynamic diameter of the particles was measured by dynamic light scattering (Zetasizer Nano ZS, Malvern, UK). The final concentration of Gd-HPDO3A in the liposome suspensions was ca. 5 mM as determined by NMR magnetic susceptibility measurements.[22]

2.2. Preparation of samples for relaxometric experiments

Protamine sulfate from salmon was purchased from Sigma–Aldrich. The protamine solutions used to aggregate the negatively charged liposomes were prepared at the proper concentration in PBS buffer.

Trypsin from bovine pancreas was purchased from Sigma–Aldrich (TPCK-treated, essentially salt-free, lyophilized powder, $\geqslant$10,000 BAEE units/mg protein). Trypsin solutions were prepared at the appropriate concentration in PBS buffer.

The association of anionic liposomes and protamine was performed always in the same order of addition, with the purpose of obtaining the highest reproducibility possible. Briefly, in several different vials, liposomes and protamine were incubated achieving the final concentration of 4 mg lipids/mL and 2 mg/mL, respectively. To this suspension, from now on named the particulate suspension, trypsin was added at different concentrations. Another set of experiments was performed using the same concentration of liposomes (4 mg lipids/mL) but a lower protamine quantity, accom-

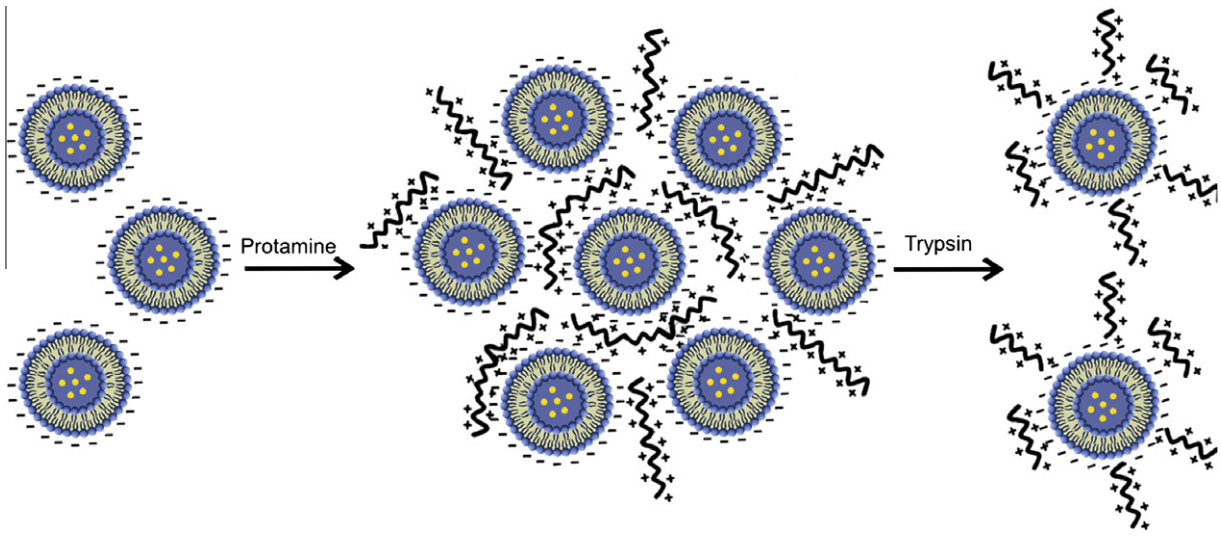

Figure 1. Illustration of the liposome aggregation induced by protamine and subsequent degradation of the supramolecular assembly by the action of trypsin.

plishing the final concentration of 0.2 mg/mL. The incubation with trypsin took place at a constant temperature of 37 °C under continuous stirring, in an Eppendorf Thermomixer.

Entrapment of the supramolecular protamine-Gd-liposome adducts into alginate capsules was performed following a previously reported experimental set-up.[23] Briefly, the supramolecular assembly to be encapsulated was re-suspended in low viscosity alginate, obtaining the final concentration of 1.5% (w/v) of alginate and 5 mg lipids/mL of liposomes. The suspension was then dripped into a 1.3% (w/v) solution of $CaCl_2$, and after that washed with HEPES buffer.

