Solid-Phase-Based Synthesis of Ureidopyrimidinone–Peptide Conjugates for Supramolecular Biomaterials

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Abstract Supramolecular polymers have shown to be powerful scaffolds for tissue engineering applications. Supramolecular biomaterials functionalized with ureidopyrimidinone (UPy) moieties, which dimerize via quadruple hydrogen-bond formation, are eminently suitable for this purpose. The conjugation of the UPy moiety to biologically active peptides ensures adequate integration into the supramolecular UPy polymer matrix. The structural complexity of UPy–peptide conjugates makes their synthesis challenging and until recently low yielding, thus restricted the access to structurally diverse derivatives. Here we report optimization studies of a convergent solid-phase based synthesis of UPy–peptide conjugates. The peptide moiety is synthesized using standard Fmoc solid-phase synthesis and the UPy fragment is introduced on the solid-phase simplifying the synthesis and purification of the final UPy–peptide conjugate. The convergent nature of the synthesis reduces the number of synthetic steps in the longest linear sequence compared to other synthetic approaches. We demonstrate the utility of the optimized route by synthesizing a diverse range of biologically active UPy–peptide bioconjugates in multimilligram scale for diverse biomaterial applications.

1 Introduction

The concept of tissue engineering was introduced in the 1980s,3 and presently comprises two approaches: the ex vivo approach, in which cells are cultivated within a (synthetic) scaffold before being implanted, and the other in vivo or in situ approach, where a scaffold is implanted that stimulates the self-healing capacity of the body. In both cases the scaffold provides mechanical support to the surrounding tissue and also has to be engineered to deliver the necessary biological signals – for example, growth factors, proteins, or peptide sequences derived from proteins – for the surrounding cells to proliferate, migrate, and differentiate.4-6 Short peptides are preferred for such purposes as they are easy to synthesize to scale and are at present easier to modify in a site-selective manner to introduce multiple identical or different groups, typically without loss of function. Furthermore, peptides are generally more resistant to chemical processing than proteins, which also facilitates their integration into biomaterials. Polymer-based scaffolds of varying degrees of mechanical strength have been generated to meet the specific mechanical demands in vivo. For both classes – stiff and soft – a variety of covalent7-10 and noncovalent11-17 polymer networks have been explored. In contrast to covalently cross-linked scaffolds, the dynamic nature of supramolecular cross-linking provides a more straightforward way to functionalize polymers through introduction of biological cues and markers conjugated to complementary supramolecular monomers.18-21 Furthermore, the use of specific interactions facilitates precise hierarchical self-organization and self-optimization,22-24 and can be used to tune the rate of biodegradation.25 Supramo-
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Supramolecular biomaterials based on polymers, cross-linked through the fourfold hydrogen bonding of ureidopyrimidinone (UPy) moieties, have shown promising results as scaffolds in cardiovascular\textsuperscript{26} and renal\textsuperscript{11,27} regenerative medicine. The introduction of extracellular matrix (ECM) derived biological cues into these materials can be realized by the noncovalent incorporation of UPy-functionalized bioactive peptides. In this way, the properties of the supramolecular biomaterial can be rapidly optimized through straightforward mixing of different peptide combinations at different ratios.

UPy functionalization of peptides has previously been performed via reaction of the N-terminus of the peptide with a carbonyldiimidazole-activated methylisocytosine, yielding an UPy–peptide without spacer between the UPy and the first amino acid (\textit{e.g.}, UPy–peptide 1 in Figure 1),\textsuperscript{20} or via a UPy–hexyl isocyanate synthon (\textit{e.g.}, UPy–peptide 2 in Figure 1).\textsuperscript{28,29} The introduction of an oligoethylene glycol (OEG) based linker group via an oxime ligation strategy (UPy–peptide 3 in Figure 1) further increased the incorporation efficiency and accessibility of the peptides within the biomaterial.\textsuperscript{30} However, for the production of functional materials a fine-tuning of the amphiphilic properties of the materials is desirable and can be achieved through the introduction of a mixture of hydrophobic and hydrophilic spacer groups within the UPy–peptide conjugates.

