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Cite this: *Chem. Commun.*, 2011, **47**, 7962–7964

www.rsc.org/chemcomm

COMMUNICATION

Drug release from hydrazone-containing peptide amphiphiles†

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Received 2nd May 2011, Accepted 27th May 2011
DOI: 10.1039/c1cc12570b

Published on 15 June 2011 on http://pubs.rsc.org | doi:10.1039/C1CC12570B
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Hydrolytically-labile hydrazones in peptide amphiphiles were studied as degradable tethers for release of the drug nabumetone from nanofiber gels. On-resin addition of the novel compound tri-Boc-hydrazido adipic acid to a lysine ε-amine allowed for precise placement of a hydrazide in a peptide sequence.

Delivery of drugs through implantable materials and devices has transformed several areas of medicine, including diabetes treatment, contraception, and cardiovascular repair.[1] However, increased control over drug release rates and greater biocompatibility and biodegradability of the delivery materials are still necessary improvements. Especially promising new varieties of implantable biomaterials are those made from biodegradable, self-assembling small molecules.[2,3] Not only are such systems more biocompatible compared with traditional polymeric gels, but all components of these systems can also be completely characterized on a molecular scale. Despite their promise as materials, sustained drug delivery from biodegradable, small molecule gels is still an underexplored area in the fields of drug delivery and regenerative medicine.[4,5]

Over the past decade, our laboratory has developed and studied peptide amphiphiles (PAs) programmed to self-assemble into high aspect ratio nanofibers.[6] These biocompatible PAs are a broad class of molecules that have recently received great attention due to their promise as bioactive materials in regenerative applications ranging from central nervous system regeneration and angiogenesis to bone, cartilage, and enamel regeneration.[7–12] PAs are synthesized by attaching a hydrophobic tail to a short peptide sequence that includes charged residues to promote solubility and control gelation through electrolyte screening. When the peptide sequence design includes a strong β-sheet forming sequence, PAs can self-assemble into long, filamentous aggregates in aqueous solution.[6] These nanostructures are approximately 10 nm in width and up to several microns in length and in most cases are always in an assembled state in aqueous solution. Charge screening by addition of salts, especially multivalent counterions, or a change in pH induces network formation and gelation at concentrations on the order of 1% by weight.[13] Previous studies on PA systems have shown the capabilities of PAs to support, signal and deliver cells through the efficient display of a bioactive peptide sequence.[7,14] Here we explore a new method for the delivery of soluble small molecules from PA gels through the use of hydrolytically labile hydrazone tethers. These small molecules may take the form of therapeutic drugs or biological signals.

Control of drug release rate can often be accomplished by covalently attaching a drug to a hydrolytically labile bond, such as a hydrazone, acetal or orthoester.[15–17] Typically, the release rate is governed by the pH of the surrounding media, with faster release observed in acidic (pH 5–6) environments compared with physiological media (pH 7.4). Hydrazone bonds are convenient hydrolytically labile bonds due to the usually facile incorporation of hydrazides into delivery materials. However, site-specific incorporation of hydrazides into peptides *via* solid phase peptide synthesis is still synthetically challenging.

We first attempted on-resin addition of protected hydrazides by the previously reported strategy of adding Boc-NHNHC(O)-CH_2CH_2COOH onto a lysine ε-amine.[18,19] However, we found couplings with this reagent to be very inefficient due to undesired intramolecular cyclization reactions, which others have also noted (see Supporting Information for details†).[20,21] A second attempted strategy was to use $(Boc)_2NN(Boc)CH_2$-COOH to reveal peptide hydrazines after cleavage, as reported by Melnyk.[22] Though the synthesis was effective, the resulting peptide hydrazines were found to undergo oxidative decomposition under the reaction conditions required for hydrazone formation. Inspired by Melnyk's synthesis, we synthesized triply-protected hydrazido acid **4** for use in SPPS. It was expected that the carbonyl of the hydrazide would provide more oxidative stability compared with the hydrazine.

Synthesis of **4** was accomplished in three steps from previously reported adipic acid monobenzyl ester (**1**) according to Scheme 1. First, *tert*-butylcarbazate was coupled to **1** using EDC and DPTS, affording monoprotected hydrazide **2** (DPTS = dimethylaminopyridinium *p*-toluene sulfonate[23]). Addition of Boc_2O to **2** yielded the triprotected derivative, **3**. Hydrogenolysis of the benzyl ester with Pd/C furnished triprotected hydrazido acid **4**. All reactions showed good to excellent yields, and no chromatography was required.

