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MMP-9 triggered micelle-to-fibre transitions for slow release of doxorubicin

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Phenylacetyl-peptide amphiphiles were designed, that upon cleavage by a disease-associated enzyme reconfigure from micellar aggregates to fibres. Upon this morphological change, a doxorubicin payload could be retained in the fibres formed, which makes them valuable carriers for localised formation of nanofibre depots for slow release of hydrophobic anticancer drugs.

Peptide self-assembly is increasingly investigated for a plethora of applications in biomedicine including drug release, tissue engineering, diagnostic studies and regenerative medicine.[1] These nanostructures are of interest as they may contain bioactive peptide ligands, as well as structural components which enable access to a variety of nanoscale morphologies dictated by the amino acid sequence[2] but also by the route of assembly[3]. Enzymatic catalysis presents an attractive way to control molecular self-assembly.[4] In this approach, non-assembling precursors that are "blocked" with enzyme cleavable groups are converted to self-assembling building blocks (including hydrogelators), enabling self-assembly on-demand under physiological conditions. The most frequently studied biocatalytic self-assembly systems are those based on aromatic peptide amphiphiles. These consist of short peptide sequences modified with aromatic groups such as phenyl, naphthyl, fluorenyl and others.[5] Different enzymes such as phosphatases[4b,6] and proteases (including matrix metalloproteinases (MMPs))[7] have been used to trigger molecular self-assembly *in vitro* and *in vivo*[6c,8].

Expression levels of enzymes dictate the difference between health and disease in many cases, including cancer. MMPs are a family of zinc dependent endopeptidases that are involved in the digestion and remodelling of the extracellular matrix.[9] Some members of this family, such as MMP-9 have been reported to be overexpressed in breast, cervical, colon and other types of cancer.[10] This makes them valuable triggers for responsive biomaterials and targeted self-assembly. Typically, MMP responsive peptide-based systems act *via* hydrolysis and dissociation of structures i.e. using enzymatic cleavage to trigger dissociation of hydrogels (containing MMP sensitive crosslinks)[11], supramolecular peptide filaments[12] and polymer-peptide hybrids[13]. The first example of the use of MMP-9 to form (rather than degrade) a peptide based, supramolecular hydrogel was presented by the Xu group.[7a] A morphology change induced by

MMP-7 was previously shown for an aliphatic, palmitylated peptide amphiphile system.[14] A very recent study shows the development of a selective assay for MMP-9 *via* gelation.[15]

Based on this knowledge we set out to develop a peptide based enzyme-responsive system that is able to undergo a morphological change from micellar aggregates to fibres in response to cleavage by MMP-9 (Fig. 1) and use it for localised formation of a depot for slow release of hydrophobic drugs (e.g. doxorubicin) at the cancer site. There are three design requirements for such a system: (i) a biocompatible fibre forming self-assembly unit (Phenylacetyl-FFAG) that also provides the hydrophobic binding region for drug candidates, (ii) the MMP-9 cleavable sequence and (iii) a hydrophilic unit (LDD) that modifies the amphiphilic balance of the precursor to favour micelle formation (Fig. 1). Thus, upon MMP-9 cleavage, the peptide micelles reconfigure into fibres, due to a change in hydrophobic/-philic balance of the sequence. In turn, this aspect may be used for hydrophobic drug entrapment into fibres, presenting a unique advantage for the development of drug delivery systems for prolonged release times after initial exposure.
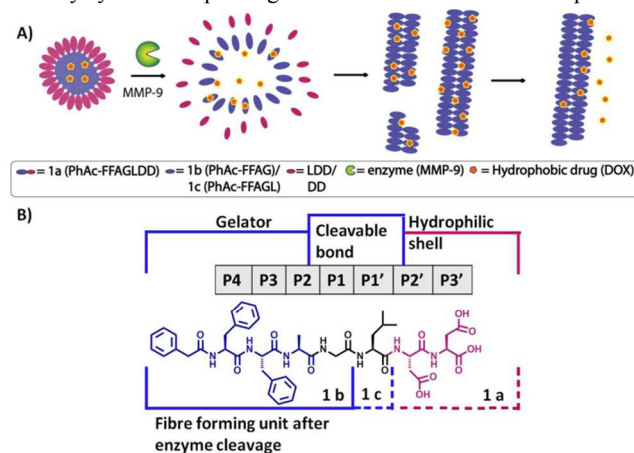

