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Aust. J. Chem. **2010**, *63*, 1169–1172

Synthesis of Self-assembling Cyclic Peptide-polymer Conjugates using Click Chemistry

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Self-assembling cyclic peptide-polymer conjugates were prepared by 'clicking' polymers (prepared by RAFT polymerization) to an azide functionalized D-alt-L cyclic octapeptide via the Huisgen 1,3-dipolar cycloaddition reaction. Due to the high graft density, the efficiency of the click chemistry conjugation reaction was found to be highly dependent on the size of the polymer. At relatively low molecular weights, as many as four polymer chains could be grafted to each 8 residue cyclic peptide ring. Evidence for the self assembly of the conjugates into peptide-polymer nanotubes was observed by TEM and IR.

Manuscript received: 18 March 2010.
Manuscript accepted: 30 June 2010.

Modern techniques of polymerisation have revolutionalized the chemists' ability to design functional materials with tailored properties,[1] but these synthetic capabilities are still far outstripped by nature's. As scientists try to synthesize more complex materials, the challenge to do so by traditional synthetic methods increases significantly and recent research has taken inspiration from the way that nature builds well defined and complex structures through the self assembly of smaller molecules.[2] This bottom-up approach has proved to be versatile and the most promising route to design materials with structures controlled at the nanoscale.

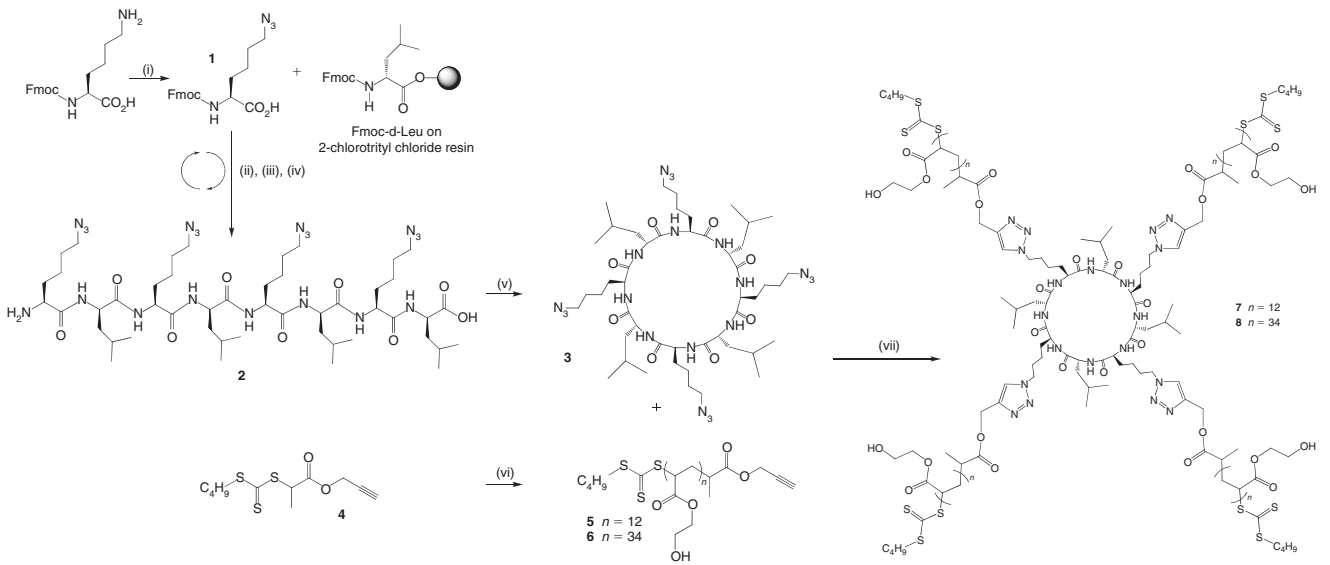
Perhaps the most interesting and well understood self-assembling molecules are β-sheet peptides, which can assemble into tapes, ribbons, fibres, and tubes.[3–6] This range of nanostructures can be exploited by conjugating polymers to such peptides, and using the peptide to drive the self-assembly of the conjugate into a nanostructure.[7–10] With the advance of modern polymer chemistry, and in particular living radical polymerization (LPR) techniques, there exists a variety of routes through which to conjugate polymeric chains to peptide segments. Approaches are generally divided between divergent synthesis,[11–15] which fully exploits the versatility of LRP by growing a polymeric chain from a peptide used as polymerization initiator, and convergent synthesis,[16,17] in which the peptide is coupled to a pre-made polymer. The self-assembly of cyclic peptide-polymer conjugates into nanotubes has been demonstrated with selected systems and offers access to well defined polymeric nanotubes. Most examples of polymer peptide nanotubes in the literature are synthesized by the divergent approach, growing polymeric chains from an ATRP initiator modified cyclic peptide, either before or after self-assembly.[12–15] While grafting to a cyclic peptide before self-assembly has been shown to work in principle through a condensation reaction,[17] click chemistry offers the ability to conjugate almost any polymer to the cyclic peptide and then self-assemble it into polymer nanotubes. Furthermore, click reactions allow access to high graft densities that are not possible with other approaches. In the present work we show that the click approach enables the conjugation of polymeric chains to cyclic peptides and that the resulting conjugates show self-assembly as evidenced by transmission electron microscopy (TEM) and IR.

