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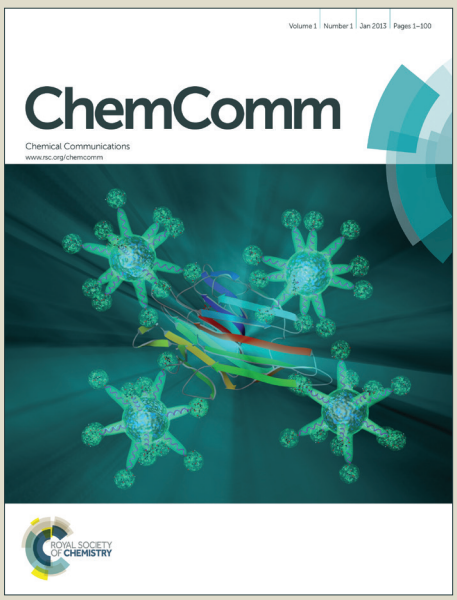

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The Formation of Biodegradable Micelles from a Therapeutic Initiator for Enzyme-Mediated Drug Delivery

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The direct grafting of amphiphilic macromolecules by sequential n-carboxyanhydride ring-opening polymerisation (NCA ROP) from a therapeutic initiator enables the formation of monodisperse drug-containing micelles. The subsequent enzyme-mediated hydrolysis of the peptide component permits the programmed release of the encapsulated drug molecules, demonstrating a controlled drug delivery platform that negates any challenging payload loading procedures.

Highly controlled stimuli-responsive release mechanisms permit the programmed release of payload molecules from a carrier vehicle on-demand.[1] Polymers are often employed as drug carriers to provide protection against premature drug metabolism and clearance *in vivo*.[2] Changes in environmental pH, temperature and ionic strength may be utilised to alter the polymer properties and mediate the controlled delivery of guest molecules at a target site.[3] In addition, proteolytic enzymes may be exploited to trigger the swelling or degradation of (poly)peptide-containing carrier vehicles, resulting in payload release.[4] Such enzyme-responsive materials may be targeted towards proteases that are over-expressed at particular disease sites, thus providing this class of biomaterial significant medicinal relevance.[5]

NCA ROP permits the generation of homopeptides that possess properties analogous to proteins, but are generated by a straightforward, controlled polymerisation that avoids the practical and economic demands associated with solid phase peptide synthesis.[6] Homopeptides are particularly useful as biomaterials as they possess the capability to form secondary structures, have general low toxicity, and may possess targeted biodegradability. Consequently, this class of polymer has been exploited for use in numerous applications including drug delivery, tissue regeneration and biomineralisation.[7]

A frequently demonstrated method of controlled drug delivery utilises therapeutic molecules that are non-covalently loaded within polymeric structures before their release upon interaction with a targeted stimulus. This approach enables relatively high loading quantities within the polymer, but the process of drug loading can be challenging and may result in the loss of valuable drug molecules. A method that restricts molecular loss during payload loading into the polymer architecture is to initiate NCA ROPs directly from the drug molecule, affording a structure capable of self-assembling into micelles. The nanoparticles formed contain drug molecules covalently bound within a core surrounded by a biodegradable shell that, upon targeted degradation, permits the release of the therapeutic initiator on-demand.

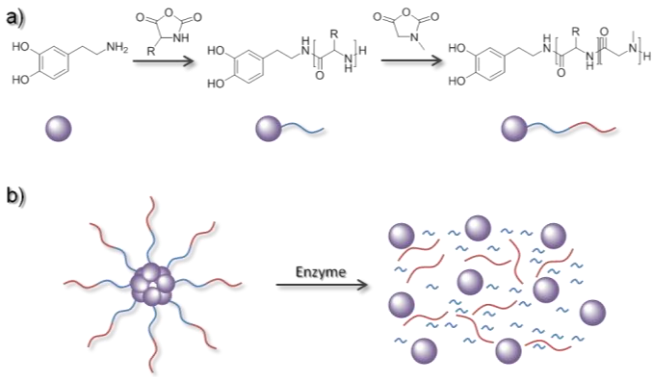

Figure 1. a) Dopamine acts as the therapeutic initiator for the sequential ROP of a hydrophobic amino acid NCA, followed by sarcosine NCA. b) Dopamine-conjugated amphiphilic block copolymers form micelles in aqueous solution. Hydrolysis of the peptide component by protease enzymes results in micelle disassembly, and the release of dopamine molecules.

