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On-resin Diels–Alder reaction with inverse electron demand: an efficient ligation method for complex peptides with a varying spacer to optimize cell adhesion
On-resin Diels–Alder reaction with inverse electron demand: an efficient ligation method for complex peptides with a varying spacer to optimize cell adhesion†

Marleen Pagel,* René Meier,* Klaus Braun,b Manfred Wiesslerb and Annette G. Beck-Sickinger∗a

Solid phase peptide synthesis (SPPS) is the method of choice to produce peptides. Several protecting groups enable specific modifications. However, complex peptide conjugates usually require a rather demanding conjugation strategy, which is mostly performed in solution. Herein, an efficient strategy is described using an on-resin Diels–Alder reaction with inverse electron demand (DARinv). This method is compatible with the standard Fmoc/tBu strategy and is easy to monitor. As a proof of concept a titanium binding peptide was modified with a cyclic cell binding peptide (RGD) by DARinv on a solid support applying different tetrazines and alkenes. The generated bulky DARinv linkers were employed to act as the required spacer for RGD mediated cell adhesion on titanium. In vitro studies demonstrated improved cell spreading on DARinv-conjugated peptides and revealed, in combination with molecular dynamics-simulation, new insights into the design of spacers between the RGD peptide and the surface. Performing the DARinv on resin expands the toolbox of SPPS to produce complex peptide conjugates under mild, catalyst free conditions with reduced purification steps. The resulting conjugate can be effectively exploited to promote cell adhesion on biomaterials.

Introduction

Peptide conjugates are perfect tools to selectively address various targets in medical applications and analytical investigations. Solid phase peptide synthesis (SPPS) provides a variety of orthogonal protecting groups, which facilitate the modification of peptides with fluorophores, drugs, carbohydrates, fatty acids or reporter molecules.1–3 Nevertheless, the efficient synthesis of multifunctional peptides with several complex bioactive moieties such as cyclic peptides is still challenging. Chemoselective click reactions offer the possibility to selectively ligate biomolecules without the use of further protecting groups in benign solvents resulting in high yields.4,5 Therefore click chemistry is a convenient method to upgrade traditional SPPS to obtain multifunctional peptides.6–8 It was shown that copper(i) catalyzed azide–alkyne cycloaddition (CuAAC), oxime ligation and recently the Staudinger–phosphite reaction are applicable for on resin synthesis to obtain branched and functionalized molecules.9–12 Click reactions on a solid support reduce purification steps, enable automation or protect sensitive amino acids like L-3,4-dihydroxyphenylalanine (DOPA) from oxidation.10 Another bioorthogonal cycloaddition is the Diels–Alder reaction with inverse electron demand (DARinv) that can be performed in water without catalysts or hazardous sideproducts and reagents.13 This irreversible reaction between an alkene (dienophile) and a tetrazen (diene) has been successfully used to synthesize peptide conjugates by us and others for various medical applications.14–17 However, solubility problems or instability of tetrazines can lead to difficulties.17,18 An on-resin approach can overcome these limitations and decrease purification and cleavage steps, thus increasing the yield. DARinv-conjugates mostly consist of bulky linker-units between two conjugated molecules with different distances and flexibilities depending on the applied dienes and dienophiles. This could be beneficial for several in vitro investigations where distances are required to enable binding of ligands to proteins or reporter molecules.19–21 RGD-peptides bind to receptors in the cell-membrane and thus can mediate
cell-adhesion on surfaces to improve healing and osseointegration of e.g. titanium implants.\(^22,23\) It could be shown that a minimum distance between the RGD-peptide and the surface is crucial for successful cell adhesion.\(^24\) Hence, DARinv-conjugated RGD-peptides present an efficient approach to promote cell attachment on surfaces such as titanium.

Herein, it is demonstrated that the DARinv can be expanded to exploit polymer-bound synthesis compatible with standard Fmoc/tBu strategy (Fig. 1). The applicability of two tetrazines and four different dienophiles was investigated by ligating a cell-adhesive- and a titanium-binding-peptide to the solid support. The obtained products with a varying linker length were then applied to improve cell adhesion on coated titanium. Moreover, molecular dynamics (MD)-simulation was used to assess the DARinv-linker conformation and thus discuss the cell response in theory.