Figure 1. A) Schematic representation of micelle to fibre transition induced by MMP-9 cleavage showing disassembly of micelles and the re-assembly into fibres after the removal of the hydrophilic group enabling prolonged drug release. B) Chemical structure of the biocatalytic gelation system and its components.

In order to design the enzyme cleavage site, the MEROPS[16] database was used, which provides cleavage patterns for peptidases based on a collection of experimental data from

literature. This can be used for the design of substrates that simultaneously meet the three criteria mentioned. MMP-9's specificity preference for P4 – P3' subsites (based on 367 cleavages reported in literature) is based on the $GPX_1G\downarrow LX_2G$ sequence with G/L (P1-P1') being the cleavable bond, X_1 (P2) being preferentially alanine or leucine, and X_2 (P2') glycine as the preferred choice.[16] MMP-9 requires longer substrates, of for *e.g.* 7 residues (i.e. P4-P3'), in order to be able to recognise and efficiently cleave the G↓L bond[17], with GPLG↓LAG being an example.[18] The length of the substrate and the presence of large substitutions (i.e. pyrene, naphthalene, etc.) at the N-terminus can lead to a shift in specificity of MMP-9.[7b] To fulfil the requirement of the gelator (fibre forming) unit in the P3 and P4 positions phenylalanine-phenylalanine[19] was used (proline and glycine preferred, but phenylalanine known to be tolerated in P3 and P4). In P2 we chose alanine, while in P2' and P3' (referred to as amino acids flanking the scissile bond towards the C-terminus) aspartic acid-aspartic acid, was used, as it is known to be tolerated in both positions, and will provide a negatively charged surface of the micellar aggregates.

First the PhAc-FFAGLDD (**1a**) and its expected product of enzyme cleavage PhAc-FFAG (**1b**) were synthesized and characterized by AFM, FTIR, DLS, rheology and fluorescence.
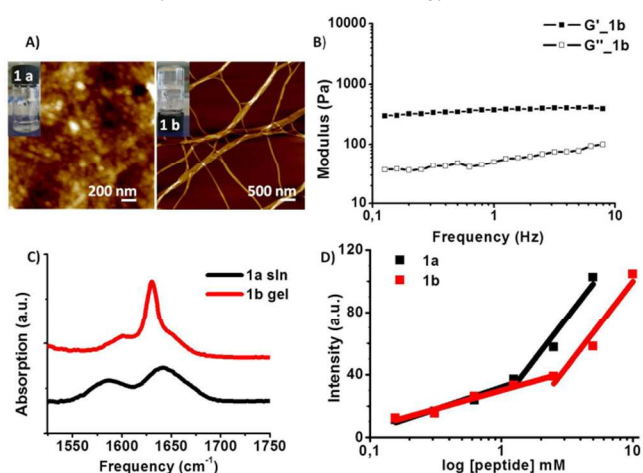

Figure 2. A) AFM showing the micellar aggregates (solution) for **1a** and fibres (hydrogels at 20 mM) for **1b**. B) Rheology data for **1b** gel showing the plot of G' (elastic modulus) and G'' (viscous modulus) against frequency. C) FTIR absorption spectrum in the amide I region (in D_2O at pH 7): **1a** (solution) and **1b** (gel). D) Critical aggregation concentration (CAC) of (1.32 mM (**1a**) and 2.88 mM (**1b**).