Here we describe the efficient solid-phase synthesis of UPy-functionalized peptide conjugates with different lengths of hydrophobic and hydrophilic linkers for use in supramolecular biomaterial applications. The synthesis of the UPy–peptide conjugates was explored using three strategies – one divergent and two convergent; the advantages and disadvantages of each strategy are discussed here. Our data suggests that a convergent strategy, using amide-coupling chemistry to introduce a suitable UPy-derived linker molecule at the N-terminus of the peptide, is the most suitable for the scaled-up synthesis of UPy–peptide conjugates. This convergent strategy in particular has enabled access to a broad range of conjugates and should find extensive use in the preparation of supramolecular biomaterials.

![Figure 1](Synlett_I_2015_Fig1.png)

**Figure 1** UPy–peptide designs, exemplified by the ECM-derived (GGG)GRGDS sequence, previously used for the functionalization of UPy-based biomaterials.\textsuperscript{20,29,30}
2 Divergent Synthesis

A solid-phase-synthesis strategy is known to significantly increase the efficiency of the synthesis by avoiding complicated workup procedures, reducing reaction times through the use of excess reagents, and by simplifying the purification due to washing steps.31 Furthermore, a divergent strategy is an effective way to build and generate structurally diverse compounds, especially peptide-based libraries. For these reasons, a divergent synthesis route was initially investigated to prepare three different UPy–peptide conjugates with hydrophobic (C12) and hydrophilic OEG spacers on a Rink amide resin in a filter-equipped syringe.

First, the peptide sequence 4 was prepared by standard solid-phase Fmoc peptide synthesis (SPPS, Scheme 1).32 After removal of the Fmoc-group, the N-terminus of the peptide was reacted with succinic anhydride and pyridine to introduce a carboxylic acid group 5, which permitted the coupling of an OEG diamine using amide-coupling chemistry. Interestingly, the use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and dimethylaminopyridine [DMAP, conditions (iv) b)] in this case gave exclusively the desired product 7, whereas activation with o-benzotriazole-\(N,N,N',N'\)-tetramethyluronium hexafluorophosphate (HBTU) and \(\text{N,N'-diisopropylethylamine} [\text{DIPEA}, \text{conditions (iv) a}]\) yielded a mixture of the desired product 7 and a cross-linked byproduct 6. The chemoselectivity observed in the EDC case is most likely due to protonation of the second amine group. Nevertheless, a double-coupling strategy using EDC and DMAP was needed to ensure complete conversion to the monocoupled product. Thereafter, the hydrophobic spacer was introduced as the Fmoc-protected amino dodecanoic acid 8 using HBTU and DIPEA, followed by Fmoc-deprotection. The UPy moiety was subsequently coupled onto the resin-bound amine by using an excess of UPy–hexyl isocyanate28 (UPy-C6-NCO) to afford the UPy-functionalized GGGGRGDS, GGGPHSRN, and GGGYIGSR peptide conjugates (Table 1, 9a–c) after resin cleavage and deprotection of peptide side-chain groups using a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS), and \(\text{H}_2\text{O} (95:2.5:2.5 \text{ v/v%})\). Overall yields of 5–12% over 23 steps were obtained for the three peptide sequences relevant for cell adhesion and migration.33,34 Purities of typically >95% were obtained after purification by reversed-phase preparative high-pressure column chromatography (RP-HPLC).
Convergent Synthesis

Though clearly applicable to the synthesis of structurally diverse UPy–peptide conjugates bearing different linker lengths and amphiphilic character, the disappointingly low yields for 9a–c prompted the exploration of other convergent synthetic strategies. In particular, a scalable, more efficient synthesis of structurally diverse UPy–peptide conjugates bearing the same hydrophobic and hydrophilic linker groups was sought. This is not possible in the divergent strategy where the entire synthesis needs to be performed from start to end.