Results and Discussion

Scheme 1 shows a summary of the synthetic approach used to create cyclic peptide-polymer conjugates. A novel four-arm azide functionalized cyclic peptide *cyclo*-[L-Lys(N_3)-D-Leu]$_4$ **3** was prepared by cyclization of the linear precursor **2**, which was synthesized by standard Fmoc SPPS protocols (HBTU coupling).[18] The precursor amino acid, azido Fmoc-L-Lysine **1** was synthesized from Fmoc-L-Lysine using the diazo transfer agent developed by Stick and coworkers (see supplementary information),[19] which was preferred over the more dangerous procedure using triflyl azide by which azido lysine is more commonly made. Polymers of hydroxy ethyl acrylate (HEA) were prepared via reversible addition fragmentation chain transfer (RAFT) polymerization,[20,21] then attached to the peptide using click chemistry. HEA was chosen in order to improve the solubility of the conjugate, and the resulting nanotubes, in polar solvents. Alkyne functionality was introduced to the polymers by using an alkyne functionalized trithiocarbonate RAFT agent **4** (see supplementary information) to polymerize the monomer, since trithiocarbonates are stable at the reaction temperatures.[22] Protection of the alkyne group (such as with TMS) during polymerization was found to be unnecessary at the times and temperatures used.

The unconjugated cyclic peptide **3** was found to be remarkably insoluble, even in very polar solvents such as DMF and DMSO, presumably due to strong self-assembly properties. While trifluoroacetic acid (TFA) could be used to break the self

 10.1071/CH10128 0004-9425/10/081169



Scheme 1. Synthesis of cyclic peptide-polymer conjugates; (i) Fmoc-L-Lys-OH, Im-SO_2-N_3, $CuSO_4$, $NaHCO_3$; (ii) Fmoc-L-Lys(N_3)-OH/Fmoc-D-Leu-OH, HBTU, Hünig's base, DMF; (iii) 20% Piperidine, DMF; (iv) TFA, TIPS, thioanisole, H_2O; (v) HOBt, HBTU, Hünig's base, DMF; (vi) HEA, AIBN, *t*-butanol; (vii) DMSO, PMDETA, Cu(I)Br.

assembly and solubilize the peptide,[17] the presence of TFA in the click reaction was found to interfere with ligation of the copper. As a result, the conjugation was carried out in a heterogeneous solution of DMSO prepared by sonication for 10 min. A two-fold excess of CuBr and 2,2′-pentamethyldiethylenetriamine (PMDETA) with respect to the cyclic peptide was used due to the large degree of binding expected between copper and peptide.[23] As the reaction proceeded, the insoluble cyclic peptide was found to dissolve, leading to a homogeneous mixture, indicating that the attachment of polymer dramatically improved the solubility of the peptide, pushing the reaction towards the product. The product **6** was purified by precipitation from aqueous EDTA (0.065 M), and subsequent washing with water. Click reactions were carried out with short (60% conversion, $M_n = 1700\ g\ mol^{-1}$, PDI 1.10) and long (35% conversion, $M_n = 4000\ g\ mol^{-1}$, PDI 1.13) polymers.

