"Uncorking" of liposomes by matrix metalloproteinase-9†

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Received (in Cambridge, MA, USA) 4th November 2004, Accepted 19th November 2004
First published as an Advance Article on the web 10th January 2005
DOI: 10.1039/b416827e

A triggered release methodology of liposomal contents *via* the enzyme MMP-9 is described.

Liposomes enjoy a special place in drug delivery for their biocompatibility and the ability to encapsulate hydrophilic molecules in the aqueous interior.[1] To the best of our knowledge, there are only a few reports on the enzyme triggered release of liposomal contents. The triggering enzymes used in such rare studies include elastase, alkaline phosphatase, trypsin and phospholipase A_2.[2] The matrix metalloproteinases (MMPs) are a family of endopeptidases, containing Zn^{2+} at their active sites.[3] Under normal physiological conditions, MMPs are responsible for embryonic development, wound healing, tissue remodeling, angiogenesis, *etc.*[3] Although many of these MMPs have been implicated in human diseases, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) have been widely recognized to be involved in the progression and metastasis in most of the human tumors.[4]

Herein, we report a triggered release methodology of liposomal contents *via* the enzyme MMP-9. To demonstrate this, triple helical collagen-mimetic peptides were conjugated to stearic acid and the resultant lipopeptides were incorporated into liposomes. These liposomes, when exposed to a catalytic amount of MMP-9, efficiently released the encapsulated fluorescent dye (5-carboxyfluorescein), in the surrounding medium. To the best of our knowledge, this is the first example of the matrix metalloproteinase mediated release of liposomal contents.

Gelatins are the natural substrates for the enzyme MMP-9.[5] For these studies, a mimetic peptide was designed with a triple helical structure, containing the cleavage site for the enzyme MMP-9 [**P1**, H_2N-GPQ**GI**AGQR$(GPO)_4$GG-OH, the cleavage site is indicated in bold]. This peptide was conjugated to stearic acid to generate the corresponding lipopeptide **LP1** [$CH_3(CH_2)_{16}$COHN-GPQ**GI**AGQR$(GPO)_4$GG-OH]. Four repeat units of the amino acid triad Gly–Pro–Hyp (GPO) were incorporated in the peptide to impart the triple helical structure.[6] **P1** and **LP1** were synthesized by the solid-phase peptide synthetic protocol. The resultant products were purified by RP-HPLC (C_{18} column), and characterized by circular dichroism (CD) and mass spectroscopy (MALDI-TOF, Electronic Supporting Information†).

In the CD spectra, the triple helical peptides are characterized by strong positive maxima centered at 220–225 nm and an intense negative band located at 196–200 nm.[6] Both the peptide and lipopeptide showed a positive peak around 225 nm, and a negative peak at 200 nm, suggesting their preponderance in the triple helical forms in aqueous solution (Fig. 1A and ESI†). The Rpn values for **P1** and **LP1** were calculated as being equal to 0.06 and 0.11 respectively (for natural collagen, Rpn = 0.13).[7] Temperature dependent CD spectra of **LP1** (Fig. 1A) showed an isosbestic point at 213 nm, suggesting its equilibrium distribution between the two alternative conformational states (*e.g.*, single stranded ↔ triple helical). The melting temperature (T_m) was calculated (by plotting the CD_{225} as a function of temperature) to be 57 °C (ESI†). Since the peptide **P1** did not show any sigmoidal melting curve, no T_m could be assigned for this peptide (ESI).[8] From the shape of the

† Electronic supplementary information (ESI) available: peptide synthesis and purification protocols; CD spectra of **P1** and **LP1** and temperature dependent CD spectra; details for the cleavage and leakage studies with MMP-9. See http://www.rsc.org/suppdata/cc/b4/b416827e/

