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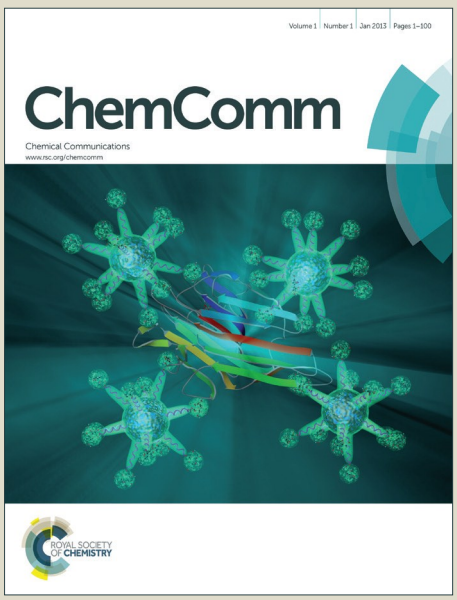

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Journal Name

COMMUNICATION

Dual-Responsive Nanoparticles Release Cargo Upon Exposure to Matrix Metalloproteinase and Reactive Oxygen Species

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Micellar nanoparticles were designed to be responsive to matrix metalloproteinases (MMPs) and reactive oxygen species (ROS), each of which is upregulated in the pathology of inflammatory diseases. The amphiphilic polymer-based nanoparticle system consists of a hydrophilic shell responsible for particle morphology change and aggregation, together with a hydrophobic block designed to release cargo in the presence of ROS.

In this paper, we describe the design of an enzyme-responsive micellar nanoparticle[1-6] carrying a latent hydrophobic protease inhibitor, whose release, and thus activation, from the hydrophobic core of the micelle copolymer is triggered by reactive oxygen species (ROS). Dual-responsive systems and enzyme-responsive cargo release systems have been of increasing interest and have well-documented in the field;[7-13] however, this work is motivated by observations of coincident upregulation of both ROS, namely H_2O_2, and matrix metalloproteinases (MMPs) in the pathology of many inflammatory diseases, including myocardial infarction,[14] arthritis,[15, 16] ischemia,[17-19] and atherosclerosis,[20] as well as during tumor cell invasion in certain cancers, including colon cancer[21] and melanoma.[22] MMPs are expressed and excreted initially as zymogens and their activity is tightly regulated in normal physiology.[23] MMPs become activated in the presence of proteases, other MMPs, or by ROS.[24] Hence, the interplay between ROS and MMPs is linked to disease progression.[18, 24]

Amphiphilic block copolymers were designed and synthesized using ring opening metathesis polymerization (ROMP), a robust polymerization technique[25] that uses a highly functional group tolerant Ru-based initiator[26-29] to generate well-defined, low dispersity polymer systems. These copolymers consist of an inactive MMP inhibitor as the hydrophobic block, coupled with a hydrophilic block consisting of a peptide MMP substrate. Hence, the latent MMP inhibitor is sequestered in the hydrophobic core, while the hydrophilic MMP substrate forms the shell of the resulting micellar nanoparticles (Figure 1). Upon exposure to the enzyme, the peptide is cleaved inducing aggregation.[1-6] As a control, a *d*-amino acid version of the peptide substrate (**PSC**, Figure 1) was synthesized, as a sequence insusceptible to enzymatic degradation.

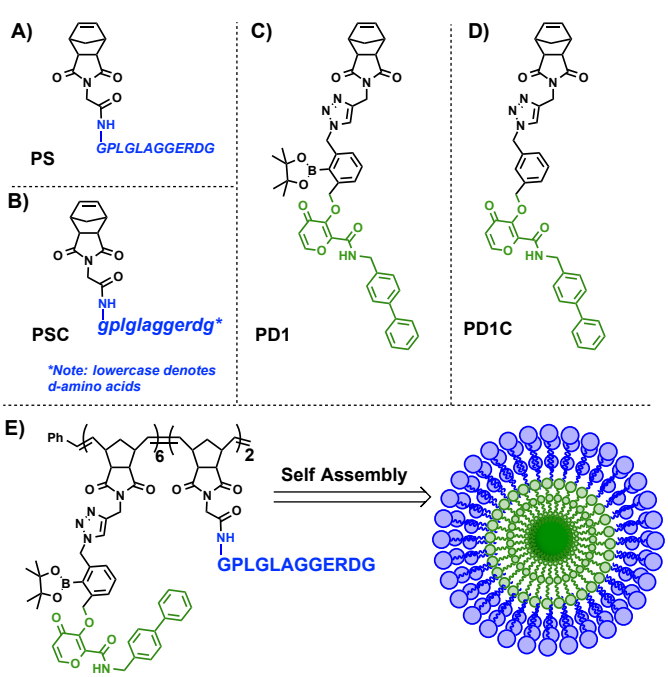

Figure 1: Monomer, polymer, and nanoparticle structures. A) **PS** peptide substrate, B) **PSC** peptide substrate control, C) **PD1** prodrug-1 and D) **PD1C** prodrug-1 control. E) Upon dialysis from DMSO into aqueous media, the polymers assemble into micelles with cores composed of the hydrophobic **PD1** or **PD1C**, and shells composed of **PS** or **PSC**.