The conjugation of short polymers to the cyclic peptide was very efficient, proceeding to near full conversion in 3 days at 60°C. Despite the persistence of an azide signal in the IR spectrum (Fig. 1a), indicating some unreacted sites on the cyclic peptide, a clear shift in the molecular weight was seen in the size exclusion chromatography (SEC) trace of the click product (Fig. 1b). The high molecular weight shoulder in the SEC trace of the conjugate suggests some small scale self-assembly of two or more cyclic peptide conjugates in DMF. While the molecular weight of such aggregates would be double or triple that of a single conjugate, the molecular weight observed by SEC only increases slightly, because the effect of aggregation on hydrodynamic volume is only slight. The conversion of the reaction was estimated to be ~70–90% by ^{1}H-NMR spectrometry (Fig. S1), by integration of the signals corresponding to the triazole proton and the CH_2 groups α to the triazole relative to the Leu-CH_3 groups. No improvement in conversion was seen after 6 days. The signal corresponding to the O-CH_2-triazole protons is notable, as it appears as a doublet of doublets rather than as a singlet as is more commonly reported for similar molecules in d_6-DMSO.[24] This indicates reduced rotation about this bond, which can be attributed to significant steric hindrance, consistent with a structure in which 3 or 4 bulky polymer chains are attached to a single, small peptide centre.

The presence of azide functionality in the final conjugates demonstrates that the reaction is not hindered by the destruction of the azide groups, and the fact that the entire mixture is soluble at the end of the click reaction rules out the possibility that the unconverted azide results from a small fraction of cyclic peptides that have not reacted at all. An analogous reaction carried out at 40°C over 6 days gave similar results, indicating that changing the temperature does not affect the efficiency of the reaction. It is therefore apparent that blocking of the reactive sites by the polymer and/or aggregation of the peptide rings are the most likely explanations for the lack of complete conversion. In order to investigate these steric effects further, the molecular weight of the polymer clicked to the cyclic peptide was increased. Increasing the weight of the polymer to $4000\ g\ mol^{-1}$ dramatically reduced the efficiency of the click reaction, as can be seen by SEC (Fig. 1c). The size of the resulting conjugate was nearly equal to that of the shorter chain conjugate despite the increase in molecular weight of the polymer, and a very strong azide signal was observed in the IR spectrum. Increasing the time of reaction to 6 days yielded some, although not significant, improvement in conversion. This demonstrates that steric effects play a significant role in the conjugation of polymers to cyclic peptides, especially to those with such a high tendency to aggregate. While high conversions can be reached with low molecular weight polymers, harsher conditions would be required to achieve the same efficiency of conjugation with higher molecular weight polymers.

To investigate the self-assembly of the conjugates, we prepared aggregates by dilution of a concentrated DMF solution of the conjugates in methanol, as previously described for similar systems.[17] The TEM images of the short chain polymer nanotubes in Fig. 2 show the tubes of ~15 nm in diameter, in the range that is expected from such conjugates.[13,15] While no spectra of the unassembled peptide could be taken due to