Therefore, two different convergent approaches were investigated with a view to increasing the overall yield and efficiency of the synthesis. For both approaches, the peptides were first synthesized by standard Fmoc SPPS on a Rink amide resin. In contrast to the divergent approach, the UPy building blocks 10–13 (Figure 2), were synthesized according to an efficient and scalable solution-phase protocol in yields ranging from 11–25% over 6–10 steps [see the Supporting Information (SI) for experimental conditions and compound characterization]. The difference between these UPy building blocks lies in their chain length and in the composition of the hydrophilic and hydrophobic spacers. Compounds 10–13 were then coupled to the resin-bound peptide using amide-coupling chemistry at the step prior to resin cleavage. The two convergent approaches explored (Scheme 2), differed at the point of connection: while the first approach – UPy–amine strategy – introduced the UPy building blocks 10 and 11 through activation of a resin-bound acid functionality, the second approach – UPy–carboxylic acid strategy – required pre-activation of compounds 12 or 13 before coupling to the peptide N-terminus.

<table>
<thead>
<tr>
<th>Synthetic strategy</th>
<th>Compound</th>
<th>Peptide sequence (R)(\text{a})</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divergent</td>
<td>9a</td>
<td>GGGGRGDS</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>9b</td>
<td>GGGPHSRN</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9c</td>
<td>GGYYGSR</td>
<td>8</td>
</tr>
<tr>
<td>Convergent 10</td>
<td>17a</td>
<td>GGRGDS</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>17b</td>
<td>GGPHTSN</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>17c</td>
<td>GSGDRG</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>17d</td>
<td>GGDGEA</td>
<td>20</td>
</tr>
<tr>
<td>Convergent 11</td>
<td>18a</td>
<td>GGRGDS</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>18b</td>
<td>GGGPHSRN</td>
<td>33</td>
</tr>
<tr>
<td>Convergent 12</td>
<td>19a</td>
<td>GGRGDS</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>19b</td>
<td>GGRGDS(\text{b})</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>19c</td>
<td>c(KRGDF)(\text{c})</td>
<td>56(\text{d})</td>
</tr>
<tr>
<td>Convergent 13</td>
<td>20a</td>
<td>GGRGDS(\text{b})</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>20b</td>
<td>GGDGEA(\text{b})</td>
<td>42</td>
</tr>
</tbody>
</table>

\(\text{a}\) UPy linker is attached to N-terminal amino group.
\(\text{b}\) Coupled using HATU.
\(\text{c}\) Solution-phase chemistry.
\(\text{d}\) Yield of the protected UPy–peptide.
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To enable coupling of UPy–amine building blocks 10 and 11, a carboxylic acid group was installed at the peptide N-terminus by reacting the α-amino group with succinic anhydride. Coupling of the functionalized peptide to UPy–amine derivatives 10 or 11 (Figure 2) was achieved via the activated ester, formed on treatment with benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the presence of DIPEA. In the final step, the UPy–peptide conjugate was deprotected and cleaved from the resin using a mixture of TFA, TIS, and H₂O (95:2.5:2.5 v/v), and purified by preparative RP-HPLC. This resulted in the successful formation of fibronectin (GGRGDS, GGPHS-RN, and scrambled control peptide GSGDRG) and collagen I (GGDGEA) derived peptide conjugates 17a–d in yields of 20–32% for building block 10. Similar yields were obtained for UPy–peptide conjugates 18a and 18b, where the UPy building block 11 was equipped with a longer OEG spacer. Furthermore, an irreversible side reaction was observed, which would contribute to the overall low yields (Scheme 3). Analogous to aspartyl and asparaginyl cyclization, pre-

**4 UPy–Amine Strategy**

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**Figure 2** Amine-terminated (10, 11) and carboxylic acid terminated (12, 13) UPy building blocks bearing different spacer lengths for convergent UPy–peptide synthesis