In this paper, we describe the formation of biodegradable micelles formed by NCA ROP initiated from a therapeutic initiator (dopamine hydrochloride). Hydrophobic peptides are grafted from the primary amine group of dopamine hydrochloride, followed by the grafting of hydrophilic sarcosine peptoids to yield an amphiphilic dopamine-terminated macromolecule, by a one-pot synthetic strategy. The micelles formed contain a peptide component that is capable of actuating dopamine release to an extent and rate, dependent on the protease enzyme that it is reacted with. To our knowledge, this is the first example of NCA ROP being carried out directly from a therapeutic initiator to afford peptide-containing self-assembled structures that may be selectively degraded by protease enzymes to release a therapeutic cargo.

Dopamine hydrochloride, a hormone and neurotransmitter that is

Table 1. The theoretical composition and molecular weights of the dopamine-conjugated block copolymers produced. Molecular weights were determined by Electrospray ionisation mass spectrometry (ESI-MS) and ^{1}H NMR. Dop signifies dopamine. Sar and Ala represent sarcosine and alanine respectively.

Theoretical Composition	Mw by ^{1}H NMR (g/mol)	Mw by ESI MS (g/mol)
Dop-$(Ala)_5$-$(Sar)_{15}$	1573	1715
Dop-$(Phe)_5$-$(Sar)_{15}$	2019	2160

prescribed to correct hemodynamic status in patients suffering from shock syndrome, was selected as the therapeutic initiator in this proof-of-concept study. Patients suffering from Parkinson's disease lose dopaminergic cells in the substantia nigra region of the brain resulting in depleted levels of dopamine. As such, dopamine is also prescribed to regulate the effects of Parkinson's disease.[8] The presence of a primary amine group capable of initiating NCA ROP, and dopamine hydrochloride's complete solubility in *N*,*N*-dimethylformamide make this therapeutic molecule particularly suited for the grafting of peptides from it. In addition, Dimitrov and Schlaad disclosed that primary amine hydrochlorides promote highly-controlled NCA ROP by negating the formation of NCA anions, which cause significant premature chain termination.[9]

Alanine and phenylalanine amino acids were independently converted to their corresponding NCAs and reacted with dopamine hydrochloride to induce their ring opening and the formation of short hydrophobic peptides conjugated to the dopamine initiator. The NCA of sarcosine, a hydrophilic but charge neutral amino acid, was then reacted with the dopamine-peptide to yield a dopamine-terminated hydrophilic-hydrophobic macromolecule capable of self-organisation into micelles (Figure 1). Sarcosine was selected as the hydrophilic component within the structure due to its reported non-toxicity, which contrasts to other peptides such as poly-L-lysine.[10] It has been demonstrated that polysarcosine possesses excellent resistance to nonspecific protein adsorption and cell attachment,[11] and biocompatibility.[12]

The length of the hydrophobic peptide segment was limited by the solubility of the growing dopamine-peptide conjugate in DMF. The molecular weight of the amphiphilic dopamine-containing macromolecules, as determined by ^{1}H NMR and ESI-MS (supporting information), is given in Table 1. The NCA ROP was limited to an average of five and four repeat amino acid units for alanine and phenylalanine per dopamine-initiated chain respectively, as determined by ^{1}H NMR. A kinetic study to determine the rate of ROP of the amino acid NCAs found that the grafting of the peptide segment was complete within 96 hours in both cases, and the desired amphiphilic architecture was created within 192 hours (supporting information). The actual compositions of the diblock copolymers produced were Dop-$(Ala)_5$-$(Sar)_{15}$ and Dop-$(Phe)_4$-$(Sar)_{18}$, according to ^{1}H NMR analysis, and Dop-$(Ala)_5$-$(Sar)_{17}$ and Dop-$(Phe)_4$-$(Sar)_{20}$, according to ESI-MS analysis. The former composition will be used to describe further exploitation of these materials.