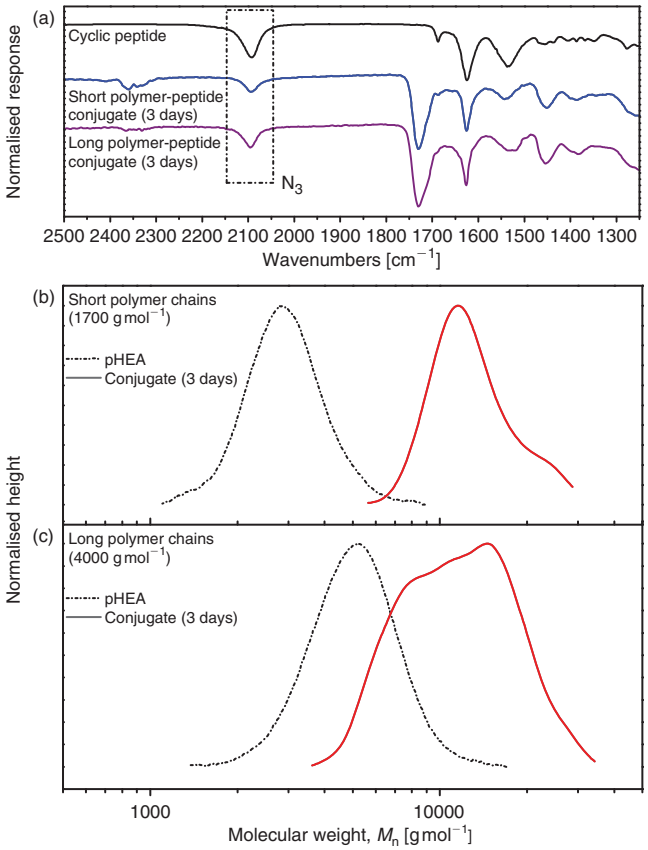


Fig. 1. (a) ATR-FTIR of the cyclic peptide and both the short (1700 g mol^{-1}) and long (4000 g mol^{-1}) chain polymer-peptide conjugates; SEC traces (DMF + 0.3% LiBr) for (b) the low molecular weight polymer and its conjugate and (c) the high molecular weight polymer and its conjugate.

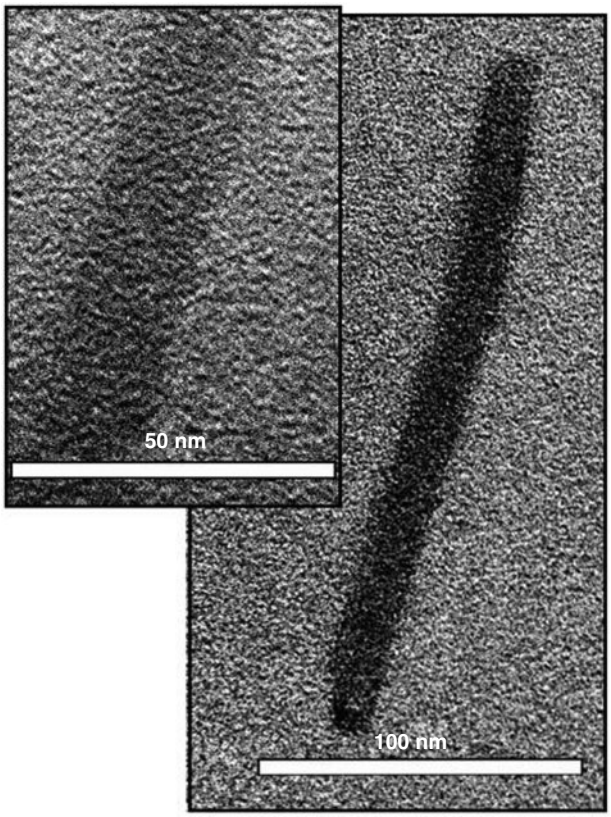


Fig. 2. TEM images of the low molecular weight peptide-polymer conjugate cast from methanol.

the low solubility and high tendency to aggregate, IR spectra of methanol cast films of both the cyclic peptide and the peptide-polymer conjugate show the presence of amide-I bands at 1628 cm^{-1} and amide-II bands at 1541 cm^{-1}, suggestive of the formation of β-sheet structure.[25,26]

In summary, we have shown that by using click reactions it is possible to graft as many as four polymer chains to a cyclic peptide, and that such a high graft density does not prevent the natural self-assembly process of the peptide into nanotubes. In principle, such an approach enables access to high graft density nanotubes from almost any polymer. These click reactions are highly dependent on steric bulk, and increasing the molecular weight of the polymer decreases the efficiency of reaction significantly.