The water proton longitudinal relaxation times of anionic liposomes and particulate suspensions were measured using the usual inversion-recovery pulse sequence, on a Stelar Spinmaster spectrometer (Stelar, Mede, Italy), at 20 MHz and 25 °C. The observed T_1 value (T_{1obs}) was correlated to the effective millimolar concentration of the Gd(III) complex in the system through Eq. 1:

$$R_{1obs} = r_1 \times [Gd] + R_{1dia} \quad (1)$$

where $R_{1obs} = 1/T_{1obs}$, R_{1dia} is the diamagnetic contribution (i.e., R_{1obs} of the same solution non containing the paramagnetic complex), and r_1 is the relaxivity, that is, the paramagnetic relaxation rate enhancement per mM concentration of the Gd(III) complex.

3. Results and discussion

Anionic liposomes loaded with Gd-HPDO3A were obtained by means of the film hydration methodology. At 25 °C and 20 MHz, the relaxivity (per Gd^{3+} ion) of the obtained suspension is 1.1 s^{-1} mM^{-1}. This finding is an unambiguous indication that the solvent water molecules do not permeate freely across the liposome membrane. In fact, in the presence of the free water exchange between inner and outer liposome compartments, a relaxivity of ca. 4.2 s^{-1} mM^{-1} would have been measured, that is, the one corresponding to the aqueous 1 mM solutions of Gd-HPDO3A. The magnitude of the relaxivity decrease operated by the water diffusivity across the liposome membrane allows the estimation of the water permeability (P_w) of the liposomes.[20] Considering a membrane thickness of 5 nm and an intravesicular concentration of Gd-HPDO3A of 200 mM, the calculated P_w for the POPC:CHOL:DPGS (2:2:1) vesicles was 7.5 μm/s, that is, ca. threefold lower than the value reported for POPC/CHOL/DSPE-PEG2000 (11:8:1).[20] Likely, the reduced permeability is related to the presence of the saturated dipalmitoyl-based DPGS that increases the membrane packaging.

Through light scattering and electrophoretic mobility measurements it was possible to determine the size and appropriate charge (*Z*-potential) of the anionic Gd-loaded liposomes, which resulted to be ca. 165 nm (diameter) and −35 mV, respectively. From the knowledge of the liposome size (under the same assumption done for the P_w calculation) one may conclude that each vesicle contains ca. 2.35×10^5 Gd(III) complexes and, consequently, the relaxivity per liposome is ca. 2.5×10^5 mM^{-1} s^{-1}.

The supramolecular protamine-Gd-liposome adducts were obtained by adding aliquots of a solution of protamine (a positively charged protein containing ca. 66 residual positive charge per molecule) to the anionic liposomes suspension. The addition of protamine to the liposome suspension resulted in the immediate formation of large aggregates, whose hydrodynamic diameter ranged from ca. 2.5 to 7.0 μm depending on the relative ratio between the negative charges brought by the anionic liposomes and the positive charges brought by the protein. Larger adducts were obtained in the presence of an excess of positive charges (i.e., on going from 1:2 to 1:4 ± charge ratios). Interestingly, no aggregation phenomena was observed using liposomes containing 2 mol % of DSPE-PEG2000 in their membrane. This finding demonstrates that the increased hydrophilicity and the steric hindrance of the DSPE-PEG2000 component were able to prevent the formation of strong electrostatic interaction between the liposomes and the cationic protein.

The aggregates were almost silent from the relaxometric point of view, and the small contribution to the relaxation enhancement measured (0.2 s^{-1} mM^{-1}) was likely due to the small soluble portion escaping the tight binding interactions that characterize the whole aggregate. Hence, the protein acts as a sort of glue towards the negatively charged liposomes, thus creating a further barrier to the exchange of water molecules across the liposomes membranes. The aggregated particles appear quite stable as the longitudinal relaxation enhancement of solvent water protons did not change over a period of several weeks.