The capability of the dopamine-containing diblock copolymers produced to self-assemble into micelles by nanoprecipitation in phosphate buffered saline (PBS) solution was assessed by dynamic light scattering. It was revealed that the micelles formed from Dop-$(Ala)_5$-$(Sar)_{15}$ possessed a mean diameter of 204.7 ± 12.7 nm and micelles formed from Dop-$(Phe)_4$-$(Sar)_{18}$ possessed a mean diameter of 250 ± 31.4nm. The stability of the micelles formed was assessed over time. It was found that after remaining in PBS solution at room temperature, the average diameter of Dop-$(Ala)_5$-$(Sar)_{15}$ nanoparticles increased by 2.4% over 14 days and the average diameter of Dop-$(Phe)_4$-Sar_{18} particles increased by 5.0% over 14 days. Scanning electron microscope images of Dop-$(Ala)_5$-$(Sar)_{15}$ and Dop-$(Phe)_4$-$(Sar)_{18}$, together with the size distribution chart obtained by DLS in each instance and a representative plot displaying the stability of Dop-$(Ala)_5$-$(Sar)_{15}$ micelles in the first 90 minutes after formation, are provided in Figure 2. DLS was then used to determine the critical micelle concentration (CMC) of both diblock colpolymers to determine the suitability of using the self-assembled materials *in vivo*. The CMC of Dop-$(Ala)_5$-$(Sar)_{15}$ was found to be 4.01×10^{-6} mol/L (i.e. 5.99 µg/mL) and the CMC of Dop-Phe_4-Sar_{18} was found to be 3.58×10^{-7} mol/L (i.e., 0.724 µg/mL) (supporting information).