Experimental

Synthesis of *cyclo*[L-Lys(N$_3$)-D-Leu]$_4$ (3): The linear precursor peptide H$_2$N-(L-Lys(N$_3$)-D-Leu)$_4$-OH **2** was assembled on 2-chlorotrityl chloride resin via standard SPPS techniques in DMF (HBTU/Hünig's base as the coupling agents). The linear peptide **2** (0.08 g, 0.074 mmol) was dissolved in DMSO (20 mL) in a sonic bath, and the resulting solution diluted to 50 mL with DMF. The mixture was cooled to 0°C in an ice bath and HBTU (1.5 equiv, 0.042 g, 0.110 mmol), HOBt (1.5 equiv, 0.015 g, 0.110 mmol) and Hünig's base (3 equiv, 0.029 g, 0.221 mmol) were added portion-wise. The mixture was stirred at room temperature for 6 h, concentrated under reduced pressure to ~3 mL, and precipitated from methanol (~30 mL). The cyclic peptide **3** (0.036 g, 46%) was recovered as a white powder after further washing with methanol (×2). ν_{max} (ATR)/cm^{-1} 3275 (NH), 2092 (N$_3$), 1623 (Amide-I), 1536 (Amide-II). δ_H (300 MHz, *d*-TFA) 0.97 (br s, 24H, Leu CH$_3$), 1.34–2.21 (m, 36H, Lys & Leu), 3.35 (m, 8H, 4 N$_3$-CH$_2$), 4.60–4.97 (m, 8H, 8 α-CH). *m/z* (APCI) 1069.53 [M + H]$^+$, calc. 1069.69.

General synthesis of polymer-peptide conjugate: Click reactions were performed in DMSO with PMDETA/Cu(I)Br.[23] The cyclic peptide **3** (15 mg, 0.014 mmol) was suspended in DMSO (2.2 mL) with the polymer (1.5 equiv/site) by sonication for 10 min. In separate vials, CuBr (4.0 mg, 0.028 mmol), and a solution of PMDETA (4.9 mg, 0.028 mmol) in DMSO (2.5 mL) were prepared. All three vials were degassed with N$_2$ for 30 min. The PMDETA solution was then transferred into the CuBr vial by cannula, and then this solution was added to the peptide and polymer solution by cannula, and allowed to react at 60°C under an atmosphere of nitrogen. The crude mixture was precipitated out of an aqueous EDTA solution (0.065 M) at 0°C to remove the copper and any unreacted pHEA, and the resulting solid washed with ice-cold water (2 × 10 mL).

Short polymer-peptide conjugate (7): Conjugation of the short pHEA chain **5** (M_n = 1700 g mol^{-1}, PDI 1.10) to the cyclic peptide **3** was performed according to the general synthesis above. ν_{max} (ATR)/cm^{-1} 3273 (NH), 2092 (N$_3$), 1728 (C=O), 1628 (Amide-I), 1541 (Amide-II). δ_H (300 MHz, d_6-DMSO + 5% D$_2$O) (see supplementary information for full spectrum): 0.77 (m, 24H, Leu CH$_3$), 0.87 (t, *J* 7.6, 9H RAFT CH$_3$), 1.01 (m, 9H, RAFT CH$_3$), 1.05–2.00 (m, pHEA backbone, Leu CH, Leu CH$_2$, Lys CH$_2$), 3.36 (t, 9H, *J* 7.2, RAFT CH$_2$),

3.53 (m, ~84H, pHEA CH_2), 3.99 (m, ~84H, pHEA CH_2), 4.73 (m, 14H, peptide backbone + ε-Lys), 4.64 (s, unclicked polymer CH_2), 4.74 (s, 3H, polymer CH-S), 4.87 (s, polymer OH), 5.01–5.13 (dd, *J* 12.0, 25.7, 8H, Lys ε-CH_2), 8.03 (s, 3H, triazole CH), 8.12 (br s, NH).

Accessory Publication

Details of the synthesis of the cyclic peptide precursors and the RAFT agent, as well as characterisation data for the polymers and polymer-peptide conjugate are given in the accessory publication, which is available on the journal's website.

Acknowledgements

R.C. thanks the Australian Government for an Australian Postgraduate Award scholarship, and the University of Sydney for funding to attend PPC11. We are grateful to Dominik Konkolewicz for experimental assistance and Polymer Laboratories for SEC equipment and technical support.

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[18] Abbreviations: 1-hydroxy-l-azabenzotriazoleuronium (HBTU), *O*-benzotriazole-l-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HOBt), triisopropylsilane (*Trends Pharmacol. Sci.*).

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