The hydrophobic block of the polymer, which forms the micellar nanoparticle core, contains a H_2O_2-sensitive prodrug (**PD1,** Figure 1). Specifically, an aryl boronic ester is covalently

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Electronic Supplementary Information (ESI) available: Synthetic schemes, NMR data, and experimental details. See DOI: 10.1039/x0xx00000x

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appended to an MMP inhibitor rendering the inhibitor inactive. This moiety, containing a self-immolative linker, is optimized to be hydrolytically stable under normal physiological conditions.[30] Nucleophilic attack by H_2O_2 results in the expulsion of a phenolate intermediate, which spontaneously releases the MMP inhibitor (previously designated as PY-2)[31] via an intramolecular cascade (see ESI). PY-2 was utilized in these studies, as it exhibits excellent potency against a variety of MMPs.[31]

Analytical HPLC was used to evaluate the sensitivity of **PD1** to H_2O_2 under simulated physiological conditions (50 mM HEPES, pH 7.4). HPLC confirmed quantitative conversion of **PD1** to PY-2 after H_2O_2 treatment, which contained a peak at the same retention time of an authentic sample of PY-2 (see ESI). A control compound, **PD1C**, a direct analog of **PD1** (Figure 1D) lacking the boronic ester motif, is completely unreactive toward H_2O_2, as evidenced by analytical HPLC.

With these results in hand, amphiphilic copolymer systems incorporating different combinations of **PD1**, **PD1C**, **PS**, and **PSC** were generated (Figure 1E) via ROMP. As the hydrophobic:hydrophilic ratio of the polymer governs the ability to form nanoparticles, the block lengths for each system were optimized individually for both cargo loading and micellization ability. It is known that proteolytic susceptibility of peptide-containing polymers decreases as brush density increases[32] thus a short hydrophilic block was most desirable. To maximize the cargo loading of the hydrophobic block while maintaining both the proteolytic susceptibility of the polymers and capability to form spherical micelles upon dialysis, each nanoparticle system was polymerized to a hydrophobic block length of ~6 and a hydrophilic block length of ~2 (Table S1). As negative control nanoparticles, *d*-amino acid containing analogues of all systems were made (**PSC**), as well as systems with H_2O_2-inactive monomers (**PD1C**).

From these polymers, a set of nanoparticle systems (**PD1-PS**, **PD1-PSC**, and **PD1C-PS**) was prepared via dialysis in phosphate-buffered saline (DPBS, 1×) against DMSO. The hydrodynamic radii of all systems were determined by dry state transmission electron microscopy (TEM, see ESI). All systems formed spherical nanoparticles with approximate hydrodynamic radii of 20 nm.

To address the effect of both MMP and H_2O_2 on the structure of the particles and the release of cargo, we probed the behavior of each system in response to three different conditions: i) MMP only, ii) H_2O_2 only, or iii) concurrent treatment of H_2O_2 and MMP. The concentrations used of H_2O_2 and MMP-12 were 6 mM and 100 nM, respectively. Each nanoparticle system (**PD1-PS**, **PD1-PSC**, and **PD1C-PS**) was subjected to each of the three treatments, with no stimulus added as a negative control (Figure 2). Catalytic amounts of MMP-12 were used, as it is known that that PY-2 is a potent inhibitor of MMP-12 (IC_{50} = 85 nM)[31]. The results of these experiments with respect to the efficacy of the particles to aggregate are shown in Figure 2. Upon exposure to MMP, both **PD1-PS** and **PD1C-PS** formed micron-scale aggregates. However, as **PD1-PSC** is MMP-inactive, no morphology change was observed upon MMP exposure. Importantly, none of the systems experienced a shift in morphology when treated with H_2O_2 alone, indicating that MMP is necessary.

To explore the ability of these systems to release their cargo and inhibit MMP activity, the particles were first incubated in a 96-well plate at 37 °C in the presence of both MMP-12 (1:1000 ratio **PS**:enzyme) and H_2O_2 (100:1 ratio H_2O_2:**PS**). After 4 hours (the incubation time necessary to reach maximum cleavage of **PS**, see ESI), a fluorogenic substrate of MMP-12 was introduced to each well and the fluorescence intensity monitored for 60 minutes. As PY-2 is liberated from the nanoparticle, the compound inhibits MMP activity. The greater the extent of release, the greater MMP inhibition will be. This is observed as a change in the rate of cleavage of a fluorogenic substrate (Figure 3). Indeed, the fastest relative increase in fluorescence is observed for **PD1C-PS**, where the drug core cannot be liberated by H_2O_2. Conversely, the fully degradable system, **PD1-PS**, shows inhibition of MMP activity nearly matching that of an authentic sample of PY-2 at the same concentration (300 mM). Interestingly, some suppression of MMP activity is observed for **PD1-PSC**, which may indicate that H_2O_2 is able to penetrate the particle core, and that PY-2 can be liberated due to H_2O_2 triggering alone.