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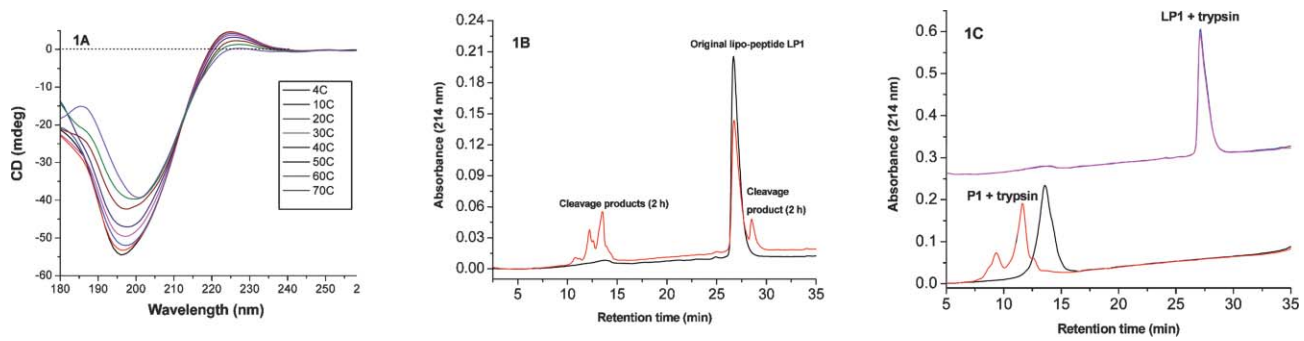


Fig. 1 The temperature dependent CD spectra for **LP1** are shown in 1A. For these experiments, the conditions are: [**LP1**] = 1 mg mL^{-1} in 10 mM phosphate buffer, pH 4.0. The peptide solution was stored at 4 °C for 12 h before recording the spectra. The sample was equilibrated for 15 minutes at each temperature before recording the spectrum. The HPLC elution profile of the lipo-peptide **LP1** before and after incubation with MMP-9 for 2 h is shown in 1B. The HPLC elution profiles of **P1** and **LP1** before and after incubation with trypsin are shown in 1C. For clarity, the elution profile of **LP1** is plotted with an offset. For the cleavage experiments, the conditions are: [**P1**] or [**LP1**] = 1 mg mL^{-1} in 25 mM HEPES buffer, pH = 8.0 containing 10 mM $CaCl_2$; [enzyme] = 5 nM.

melting curve (ESI), it appears that **LP1** forms aggregates of triple helices in solution.[8] Detailed structural studies on these aggregates are in progress and will be reported in the future.

Cleavage studies were performed using the recombinant form of human MMP-9, containing the catalytic and fibronectin domains of the enzyme (ESI†). Solutions of **P1** (or **LP1**) were incubated with catalytic amounts of the enzyme and the reaction was stopped at defined intervals by adding trifluoroacetic acid to the reaction mixture. The products were analyzed by RP-HPLC. The peptide **P1** (Rpn = 0.04) was efficiently cleaved by the target enzyme MMP-9 (ESI) as well as by a non-specific proteolytic enzyme, trypsin (Fig. 1C). However, the lipopeptide **LP1** (Rpn = 0.11) was partially cleaved by MMP-9 (in 2 hours, Fig. 1B), but was *not* cleaved at all by trypsin (Fig. 1C). This suggests that unlike trypsin, MMP-9 specifically cleaves the above lipopeptide. Since the amino acid sequences in both **P1** and **LP1** are the same, the inability of trypsin to cleave the lipopeptide (**LP1**) is presumably because the enzyme fails to unwind its triple helical structure in order to effect the cleavage.[9] In addition, in the matrix metalloproteinase family, MMP-9 is known to preferentially cleave triple helical peptides.[10]

The peptide sequence of **LP1** is derived from the amino acid sequence of type I collagen. From literature reports, it is expected that collagenases will cleave **LP1** faster than the gelatinases.[11] Since the focus of these studies is gelatinase-B (MMP-9), no cleavage studies were performed involving **LP1** and the collagenases (MMP-1, -8 and -13).

Liposomes were prepared (1 mg mL^{-1} of total lipid in 25 mM HEPES buffer, pH = 8.0) with the synthetic lipopeptide **LP1** (10 mol%) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (90 mol%) by following the standard procedure.[1] The liposomes were encapsulated with a self-quenching dye, 5-carboxy fluorescein.[12] The dye has the excitation maximum at 495 nm, and the emission maximum at 527 nm.

In the lipid bilayers of the liposomes, due to the proximity, the peptide groups of **LP1** are expected to form triple helices. Structural studies on the conformation of **LP1** in the liposomes (employing CD spectroscopy and atomic force microscopy) are in progress and will be reported elsewhere. The peptides on the outside surface of the liposomes will be recognized and cleaved by MMP-9. After the cleavage, the liposomes will be destabilized, leading to "uncorking" and release of the encapsulated carboxyfluorescein dye.