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† Electronic supplementary information (ESI) available: Full experimental details and characterization data on new compounds, SAXS fittings and aggregate parameters. See DOI: 10.1039/c1cc12570b

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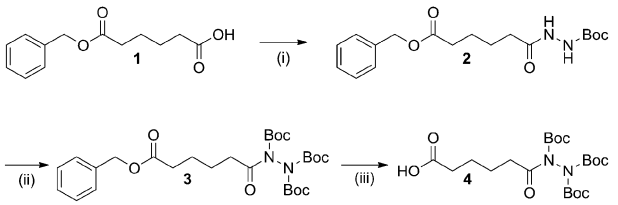


Scheme 1 Synthesis of triply-protected hydrazide building block for solid phase peptide synthesis. Reagents and conditions: (i) *tert*-butyl-carbazate, EDC, DPTS, CH_2Cl_2, 2 h, 83%; (ii) Boc_2O, DMAP, NEt_3, CH_2Cl_2, 2 h, 94%; (iii) H_2 (1 atm), Pd/C, EtOH, 4 h, 86%.

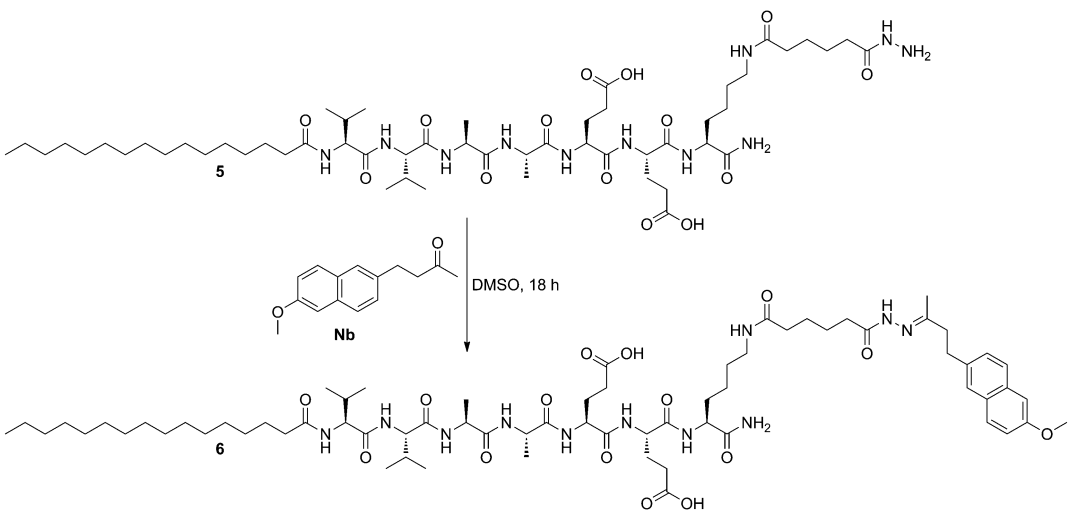
Applying building block **4** to the synthesis of potentially bioactive materials, hydrazide-containing PA **5** was produced using standard Fmoc-based solid-phase peptide synthesis conditions. A free amine was generated on-resin by addition of a Lys(Mtt) residue (Mtt = 4-methyltrityl) with selective removal of the Mtt protecting group using 5% TFA in CH_2Cl_2. Hydrazino acid **4** was activated using HBTU and DIEA and added on-resin to the exposed lysine ε-amine. Following cleavage using 95% TFA, which removed all protecting groups, PA **5** was purified by HPLC. The unprotected hydrazide was found to be oxidatively stable in air in organic solvents at 40 °C for at least 24 h.

The ketone-containing, non-steroidal anti-inflammatory drug nabumetone (**Nb**) was chosen as the model drug to serve as a release probe. Condensation of **Nb** with PA **5** was carried out in DMSO at room temperature to form hydrazone-containing PA **6**, which was purified by HPLC (Scheme 2). Structural characterization of **5** and **6** was then carried out to assess the self-assembly of the PAs.

PAs **5** and **6** were characterized in aqueous solution by conventional transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS) (Fig. 1). TEM of PA **5** showed long, cylindrical nanofibers typical of many PAs synthesized in our laboratory.[3] Some bundling of fibers can be observed in the TEM image. TEM of PA **6** showed similar cylindrical nanofibers, though they appeared shorter and less bundled than those of PA **5**. SAXS in aqueous solution was used to further investigate the aggregates formed from PAs **5** and **6**. A slope of −1 in the low q region is indicative of cylindrical aggregates and would be expected;[24] however, the slight deviation from the −1 slope observed in the SAXS curve of PA **6** (Fig. 1D) is likely a result of a small contribution of bundled aggregates. The even steeper slope in the low q region seen in the PA **5** scattering curve (Fig. 1C) is consistent with the higher-order aggregates (bundles) seen in the TEM. We attribute the bundling observed in PA **5** to interfiber hydrogen bonding between surface hydrazide groups. Addition of **Nb** yields a hydrazone that is less capable of hydrogen bonding, which may explain the reduced number of higher-order aggregates observed in PA **6**. Excluding the low q region, the SAXS curves can be reasonably fitted to a polydisperse core-shell cylinder form factor (Supporting Information†). The best fit parameters yielded an average fiber diameter of 7.1 nm for PA **5** and 7.0 nm for PA **6**, supporting TEM results that addition of **Nb** did not alter fiber dimensions.