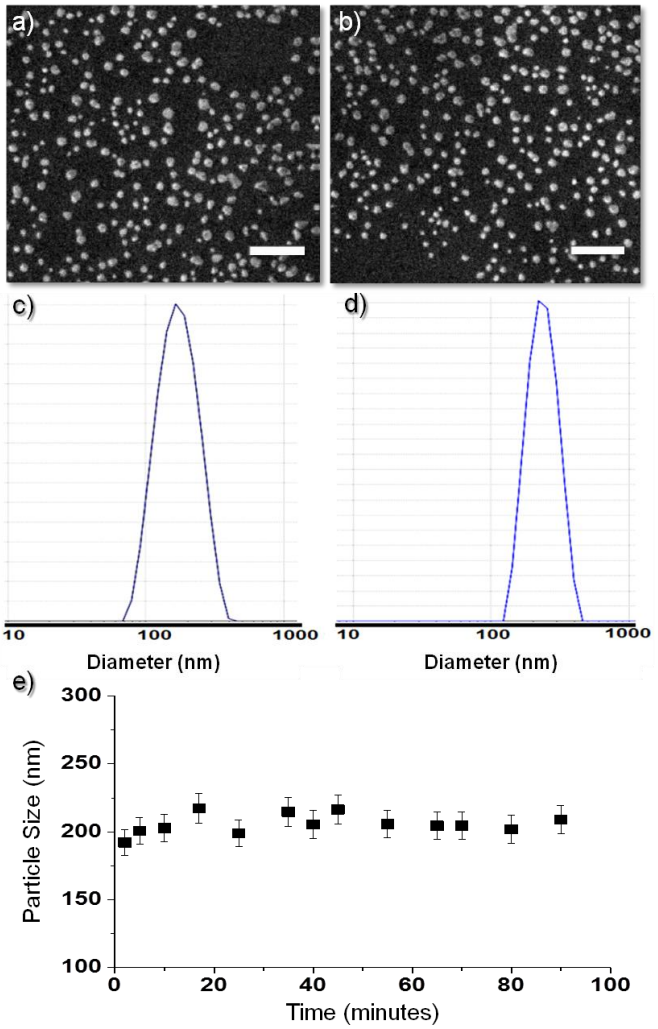

Figure 2. SEM images of a) Dop-$(Ala)_5$-$(Sar)_{15}$ and b) Dop-$(Phe)_4$-$(Sar)_{18}$ micelles formed following nanoprecipitation into PBS. Scale bars represent 1 µm. DLS charts revealing the size distribution of c) Dop-$(Ala)_5$-$(Sar)_{15}$ and d) Dop-$(Phe)_4$-$(Sar)_{18}$ micelles in PBS. The polydispersity indices were measured as 0.24 and 0.22 respecitvely. e) A representative chart detailing the stability of Dop-$(Ala)_5$-$(Sar)_{15}$ particles stored in PBS for 90 minutes.

Following the formation of monodisperse micelles, studies were undertaken to demonstrate the controlled release of covalently encapsulated dopamine upon targeted peptide degradation by targeted protease enzymes. As a proof-of-concept study, the activities of three serine protease enzymes were analysed against dopamine-containing nanoparticles. It has been demonstrated that serine proteases may be employed as highly effective triggers to activate enzyme-responsive materials, particularly to mediate the release of payload molecules in controlled drug delivery mechanisms.[13] Chymotrypsin from bovine pancreas possesses the selectivity to cleave peptide bonds flanked by aromatic amino acids

located at the P1 position, but is rather non-specific for the P′1 amino acid.[14] Thermolysin from *Thermoproteolyticus rokko* possesses low cleavage selectivity,[15] but elastase from bovine pancreas selectively cleaves peptide bonds flanked by small amino acids, for example alanine, overwhelmingly.[16] Dopamine release from particles incubated independently with chymotrypsin, elastase and thermolysin was monitored over a 48 hour period. It may be hypothesised that elastase should demonstrate extensive activity against Dop-$(Ala)_5$-$(Sar)_{15}$ compared to Dop-$(Phe)_4$-$(Sar)_{18}$, chymotrypsin should cleave more extensively Dop-$(Phe)_4$-$(Sar)_{18}$ compared to Dop-$(Ala)_5$-$(Sar)_{15}$ due to the presence of aromatic phenylalanine and thermolysin should demonstrate activity against both sets of particles. Conversely, extremely limited levels of dopamine are anticipated to be detected in response to incubation in PBS that lacks a protease enzyme.

The self-assembled nanoparticles were maintained within PBS solutions (0.1 mg/mL, pH 7.4, temperature = 37 °C) containing 20 units of the appropriate enzyme, within a dialysis tubing possessing a molecular weight cut-off value of 1,200 Da. Any dopamine released through the dialysis tubing could then be isolated and quantifiably assessed by HPLC analysis. This methodology enables a comparison of relative release rates to be made as, unlike the micelles formed, free dopamine is able to pass to the exterior of the dialysis tubing upon peptide hydrolysis. The values given by HPLC are not absolute as it is highly likely that a proportion of liberated dopamine will remain within the dialysis tubing and so cannot be analysed. However, a good comparison between the effect of protease enzymes, and the differences in their activities, can be made using this experimental procedure which demonstrates the enzyme-mediated release of the therapeutic initiator.

Dopamine release was detected to some extent when Dop-$(Ala)_5$-$(Sar)_{15}$ and Dop-$(Phe)_4$-$(Sar)_{18}$, particles were incubated in the enzymatic solutions. In contrast, the detection of dopamine released from the two sets of particles incubated in PBS solution that lacked a protease enzyme was significantly less in both cases (Figure 3). The different selectivities that the enzymes possess for the peptides created was validated and demonstrated that the system may be tuned to target a particular protease enzyme. Dopamine release from Dop-$(Ala)_5$-$(Sar)_{15}$ was greatest when the particles were incubated with elastase, demonstrating the relatively high activity that elastase has to cleave peptide bonds flanked by small amino acids such as alanine. In excess of 79% of total dopamine content was released to the exterior of the dialysis tubing for detection after 48 hours. In excess of 59% of dopamine was released from the dialysis tubing after incubation with thermolysin, and approximately 32% of dopamine was released from the particles when they were incubated with chymotrypsin. Dopamine release from Dop-$(Phe)_4$-$(Sar)_{18}$ particles was greatest (in excess of 78%) when the particles were incubated with chymotrypsin, demonstrating this enzyme's preference to cleave peptide bonds linking one or more aromatic amino acid. Both elastase and thermolysin showed some activity towards Dop-$(Phe)_4$-$(Sar)_{18}$ particles, but less than that observed for chymotrypsin (46% and 50% release respectively). In all cases, each time point plotted on the graph represents an independent experiment that was repeated to confirm method reproducibility.