The release of carboxyfluorescein was monitored as a function of time after adding the enzyme, MMP-9 (Fig. 2; experimental details are provided in the ESI†). The liposome solution was excited at 480 nm, and the increase in the fluorescent intensity was monitored at 518 nm. There was a time lag of about 5 minutes

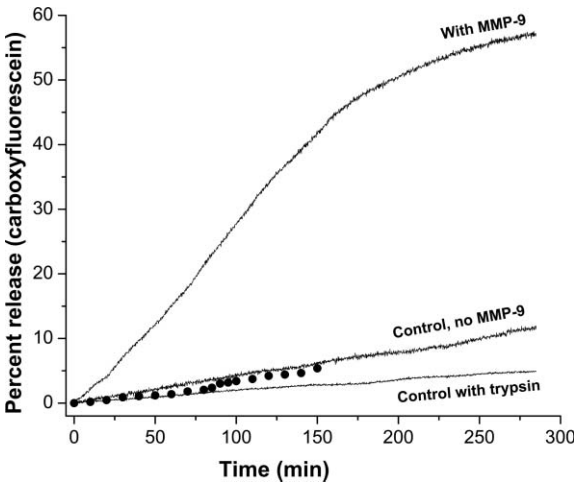
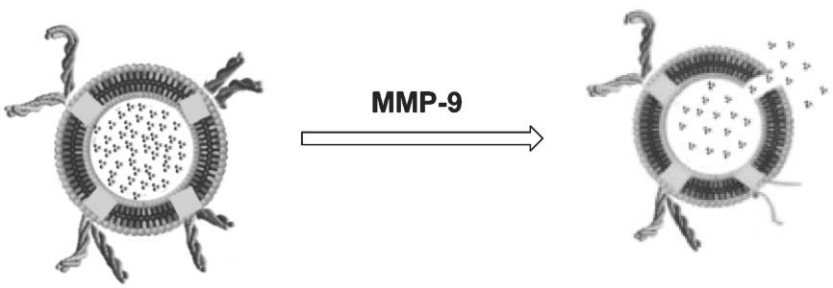


Fig. 2 Increase in fluorescence intensity due to the release of carboxyfluorescein is shown. MMP-9 released 55% of the encapsulated dye. The background release observed without any enzyme or in the presence of trypsin are also shown. The circles indicate the release profile from DSPC liposomes (containing no **LP1**) in the presence of MMP-9. For these experiments, the enzyme (10 μL of a 200 nM solution) was added to a 2 mL of liposome solution in 25 mM HEPES buffer, pH = 8.0, containing 10 mM $CaCl_2$.

prior to attainment of a steady-state phase in the fluorescence emission intensity. In five hours, about 55% of the encapsulated dye was found to be released (Fig. 2). In contrast, only 10% of dye was released from the liposomes during this time without any enzyme. The proteolytic enzyme, trypsin, once again failed to release the dye from the liposome, presumably due to its inability to cleave the liposomal triple helical peptides. As an additional control, liposomes were prepared from DSPC only. These liposomes did not release any dye when treated with either MMP-9 (Fig. 2, circles) or with trypsin (data not shown).

Based on literature reports,[13] it is anticipated that the liposome bilayers exist as a mixture of coexisting phases. After the cleavage of **LP1** on the outside surface of the liposomes by MMP-9, the lipids lose the hydrophilic head group. This possibly increases the phase heterogeneity of the liposome bilayer with concomitant increase in the liposomal permeability. Detailed studies on the molecular mechanism of "uncorking" are currently in progress.

In conclusion, we have demonstrated that the enzyme MMP-9 can be used as a trigger to release liposomal contents. The triple helical peptides act as "baits" for the enzyme. A non-specific proteolytic enzyme (*e.g.*, trypsin) fails to cleave the lipopeptides from the liposomes, and thus no dye release takes place (illustrated



Scheme 1 The release of liposomal contents (containing the lipopeptide **LP1**) mediated by MMP-9 is schematically illustrated.

in Scheme 1). If the liposomes contain encapsulated inhibitors of MMP-9, this triggered release methodology can be employed to attain the "suicidal" inhibition of the enzyme.[14] A detailed account of such studies to decipher the molecular mechanism of the liposome uncorking, its content release, and the suicidal inhibition of MMP-9, is currently being pursued, and the results will be reported in the future.

This research was supported by the National Institutes of Health grants 1R15 DK56681-01A1 to DKS and 1P20 RR 15566-01 to SM.

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14 A provisional patent application has been filed by the North Dakota State University.