### Experimental

#### Synthesis of TBP (1) and TBP 2

Titanium binding peptides (TBP (1) and TBP 2) were manually synthesized on a Tentagel S RAM (Iris Biotech) resin using the Fmoc/tBu strategy. Deprotection of \(\alpha\)-amino groups was performed twice with 30% (v/v) piperidine (Sigma Aldrich) in dimethylformamide (DMF, Biosolve) for 10 min. The peptide was elongated using 2 eq. hydroxybenzotriazole (HOBt, Novabiochem) and diisopropylethylamine (DIPEA, Roth) and respectively 2 eq. of Fmoc-Asp(Alloc)-OH, Fmoc-Pra-OH (Iris Biotech), Fmoc-DOPA(acetonide)-OH (Novabiochem), Fmoc-Lys(Mtt)-OH (Bachem) or Fmoc-Lys(Dde)-OH (Sigma Aldrich) in DMF for 10 min. The peptide was elongated on an acid labile 2-chlorotrityl-resin (Novabiochem) to obtain Fmoc-\(\alpha\)-Phe-Lys(Dde)-Arg(Pbf)-Gly-Asp(Bu)-resin. After Fmoc removal, the N-terminus was protected with triphenylmethyl chloride by DIPEA in DMF. Subsequently, Dde was cleaved from the Lys-side chain, as described, and modified with either the Reppe-dienophile (synthesized according to Pipkorn et al.) or 6-maleimidohexanoic acid by HOBt/DIC activation.\(^13\) After cleavage of the protected peptide from the resin with glacial acetic acid and 2,2,2-trifluoroethanol in DCM (1 : 1 : 8, v/v/v), the linear peptide was cyclized with HOBt/DIC in DCM for 16 h. Next, DCM was evaporated and the peptide was fully deprotected with TFA and a scavenger followed by purification. The peptides c[RGDFK(Alloc)] and c[RGDFE(Allyl)] were synthesized by the same protocol (Fmoc-\(\alpha\)-Phe-Lys(Alloc)-Arg(Pbf)-Gly-Asp(Bu)-resin, Fmoc-\(\alpha\)-Phe-Glu(Allyl)-Arg(Pbf)-Gly-Asp(Bu)-resin), however without side chain modification and trityl-protection.

#### Diels–Alder reaction with inverse electron demand (DAR inv) on resin to yield peptides 8a–c

TentaGel resin loaded with 1a and respectively 1b was swollen in \(\text{H}_2\text{O}\). Then the resin was incubated with 1.5 eq. of a 30 mM aqueous solution of peptides 3–6 at room temperature on a shaker in an open reaction vessel (\(\text{N}_2\) release). When the reaction was complete, the resin was washed with water, DMF and DCM. If the N-terminus was protected by Fmoc, a deprotection step with piperidine, as stated above, followed. The peptide was cleaved from the resin and isolated as described.

#### Monitoring of the Diels–Alder reaction with inverse electron demand (DAR inv) on resin by photometry

Diene modified resin (approximately 1 µmol) was transferred into a 96-well plate and swollen in water. Subsequently, the water was removed and the dissolved dienophile (30 mM; 100 \(\mu\)L) was added. An absorption scan from 430–600 nm of the diene modified resin was carried out with a plate reader (Tecan, Infinite® 200 PRO series). If the absorption maximum at 540 nm was diminished, the reaction was complete (8a: 5 h, 8b: 2 h, 8c: 7 d).
**Thiol-maleimide Michael addition to yield peptide 8d**

Peptide 8d was synthesized by ligation of TBP 2 (sequence: Ser-PEG-DOPA-Cys-DOPA-PEG-Pra-β-Ala) and c[RGDFK(maleimide)] (6) in H$_2$O/BDuOH for 20 h. The peptide was purified as described.

**Cleavage of peptides from the resin and purification**

Final and sample cleavage of the peptides was performed with TFA/scavenger (9:1 v/v) by shaking for 2 h at RT. Scavenger mixtures were used as follows: TIS/H$_2$O (1:1 v/v) for peptide 6 and TBP 2, thioanisole/thiocresol (1:1 v/v) and thioanisole/1,2-ethanediethiol (7:3 v/v) for 3–5, conjugates 8a,b and H$_2$O for peptides 1a,b and 8c. Peptides were precipitated and washed with diethyl ether. The dissolved peptide was analyzed and lyophiliized. Isolation of the obtained peptides was carried out by RP-HPLC on a Phenomenex Jupiter Proteo column (90 Å/4 μm, 22 mm × 250 mm) using linear gradients of eluents A and B. The identity and purity of the isolated products was verified by MALDI-ToF (Bruker Daltonics) and ESI-HCT (high-capacity ion trap, Bruker Daltonics) mass spectrometry and analytical MALDI-ToF (Bruker, Daltonics) and RP-HPLC. The peptide purity was evaluated by two different HPLC systems Figures48 and49. The purity of the identified products was confirmed by comparing the obtained signals with the calculated molecular weights and the peptide products were obtained with a purity of ≥90% (Table 1).