To assess whether the particles are responsive in terms of changes of water proton relaxation rate upon progressive cleavage of peptide bonds of the protein, the liposomal aggregates were added with variable amounts of trypsin, a common serine protease. This enzyme predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine and arginine, except when they are followed by proline. After the addition of trypsin to the substrate, the adduct diameter decreased down to ca. 300 nm, that is, to a value close to the original size of Gd-loaded liposomes. Conversely, no effect was observed when trypsin was added to free liposomes. Thus, trypsin yielded a progressive reduction of liposomes–protamine aggregates through the cleavage of protamine.

Figure 2 shows the effect of the addition of protamine on the r_1 values of liposomes suspension at two ± charge ratios and the effect of subsequent addition of trypsin.

One hour after the addition of trypsin, the relaxivity markedly increased to a value (1.8 s^{-1} mM^{-1}) slightly higher than the one measured for free liposomes incubated at 37 °C for the same time (1.3 s^{-1} mM^{-1}). These data support the view that trypsin, through the cleavage of protamine, restores the conditions existing before the addition of the cationic protein. Furthermore, the slight increase in the observed r_1 values with respect to the control vesicles may reflect some changes in the liposome properties (water permeability, vesicle stability) occurring after their interaction with protamine and/or upon the addition of the proteolytic enzyme.

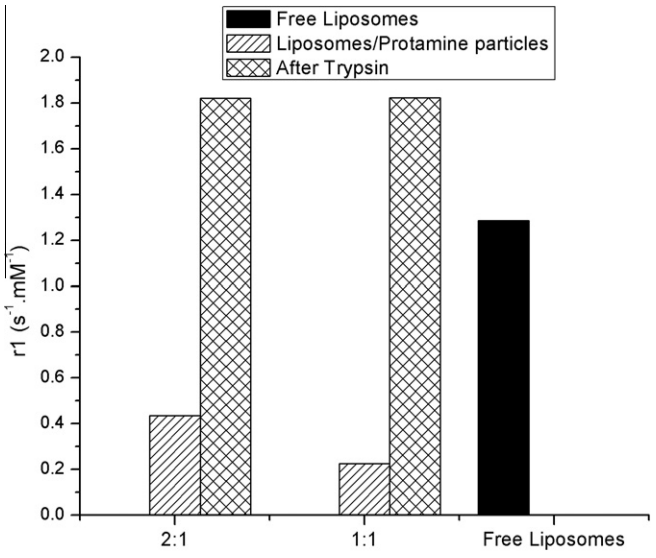

Figure 2. Effect of liposome–protamine ratio (represented by ± charge ratio) on the r_1 relaxivity of aggregates formed between negatively charged liposomes encapsulating Gd-HPDO3A and protamine. The effect of trypsin (1 mM, 1 h incubation under continuous agitation at 37 °C) is also presented. Both effects are compared with those obtained for the liposomes in the absence of protamine. The liposomal membrane is formed of a mixture of POPC:CHOL:DPGS in the molar proportion 2:2:1.

Figure 3 reports the dependence of the r_1 values (measured at 20 MHz and 25 °C) on the trypsin concentration (60–150 nM range) 6 h after the addition of the enzyme to the liposomal aggregates. The enzymatic degradation of protamine was carried out at the enzyme optimum temperature (37 °C) and a good linearity has been observed between r_1 values and the concentration of the enzyme.

This finding demonstrates that it is possible to determine protease activity by a simple relaxometric assay that may be translated into the design of a MRI-based method to determine local enzyme concentrations in vivo.

The size of these protamine/liposomes aggregates is not compatible with their in vivo use based on intravenous administration, because in this case one needs to deal with particles that have to be (max) in the hundred(s) of nanometer dimension. However, the herein reported particles may find potential application as in vivo MRI sensors of protease activity when they are suitably enclosed in given devices and located at the biological site of interest. For instance, the herein developed responsive agents may report about the protease activity in the surroundings of transplanted cells such as stem cells or pancreatic islets. To get more insight into this potential application, the aggregates were encapsulated into alginate capsules, a vehicle that is currently under intense scrutiny in the field of transplantation and cell-based therapy. An analogous approach might be tackled to assess enzymatic activities in the microenvironment of a scaffold designed for homing and differentiating stem cells.