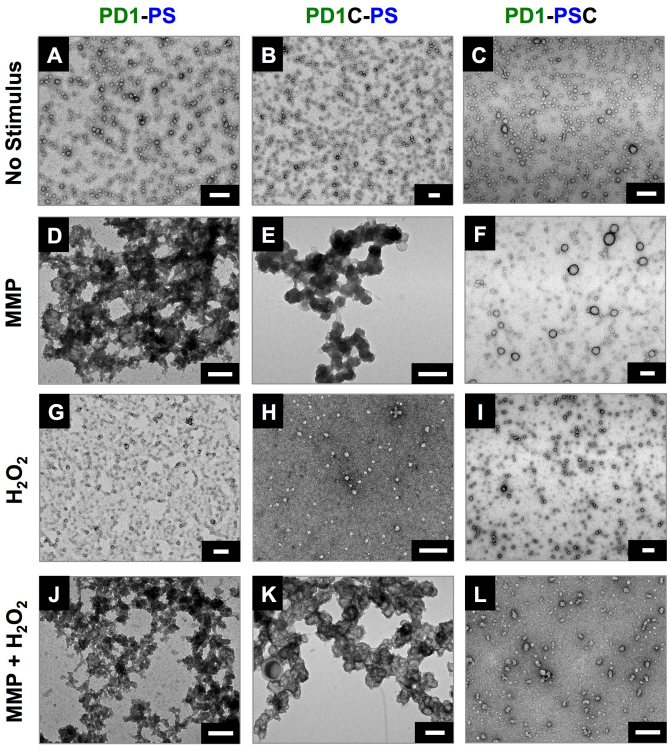

Figure 2: Effect of stimuli on nanoparticle structure. When no stimulus is applied, all polymer systems, **PD1-PS**, **PD1C-PS**, and **PD1-PSC** (**A**, **B**, and **C**, respectively) form spherical micelles. All scale bars are 100 nm. Upon exposure to MMP only, **PD1-PS** (**D**) and **PD1C-PS** (**E**) form micron-scale aggregates, while **PD1-PSC** (**F**) does not. When only H_2O_2 is applied, all systems remain as spherical nanoparticles (**G**, **H**, **I**). When both MMP and H_2O_2 are introduced into the systems, again only **PD1-PS** (**J**) and **PD1C-PS** (**K**) form micron-scale aggregates, while **PD1-PSC** (**L**) remains as spherical nanoparticles.

In summary, exposure of our micellar system to MMP

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results in a drastic shift in particle size and morphology. This change, together with the presence of H_2O_2, results in cargo release. Further, the payload released is itself an inhibitor of MMP enzymatic activity, giving this material the potential to not just respond to the inflammatory environment, but to directly address inflammation by release of MMP inhibitors. Future studies will center on the optimization of this system and the investigation of its applicability as a drug delivery vehicle for malignancies in which both MMPs and ROS are upregulated.

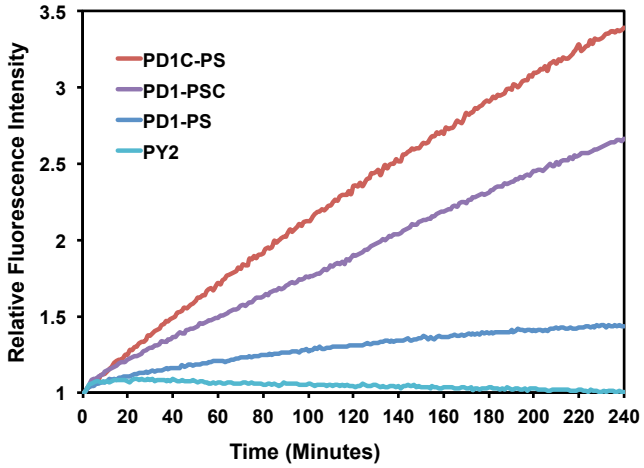

Figure 3: Effect of nanoparticles on enzyme activity after incubation with both MMP and H_2O_2. **PD1-PS**, **PD1-PSC**, **PD1S** and an authentic sample of the MMPi **PY-2** were incubated concurrently with MMP and H_2O_2 for 4 hours, at which point a fluorogenic substrate of MMP was introduced (SI). Rate of increase in fluorescence is directly correlated to MMP activity.

Notes and references

We thank the UC San Diego Molecular Mass Spectrometry Facility (MMSF) for assistance with experiments. This work was supported by a grant from the National Institutes of Health, National Institute of General Medical Sciences (R01 GM098435; K.B.D., S.M.C.), as well as the National Institute for Biomedical Imaging and Biomedical Engineering (R01 EB011633; C.E.C., N.C.G.). In addition, we thank the Army Research Office (ARO) for their support for this work (W911NF-14-1-0169). C.E.C. thanks the Cancer Researchers in Nanotechnology Program at UCSD for fellowship support.

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