**Cellular assays**

SaOS-2 cells (Sarcoma osteogenic, kindly provided by Prof. Scharnweber, Dresden, Germany) were cultured under humidified atmosphere at 37 °C and 5% CO$_2$ in McCoy’s 5A containing 15% heat inactivated fetal calf serum (FCS), 1% (v/v) glutamine and 1% (v/v) penicillin/streptomycin (by PAA, Lonza and Biochrome).

Titanium foil (Sigma Aldrich, thickness: 0.127 mm) was cut into round pieces and etched in H$_2$SO$_4$ (30%)/H$_2$O$_2$ (1:1, v/v) for 20 h. The peptide was purified as described. The dissolved peptide was analyzed by MALDI-ToF (Bruker Daltonics) and ESI-HCT (high-capacity ion trap, Bruker Daltonics) mass spectrometry and analytical MALDI-ToF (Bruker, Daltonics) and RP-HPLC. The peptide purity was evaluated by two different HPLC systems. The peptide purity was confirmed by comparing the obtained signals with the calculated molecular weights and the peptide products were obtained with a purity of ≥90% (Table 1).

**Table 1: Analytical data of purified peptides**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence</th>
<th>M [Da]</th>
<th>[M + H]$^+$</th>
<th>Elution (％ACN)</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>c[RGDFK(Reppe)]</td>
<td>915.5</td>
<td>916.5</td>
<td>44$^b$</td>
<td>&gt;95</td>
</tr>
<tr>
<td>4</td>
<td>c[RGDFK(Alloc)]</td>
<td>687.3</td>
<td>688.0</td>
<td>40</td>
<td>&gt;95</td>
</tr>
<tr>
<td>5</td>
<td>c[RGDE(Allyl)]</td>
<td>644.3</td>
<td>645.0</td>
<td>40</td>
<td>&gt;95</td>
</tr>
<tr>
<td>6</td>
<td>c[RGDFK(maleimide)]</td>
<td>796.4</td>
<td>797.5</td>
<td>37</td>
<td>&gt;95</td>
</tr>
<tr>
<td>8a</td>
<td>C-PEG-Y*·K[2a·c[RGDFK(Reppe)]]-Y*·PEG-G*·βA-NH$_2$</td>
<td>2349.1</td>
<td>2350.1</td>
<td>36</td>
<td>&gt;90</td>
</tr>
<tr>
<td>8b$^c$</td>
<td>C-PEG-Y*·K[2b·c[RGDFK(Reppe)]]-Y*·PEG-G*·βA-NH$_2$</td>
<td>2326.1</td>
<td>2327.0</td>
<td>41$^c$</td>
<td>&gt;95</td>
</tr>
<tr>
<td>8c</td>
<td>C-PEG-Y*·K[2a·c[RGDFK(maleimide)]]-Y*·PEG-G*·βA-NH$_2$</td>
<td>2230.0</td>
<td>2231.0</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>8d</td>
<td>S-PEG-Y*·C[c[RGDFK(maleimide)]]-Y*·PEG-G*·βA-NH$_2$</td>
<td>1933.9</td>
<td>1935.0</td>
<td>33$^d$</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

Peptides were analyzed by MALDI-ToF MS (Bruker, Daltonics) and RP-HPLC. The peptide purity was evaluated by two different HPLC systems using different columns and gradients of eluent B (0.08 % TFA in ACN) in eluent A (0.1 % TFA in H$_2$O) on a Phenomenex Jupiter Proteo (90 Å, 5 μm)$^b$, Phenomenex Jupiter Proteo 300 Å, 4 μm)$^b$ and Varti Tide (200 Å, 6 μm)$^c$. Oxidized product isolated, PEG (polyethylene glycol), Y* (l-3,4-dihydroxyphenylalanine), G* (propargyglycine), βA (beta alanine). n.i. = not isolated.

To investigate the initial cell behavior to the synthesized peptide coatings, cells were resuspended in media without FCS (to prevent adsorption of serum proteins) and seeded on coated and washed Ti-plates. After adhesion for 6 h, the plates were washed twice with PBS and subsequently the cells were fixed with 4% paraformaldehyde in PBS for 30 min. The plates were washed with PBS and the cells were permeabilized with 1% triton X-100 in PBS for 1 min. Next, the slides were washed and cells were stained with phalloidin-tetramethylrhodamine-isothiocyanate (phalloidin-TRITC, Sigma Aldrich) and HOECHST 33342 (Sigma Aldrich). Fluorescence microscopy (Axio Observer microscope, Zeiss) was performed with mounted Ti-plates on glass slides. For each plate, three representing pictures were taken at 20 fold magnification. The average cell area was determined by manually outlining each cell on one representing picture of each triplicate (using the Software Axio Vision 4.8, Zeiss). Data were analyzed from at least 3 independent experiments and presented as mean ± SEM.