The protamine/liposomes aggregates, generated as described above, were trapped in an alginate matrix following the same procedure previously reported for cells and liposomes entrapment.[24,25] The pore size of the alginate matrix can be tailored according to the environment to be engineered. The herein formed matrix contains pores that are large enough to allow trypsin to enter the intravesicular space and small enough to prevent the release of liposomes once the particles have been de-assembled. Figure 4 reports the observed R_{1obs} values of alginate capsules entrapping liposome/protamine aggregates upon addition of trypsin at two concentrations (0.4 and 4 mM). R_{1obs} increased to reach the saturation value after approximately 5 h from the addition of the enzyme to the alginate suspending medium. Overall, the observed behavior closely parallels what is described above for the particles suspended in aqueous medium.

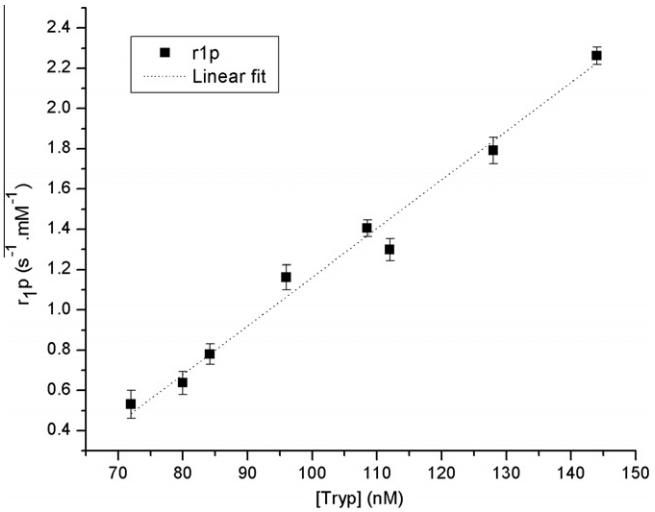

Figure 3. Linear dependence of the r_1 relaxivity of the complexes formed between negatively charged liposomes encapsulating Gd-HPDO3A and protamine, after incubation (6 h under continuous agitation at 37 °C) with different concentrations of trypsin. The liposomal membrane is formed of a mixture of POPC:CHOL:DPGS in the molar ratio 2:2:1.

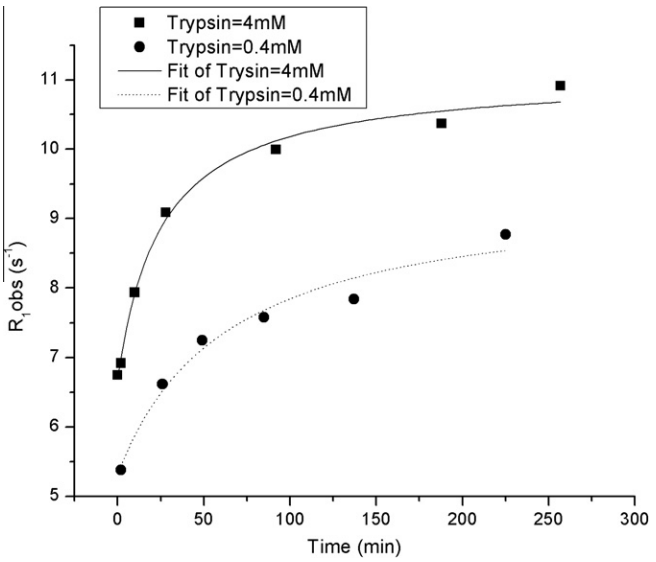

Figure 4. Observed R_1 values of a suspension containing protamine/liposomes aggregates entrapped in alginate capsules upon trypsin addition (0.4 and 4 mM). The amount of liposomes and protamine in the solution added to alginate was 5 mg lipids/mL and 0.125 mg/mL, respectively. The curves represent a simple hyperbolic fit for guiding-eye.

The performance of the proposed concept was also assessed in vitro on an imaging scanner. To this purpose, the T_1 contrast of a preparation of alginate capsules entrapping aggregated liposomes externally added with 4 mM of trypsin was compared with a control sample added with PBS. The two systems were put in two 5 mm glass tubes surrounded by agarose gel and imaged at 7 T at room temperature. The result shown in Figure 5 clearly indicates the significant contrast enhancement detected in the sample treated with the enzyme.