To investigate the release of **Nb** from PA **6**, a mixture of PA **6** with diluent PA $C_{16}V_2A_2E_2$ (1 : 1 w/w, see Supporting Information†) was prepared and gelled with $CaCl_2$. Scanning electron microscopy (SEM) of the gel (Fig. 2A) shows the expected fibrous morphology. The release kinetics of **Nb** were measured into a sink solution of buffer by monitoring absorbance at 330 nm (Fig. 2B). As we are interested in using drug-delivering PA materials as injectable gels for the long-term, sustained delivery of drugs, signals, and cells, we chose to investigate release kinetics at physiological pH. Although hydrazones are often designated as exhibiting high hydrolytic stability at physiological pH, slow hydrolysis is often still observed.[25] Release was observed over 24 d, with an initial burst release but eventual conversion to a zero-order release profile with $t_{1/2}$ = 33.9 d, as calculated from the near-linear region in Fig. 2B. Burst release is likely due to free **Nb** in the gel that occurs as a result of sample preparation. Future work will focus on developing methods to control drug release kinetics.

In summary, a small molecule building block (**4**) for incorporation of hydrazides into peptides has been synthesized. Under standard coupling conditions, **4** can be added on-resin



Scheme 2 Synthesis of drug-tethered PA by hydrazone formation.

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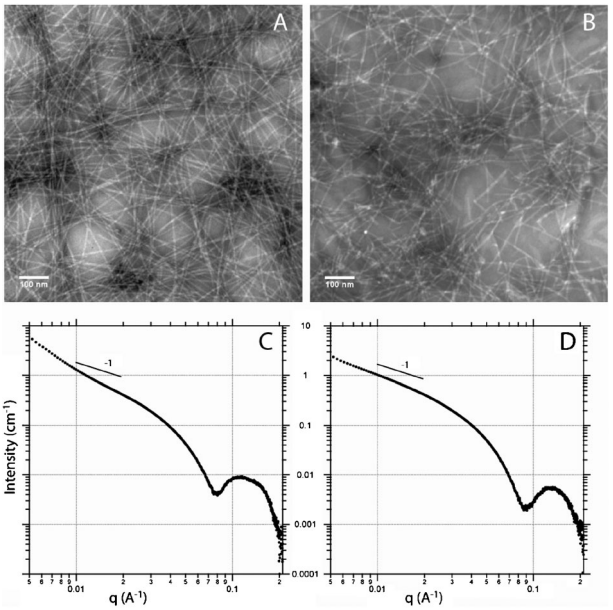

Fig. 1 TEM and SAXS of PA **5** (A and C) and PA **6** (B and D).

to a lysine ε-amine, revealing a free hydrazide upon cleavage from the resin. We demonstrated an application of this chemistry by developing a hydrazide-containing PA, to which a small molecule drug could be tethered *via* hydrazone formation. Drug tethering was found not to alter the assembly of the PA into filamentous aggregates. The small molecule drug could then be slowly released from the PA gel into aqueous solution. Further studies on small molecule drug release from PA gels are currently underway in our laboratory. We expect that building block **4** will be useful in several areas of peptide and polymer-based medicine, including peptide-based biomaterials, peptide labeling and polymeric drug conjugation.

This work was supported by the NIDCR, grant No. 2R01DE015920-06 and the NIBIB, grant No. 2R01EB003806-06A2. JBM was supported by a Baxter Early Career Development Award in Bioengineering. We are grateful to the Peptide Synthesis Core in the Institute for BioNanotechnology in Medicine (IBNAM), the Biological Imaging Facility (BIF), the Integrated Molecular Structure Education and Research Center (IMSERC) and the Electron Probe Instrumentation Center (EPIC) of the Northwestern University Atomic- and Nanoscale Characterization Experimental Center (NUANCE) for instrument use. NUANCE Center is supported by NSF-NSEC, NSF-MRSEC, Keck Foundation, the State of Illinois, and Northwestern University. We acknowledge Dr Steven Weigand and the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) Synchrotron Research Center at the Advanced Photon Source (APS) at Argonne National Lab for assistance with SAXS measurements. Use of the APS was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. Matt Webber and Dr Ronit Bitton of the authors' laboratory are acknowledged for help with SEM and SAXS, respectively.

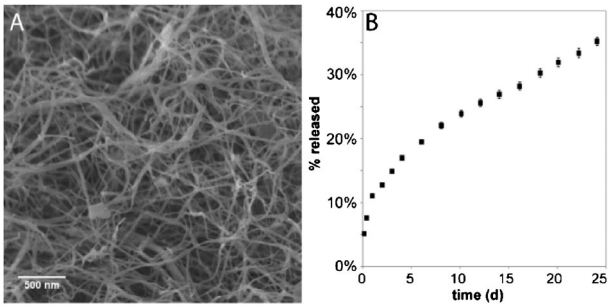

Fig. 2 SEM (A) and release profile (B) of **Nb** from gel of PA **6** mixed 1 : 1 with diluent PA into buffer at physiological pH (n = 5).

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