**Molecular dynamics of the linker**

Molecular dynamics simulations were carried out using the Gromacs-4.6 package.$^{25}$ The simulation system consisted of the respective linker with a C-terminal amidated and N-terminal acetylated lysine on the RGD-peptide side and a C-terminal amidated and N-terminal acetylated lysine/eyostine on the titanium binding site. The linker was parameterized with antechamber$^{26}$ and acype$^{27}$ for the GAFF force field. The charges were derived with the antechamber sqm method. The simulations were carried out under periodic boundary conditions in TIP3P water with 150 mM NaCl at 300 K for 50 ns after equilibration with NVT and NPT ensemble for 50 ps each. The trajectories were analyzed with the g_dist tool by measuring the C$_α$-C$_α$ distance and plotting the values in a histogram with a bin size of 0.5 Å.
Results and discussion

Peptide modification with tetrazines

The titanium binding peptide (TBP, 1) was synthesized on a Tentagel resin to enable the DARinv on the resin in aqueous solution. The sequence was built up of spacer units such as polyethylene glycol (PEG) and β-Ala. DOPA (L-3,4-dihydroxyphenylalanine), which shows high affinity and stability to titanium and other surfaces, was used to anchor the peptide to the biomaterial.28 Functional groups were introduced by using Lys, Pra (L-propargylglycine) and Cys to allow the multifunctional modifications of TBP.29 Two dienes (2a,b) were coupled to TBP after selective side chain deprotection of Lys (Fig. 2). The result of the reaction was studied by RP-HPLC and MALDI-ToF-MS analysis (Fig. 4d). The tetrazine stability in 1a and 1b was tested in solution and on resin over 7 d in an aqueous environment and yielded low fragmentation (see the ESI†). As a side reaction, the undesired DARinv between the alkyne functionality of Pra and the applied tetrazines was observed, mainly for peptide 1a in solution. On resin, this reaction occurred only to a small extent. Cleavage of 1b from the resin resulted in fragmentation induced by water, which was slightly increased after incubation of the resin in water for 7 d.10 Nevertheless, possible degradation of tetrazines by the cleavage of the peptide from the resin is circumvented by the DARinv on resin.

Peptide modification with dienophiles

Different dienophiles were introduced to the cyclic cell binding peptide c[RGDfK]. The peptide was synthesized on an acid labile chlorotrityl resin (Fig. 3). The modification with different dienophiles was either realized by the incorporation of alkene containing amino acids (4, 5) or by Lys-side chain modification (3, 6). The dienophile functionalized peptides were cleaved under mild acidic conditions to retain side chain protecting groups. Subsequently, the selective head to tail cyclization was performed in solution. Peptides were finally purified by RP-HPLC after complete deprotection by TFA. The Reppe-anhydride, which has already been used in previous studies, was coupled at the Lys-side chain of the c[RGDfK]-peptide to yield compound 3.31–33 To facilitate the introduction of dienophiles during SPPS, we used terminal alkenes, which are present in common protecting groups like Alloc and Ally. A 6-maleimidohexanoic acid modified peptide (6) was synthesized to explore the feasibility of this easily accessible dienophile for the DARinv on resin (Fig. 3).

Ligation of peptides by DARinv on resin

To perform the DARinv on the solid phase, the dienophile 3 was dissolved in H2O (c = 30 mM) and added to the resin bound peptides 1a and b. A disadvantage of reactions on the solid phase is the often complicated monitoring of the reaction. However, herein we demonstrate a direct photometric measurement of the reaction process on resin. The DARinv was monitored by measuring the specific absorbance of the tetrazines at 540 nm directly on the resin by photometry (Fig. 4b and c). The cycloaddition with peptide 1b was complete after 2 h. Tetrazine 2a resulted in a slower reaction with full conversion after 5 h (see the ESI†), which can be explained by the presence of stronger electron withdrawing groups in 2b compared to 2a. Higher reactivity of tetrazines often results in lower stability.34 During the DARinv on resin with peptide 3 we could not detect the differences in diene stability. However, for reactions with longer incubation times, as for peptide 6, lower stability of tetrazine 2b compared to 2a was observed (see the ESI†). Furthermore, combining the DARinv with additional
reactions as the CuAAC lead to stability problems for compound 8b originated from tetrazine 2b as well (see the ESI†). Contrarily, 8a was stable under the CuAAC on resin and was recently used to synthesize a multifunctional cell adhesive titanium coating.29 This suggests that the choice of the tetrazine can strongly influence the stability of the resulting DARinv-product as shown also for metabolic degradation.14 After completion of the reaction, the dienophile containing peptide 3, which was used in excess, was fully recovered by lyophilization. Cleavage from the resin and subsequent analysis showed the generation of the desired dihydropyridazine 8b (Fig. 4e). Notably, the DARinv-product was stable under the conditions of the Fmoc-cleavage with piperidine in DMF, which underlines the compatibility with Fmoc/tBu-based-SPPS.