4. Concluding remarks

The main existing methods used to determine enzymatic activities are based on the assessment of the rate of a reaction catalyzed by the given enzyme, using either natural or synthetic substrates, such as BAEE (Nα-Benzoyl-L-Arginine Ethyl Ester) or the Biuret reagent. For these rate measurements, the concentration of protein is determined by ultraviolet spectrophotometric absorption, as it has been described extensively over the years by several research groups.[26] As a proof of concept, a new relaxometric approach is hereby presented, which allows the determination of an enzyme concentration using as substrate a complex formed between negatively charged liposomes and a small cationic protein, available for both

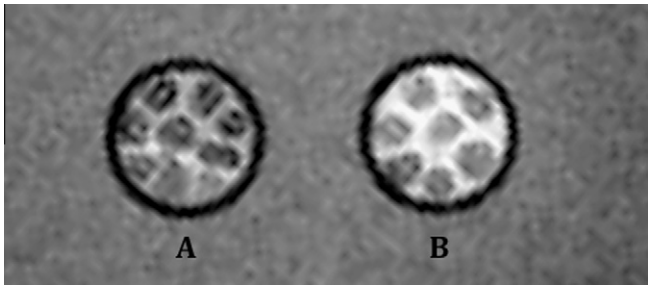

Figure 5. MRI-T_1 weighted image of two capillaries (A and B) containing alginate capsules entrapping liposomes' particles. (B) Report about the trypsin action on the Protamine/Gd-loaded liposome supramolecular adducts entrapped in the alginate matrix. The image was obtained using a standard T_1-weighted multislice multiecho sequence (TR/TE = 250/3.3).

MRI and in vitro assessments. It is known that the concentration of several proteases of biological and pathological relevance is comprised within a range that can be considered with the herein reported method. For instance, human prostatic epithelial cells continuously secrete prostate-specific antigen (PSA), a kallikrein-like serine protease, to the seminal plasma, constituting one of the most abundant proteases in this fluid, in a concentration of about 1.0 mg/ml (~30 nM).[27] Another protein that may be taken into account is trypsinogen, the precursor of trypsin. It has been reported that the concentration of two different types of trypsinogen is 26 μg/L (~1.1 nM) for trypsinogen-1 and 50 μg/L (~2.1 nM) for Trypsinogen-2 in transplanted patients having cholangiocarcinoma.[28]

Overall, the methodology presented here appears suitable for a conventional in vitro quantification of protease enzymes, being based on the capacity of protamine to bind a negatively charged liposomal formulation. Upon interaction with the negatively charged liposomes, this small cationic protein causes their association, which may finally lead to their precipitation, reducing the r_1 relaxivity of the solution. The ability to enhance water proton relaxation rates is recovered once the macromolecular association is cleaved by the serine protease trypsin, leading to a relaxivity enhancement. Another point of interest concerns the versatility of the method hereby presented. Selecting the appropriate substrate, the work presented here could be expanded to any enzyme of interest. Moreover, the limiting concentration of serine protease that can be detected is dependent mainly on the substrate concentration (small cationic protein protamine).

Finally, an in vivo exploitation of the herein described particles has been proposed. It relies on the possibility of entrapping micron-sized particles into an alginate matrix that has often been considered as a bio-compatible device in cell-based therapies. The protamine/liposomes particles may act as a protease sensor of the local microenvironment in which the device is located. The characteristics of the alginate matrix are such that one can easily modulate the size of the holes in order to get a good control of the way-in/way-out of the substances according to their size.

The herein reported methodology may be easily extended to assess other types of enzymatic activity through the proper selection of the complementary recognition characteristic of the given enzyme–substrate and the liposome outer surface.