The peptide (**1a**) was directly dissolved in deionized water, the pH adjusted to 7.4 and its self-assembly behaviour investigated after a cycle of alternating sonication and vortexing. For the expected enzyme cleavage peptide fragment i.e. **1b** the peptide was dissolved in DI water and the pH was increased (NaOH 0.5 M) to solubilise **1b**, followed by a slow decrease of pH achieved by addition of HCl 0.5 M, to a final pH of 6.5 – 7. This slightly acidic pH corresponds to that of the tumour microenvironment.[20] Gelation was observed for the expected MMP-9 cleavage product **1b** (Fig. 2A).

The AFM characterisation of the peptides revealed spherical aggregates for **1a** (d=43.6 ± 6.2 nm) while fibres were found for **1b** having micron scale length and 20-50 nm range diameter which corresponds to the size of peptide based fibres reported in literature.[2c] Alternative supramolecular organisation was further supported by infrared (IR) spectroscopy data (Fig. 2C) suggesting the presence of ordered structures for the examined peptides due to aggregation *via* intramolecular hydrogen bonding.[21] Peptide **1a** shows a red shift and a broad peak at 1643 cm^{-1} compared to 1650-1655 cm^{-1} absorption values, typical for free peptides in solution. The 1570-1580 cm^{-1} absorption band is attributed to the aspartic acid side chain carboxylate group present in **1a**.[21] Extended structures are observed for **1b** (transparent gel) characterised by a pronounced narrowing of the peak typical for short peptide β-sheets at 1630 cm^{-1}, while the 1595 cm^{-1} characteristic of the C-terminus carboxylate group has a low intensity.[21-22] Rheology measurements of the **1b** hydrogel show the elastic modulus (G') of 360 Pa, an order of magnitude higher than its viscous component (G''= 32 Pa) which is characteristic of entangled networks (Fig. 2B). Furthermore, DLS experiments (Fig. S3 and S4, ESI) were performed for peptide solution samples **1a** and **1b** at various concentrations ranging from 0.625 mM to 5 mM. The diffusion coefficients (D) of samples 1a (micellar aggregates) and 1b (fibres) at 0.625 mM are 1.5×10^{-12} m^2s^{-1} and 6.8×10^{-13} m^2s^{-1} corresponding to R_H values of 165 nm and 358 nm, respectively. The higher values of R_H compared to AFM (dry samples) indicate that in the solution state the aggregates are bigger than the collapsed, dried ones.

In order to further investigate the self-assembly behaviour of peptide amphiphiles the critical aggregation concentration (CAC) in water was determined by using the fluorescence intensity of the 8-Anilino-1-naphthalenesulphonic acid (ANS probe) as a function of the peptide concentration (Fig. S5, ESI). The determined CAC values were 1.32 mM for PhAc-FFAGLDD and 2.88 mM for PhAc-FFAG. Furthermore, the critical micelle concentration (CMC) for **1a** was determined by using pyrene as fluorescent probe. The ratio of the fluorescence intensity of the first (λ_{em}=372 nm) and the third peak (λ_{em}=384 nm) was plotted as the function of the peptide concentration. The value calculated to be 1.25 mM (Fig. S6, ESI) is in the same range of the CAC values for **1a**.

After the designed peptides **1a** and **1b** were shown to be successful for controlling the morphology of the supramolecular aggregates based on the peptide length *i.e.* hydrophobicity, the enzyme triggered micelle to fibre transition for **1a** peptide amphiphiles was explored. Peptide amphiphile **1a** was treated with 50 ng/mL MMP-9 for 96 h and the morphological change was monitored by AFM, where fibre formation was observed (Fig. 3B). This enzyme concentration was chosen based on MMP-9 quantification *in vitro* of human cancer cell lines. Peptide **1a** showed complete conversion after 96 h to PhAc-FFAGL (**1c**) fragment (Fig. S7, ESI) indicating the shift of the MMP-9 specificity for this heptapeptide to GL↓D instead of the expected G↓L in accordance with reported observations that cleavage sites of heptapeptides catalysed by MMP-9 differ from those of longer peptide sequences.[7b] According to MEROPS leucine and aspartic acid are tolerated in P1 and P1' positions respectively, but they do not seem to be preferentially recognised and reported as a cleavage site for MMP-9.