The results of the release experiments demonstrate how the release mechanism may be adapted to target particular protease enzymes. For instance, grafting $(Ala)_5$-$(Sar)_{15}$ from a therapeutic initiator may be employed to target diseases that are linked with enhanced elastase concentrations at the disease site, including non-healing chronic wounds, atherosclerosis, pulmonary emphysema and cystic fibrosis.[17] Alternatively, an appropriate enzyme may be encapsulated within the micelles that is capable of hydrolysing the peptide bonds present within the structure to release the therapeutic initiator in a controlled manner.

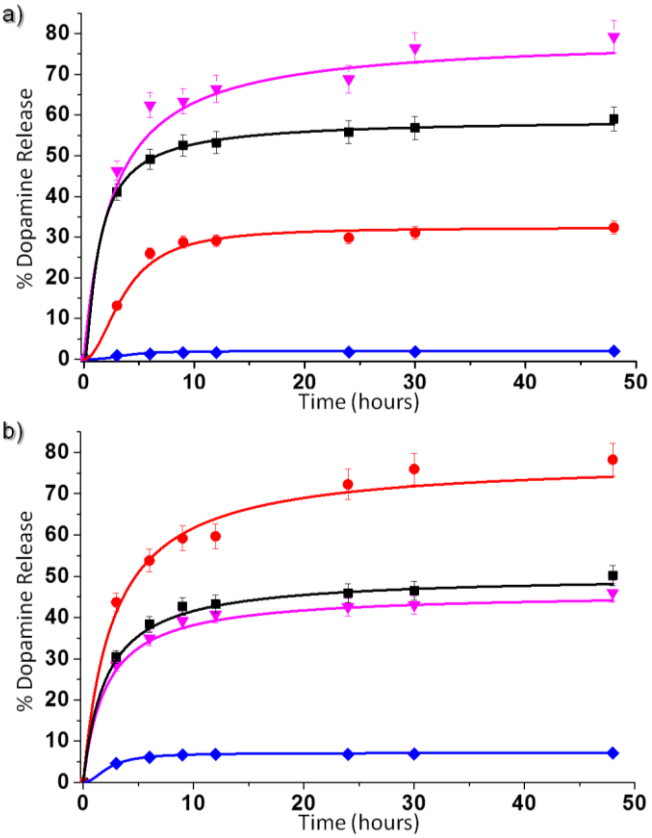

Figure 3. The release of dopamine from a) Dop-$(Ala)_5$-$(Sar)_{15}$ and b) Dop-$(Phe)_4$-$(Sar)_{18}$ particles in response to elastase (▼), thermolysin (■) chymotrypsin (●) and PBS only (♦). The % of dopamine release is based on the quantity used to initiate the initial polymerisation.

In summary, NCA ROP from a therapeutic initiator has been utilised as a highly effective methodology to create monodisperse micelles capable of selectively releasing dopamine in a controlled manner. The direct, one-pot, grafting of amphiphilic block copolymers from dopamine enables facile payload loading within the self-assembled structures. The biodegradable shell that the structures boast is susceptible to selective degradation by hydrolysis, resulting in dopamine release upon protease interaction and activity. The system is tuneable, allowing control over the release profile in response to particular target proteases by simply altering the peptide component of the structures formed. It is anticipated that this NCA ROP-based delivery strategy may be applied for the encapsulation and subsequent controlled delivery of a wide-range of therapeutic initiators associated with the treatment of numerous diseases.

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