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References and notes

1. Aime, S.; Botta, M.; Fasano, M.; Terreno, E. *Chem. Soc. Rev.* **1998**, *27*, 19.
2. Aime, S.; Botta, M.; Terreno, E. *Advances in Inorganic Chemistry—Including Bioinorganic Studies*, van Eldik, R., Bertini, I., Eds., Elsevier: San Diego, CA, USA, 2005; Vol. 57, p 173.
3. Strijkers, G. J.; Mulder, W. J.; van Tilborg, G. A.; Nicolay, K. *Anticancer Agents Med. Chem.* **2007**, *7*, 291.
4. Aime, S.; Castelli, D. D.; Terreno, E. *Methods Enzymol.* **2009**, *464*, 193.
5. Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293.
6. Geraldes, C. F.; Laurent, S. *Contrast Media Mol. Imaging* **2009**, *4*, 1.
7. Aime, S.; Delli Castelli, D.; Gianolio, E.; Geninatti Crich, S.; Terreno, E. *Acc. Chem. Res.* **2009**, *42*, 822.
8. Caravan, P. *Chem. Soc. Rev.* **2006**, *35*, 512.
9. Esposito, G.; Geninatti Crich, S.; Aime, S. *ChemMedChem* **2008**, *3*, 1858.
10. Terreno, E.; Cabella, C.; Carrera, C.; Delli Castelli, D.; Mazzon, R.; Rollet, S.; Stancanello, J.; Visigalli, M.; Aime, S. *Angew. Chem., Int. Ed.* **2007**, *46*, 966.
11. Terreno, E.; Delli Castelli, D.; Violante, E.; Sanders, H. M.; Sommerdijk, N. A.; Aime, S. *Chem. Eur. J.* **2009**, *15*, 1440.
12. Mulder, W. J.; Strijkers, G. J.; van Tilborg, G. A.; Griffioen, A. W.; Nicolay, K. *NMR Biomed.* **2006**, *19*, 142.
13. Delli Castelli, D.; Terreno, E.; Carrera, C.; Giovenzana, G. B.; Mazzon, R.; Rollet, S.; Visigalli, M.; Aime, S. *Inorg. Chem.* **2008**, *47*, 2928.
14. Torchilin, V. P. *J. Mol. Recognit.* **1996**, *9*, 335.
15. Maeda, H.; Bharate, G. Y.; Daruwalla, J. *Eur. J. Pharm. Biopharm.* **2009**, *71*, 409.
16. Torchilin, V. P. *Eur. J. Pharm. Sci.* **2000**, *11*, S81.
17. Mendonca, L. S.; Firmino, F.; Moreira, J. N.; Pedroso de Lima, M. C.; Simoes, S. *Bioconjugate Chem.* **2010**, *21*, 157.
18. Santos, A. O.; da Silva, L. C.; Bimbo, L. M.; de Lima, M. C.; Simoes, S.; Moreira, J. N. *Biochim. Biophys. Acta* **2009**.
19. Strijkers, G. J.; Hak, S.; Kok, M. B.; Springer, C. S., Jr.; Nicolay, K. *Magn. Reson. Med.* **2009**, *61*, 1049.
20. Terreno, E.; Sanino, A.; Carrera, C.; Castelli, D. D.; Giovenzana, G. B.; Lombardi, A.; Mazzon, R.; Milone, L.; Visigalli, M.; Aime, S. *J. Inorg. Biochem.* **2008**, *102*, 1112.
21. Terreno, E.; Delli Castelli, D.; Viale, A.; Aime, S. *Chem. Rev.* **2010**, *110*, 3019.
22. Corsi, D. M.; Platas-Iglesias, C.; van Bekkum, H.; Peters, J. A. *Magn. Reson. Chem.* **2001**, *39*, 723.
23. Dhoot, N. O.; Wheatley, M. A. *J. Pharm. Sci.* **2003**, *92*, 679.
24. de Vos, P.; Faas, M. M.; Strand, B.; Calafiore, R. *Biomaterials* **2006**, *27*, 5603.
25. Dai, C.; Wang, B.; Zhao, H.; Li, B.; Wang, J. *Colloids Surf., B Biointerfaces* **2006**, *47*, 205.
26. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
27. Webber, M. M.; Waghray, A.; Bello, D. *Clin. Cancer Res.* **1995**, *1*, 1089.
28. Lempinen, M.; Isoniemi, H.; Makisalo, H.; Nordin, A.; Halme, L.; Arola, J.; Hockerstedt, K.; Stenman, U. H. *J. Hepatol.* **2007**, *47*, 677.