Following this it was investigated whether the micelles were capable of performing as mobile vehicles for encapsulation to immobilised fibre networks for release of hydrophobic drugs. For

this purpose, the release of an anticancer drug, doxorubicin, was studied. Doxorubicin was loaded into the micelles and its release by passive diffusion was monitored over time by fluorescence microscopy. Fluorescence intensity of doxorubicin at 596 nm which corresponds to the maximum intensity (λ_{ex}=480 nm) was monitored over 96 h when incorporated into the **1a** peptide system. A control experiment with free doxorubicin shows a decrease in fluorescence intensity over time, probably due to aggregation. Due to its poor solubility in water, fluorescence quantification of doxorubicin is not reproducible however it becomes more stable when incorporated into peptides, becomes more stable. The interaction of doxorubicin with the hydrophobic environment of **1c** results in higher values of fluorescence intensity compared to free doxorubicin in water (doxorubicin emission in solvents of different polarity is shown in Fig. S8, ESI). For **1a** doxorubicin fluorescence intensity drops only slightly over 48 h suggesting that the payload stays incorporated into micelles over that time period showing only low release, followed by significant decrease after 72 h. When treated with MMP-9 there is a release from micelles (significant drop in fluorescent intensity upon exposure to water) followed by entrapment into fibres (resulting in an increase in fluorescent intensity). A similar discontinuous behaviour upon phosphatase triggered gelation was recently reported for a related system.[23] Even if the overall release after 48 h is similar for the MMP-9 treated and untreated **1a** the advantage of the enzymatic approach is that a localised effect is obtained with fibres expected to be significantly less mobile that the micelles.

After fibre formation, the increase of fluorescence intensity suggests that the doxorubicin re-enters a hydrophobic environment by becoming entrapped in the fibres, confirming the possibility of the system to temporarily retain the payload. TEM images were obtained on enzyme treated doxorubicin loaded peptides that confirm fibre formation and show that the presence of doxorubicin did not disrupt fibre formation (Fig. 3C).
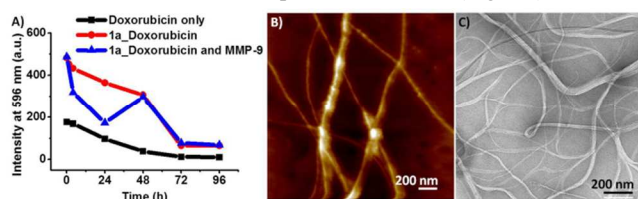

Figure 3. A) Fluorescence intensities of doxorubicin monitored over time for doxorubicin only, doxorubicin loaded into precursor peptide (**1a**) micelles and MMP-9 treated precursor peptide (**1a**) micelles loaded with doxorubicin. B) AFM images of MMP-9 induced fibre formation (PhAc-FFAGL) (**1c**) after 96 h C) TEM images of doxorubicin loaded samples treated with MMP-9 for 72 h showing that fibre formation was not disrupted by the presence of the drug.

In conclusion, an MMP-9 responsive peptide amphiphile is shown here that self-assembles into spherical aggregates. Enzyme triggered micelle to fibre transition shows that it is a substrate of MMP-9 and is capable of encapsulation and controlled release of doxorubicin. These observations suggest the use as a mobile carrier for the anticancer drug that in turn is expected to be selectively delivered to tumor tissues, where it assembles to form a localised fibre-based depot by exploiting local MMP-9 overexpression. Furthermore, the assembled fibres provide a scaffold for localised drug delivery due to the partial entrapment

of the drug and the intrinsic biodegradable nature of peptide carriers themselves. These systems are now studied in animal models. It should be noted that when used *in vivo* the system may also respond to other MMPs and specifically to MMP-2 due to some overlapping in specificity profiles.[24]

Notes and references

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