**Scheme 2** Convergent synthesis of UPy–peptide conjugates on a Rink amide resin, as exemplified by GGRGDS peptide conjugates 18a via UPy–amine strategy and 20a via UPy–carboxylic acid strategy. (i) piperidine–NMP (20% v/v), 2 × 5 min, r.t.; (ii) Fmoc-protected amino acid, HBTU, DIPEA, NMP, 30 min, r.t.; (iii) succinic anhydride, pyridine, DMF, 16 h, r.t.; (iv) UPy building block 11, PyBOP, DIPEA, DMF 2 × 16 h, r.t.; (v) TFA–H₂O–TIS (95:2.5:2.5 v/v) 4 h, r.t.; (vi) RP-HPLC (H₂O–MeCN–0.1% TFA); (vii) UPy building block 13, HATU, DIPEA, DMF 2 h, r.t.; (viii) TFA–H₂O–TIS–EDT (94:2:2:2 v/v) 4 h, r.t.
sumably, the 1-hydroxybenzotriazol activated ester 14 undergoes an intramolecular ring-closure reaction with the amide at the peptide N-terminus, forming the succinimide adduct of the peptide 15 on the resin while releasing the benzotriazole 16 into solution.\textsuperscript{33,36,37} Though this convergent UPy–amine strategy is more practical than the divergent route for the preparation of structurally diverse peptide sequences bearing the same UPy linker group, we decided to explore another convergent strategy to further improve the yields.

In conclusion, we report the development of divergent and convergent strategies for the synthesis of UPy–peptide conjugates. The highest yields were obtained using a convergent approach, utilizing carboxylic acid functionalized building blocks 12 and 13, which were readily synthesized with high overall yields. The convergent strategy enables a fast optimization of the peptide sequence and an efficient coupling of the preprepared UPy linker moiety, facilitating the access to diverse libraries of UPy–peptide conjugates. These can be readily mixed with the UPy-functionalized polymeric material in different compositions and at different ratios, allowing future application of these conjugates in the biomedical field.

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Supporting Information

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References and Notes

1. These authors contributed equally.
2. These authors contributed equally.

(38) General Experimental Procedure for the Coupling of the Carboxylic Acid Terminated UPy Building Block to the Peptide Using HATU (Solid Phase)

Rink amide resin (ca. 100, μmol) with the attached peptide was allowed to swell in N,N-dimethylacetamide (DMAc) for 3 h, rinsed with DMAc and combined with a solution of carboxylic acid terminated UPy building block (0.15 mmol), DIPEA (0.3 mmol), and HATU (0.13 mmol) in DMAc that was preactivatied for 30 min. The resin was agitated in the reaction mixture for 90 min, rinsed with DMAc, and dried for 30 min. The resin was agitated in the reaction mixture for 90 min, rinsed with DMAc and combined with a solution of carboxylic acid terminated UPy building block (0.15 mmol), DIPEA (0.3 mmol), and HATU (0.13 mmol) in DMAc in a mixing vessel for 2 h. After a test cleavage showed complete conversion to 19b, the resin was stirred in 5 mL cleavage mixture (TFA–H2O–TIS, 95:2.5:2.5 v/v) for 2 h. The resulting solution was collected, and the resin was washed with the cleavage mixture (2 × 2.5 mL) and CH2Cl2 (2 × 2.5 mL), and dried in vacuo. After a test cleavage showed complete conversion to 19b, the resin was stirred in 5 mL cleavage mixture (TFA–H2O–TIS, 95:2.5:2.5 v/v) for 2 h. The resulting mixture was precipitated in 50 mL ice-cold Et2O resulting in a white precipitate that was collected by centrifugation and dried. The product was purified using preparative RP-HPLC-MS on a C18 column using a gradient of MeCN in H2O containing 0.1% formic acid.