Conjugate 8b was partially oxidized to the corresponding pyridazine (Fig. 1). Complete oxidation can be achieved by incubation with isoaconit nitrite and acetic acid, which can be performed on resin and in solution (Fig. 5).35

Terminal alkenes were successfully applied to react with tetrazines by a DARinv.36,37 Incubation of the tetrazine containing peptide 1a and dienophile 4 or 5 respectively could show the formation of the desired DARinv product, which was however cleaved after cycloaddition (for the hypothesized mechanism see the ESI†). The reaction of these terminal alkene bearing peptides with tetrazine 1b resulted only in low conversion of around 10% as a result of low reactivity and stability of the tetrazine (see the ESI†). Alternative tetrazines could be applied to overcome this problem to establish an easy introduction of dienophiles in SPPS. Moreover, the suggested mechanism could

be probably applied as a decaging method as described recently for proteins or drugs, since the active chemotherapeutic RGD-peptide is released upon fragmentation.38,39 Maleimides are classical dienophiles in normal Diels–Alder reactions.40 However, the DARinv between peptide 1a and the
maleimide modified RGD (6) resulted in the stable product 8c. The reaction completed rather slowly after 7 d (Fig. 6). This reactivity towards tetrazines should be considered when using maleimide modified tetrazines or a combination of DARinv with a thiol-maleimide addition. Moreover, this finding gives rise to the potential use of maleimides as easily accessible dienophiles in the DARinv. However, alternative tetrazines should be tested to increase the reaction rate. The outcome of all the studied reactions between the different tetrazines and dienophiles is summarized in Table 2.

The DARinv on a solid support displays the following advantages. Tetrazines are often hardly soluble in aqueous solution. Therefore, the reaction has to be carried out in organic solvents or the synthesis strategy has to be changed by the introduction of solubility increasing molecules such as short PEG units. Performing the DARinv with a tetrazine containing peptide bound to a solid phase overcomes this problem. Besides the advantages of water as a solvent for environmental and biochemical issues it is also favorable for kinetic reasons. It is known that water can increase the proximity of hydrophobic molecules like the used diene and dienophiles. Hence the reaction can be accelerated. Additionally, the water-swellable TentaGel resin facilitates the synthesis of rather long or branched peptides compared to the synthesis carried out on non-PEG-based resins. Furthermore, cleavage and purification steps are reduced since the crude resin bound peptides can be directly used for the ligation. This is especially beneficial for peptides that consist of rather cost intensive amino acids such as DOPA. DARinv-linkages as the dienophile carrying RGD peptide are easily recovered by lyophilization and can be thus used in excess without loss of yield owing to purification steps.

### Table 2 Summary of reactivity between different diene/dienophile combinations

<table>
<thead>
<tr>
<th>Tetrazine</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
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<tbody>
<tr>
<td>1a</td>
<td>Stable product 8a</td>
<td>Unstable product*</td>
<td>Unstable product*</td>
<td>Stable product 8c</td>
</tr>
<tr>
<td>1b</td>
<td>Stable product 8b</td>
<td>Low conversion*</td>
<td>Low conversion*</td>
<td>Low conversion*</td>
</tr>
</tbody>
</table>

* Unstable products and low conversion of reactions are discussed in more detail in the ESI.

**Fig. 6** DARinv on resin with c[RGDFK(maleimide)]. (a) Reaction scheme of the DARinv. The tetrazine bearing resin was incubated with the dienophile 6 dissolved in H2O/tBuOH. The peptide product was cleaved off from the resin by TFA. (b) Absorption scan of resin bound tetrazine 1a during the DARinv to monitor the progress of the reaction. (c) Crude from the resin by TFA. (d) Absorption scan of resin bound tetrazine 1b during the DARinv to monitor the progress of the reaction. (e) Crude product 8c from the resin by TFA. (f) RP-HPLC chromatogram and MALDI-ToF MS of the DARinv product 8c.
suggested that the three-dimensional length is evaluated. We suggest that a suitable linker consists of two short flexible ends, connected by a long rigid structure (see the ESI†). The flexible parts in the spacer may be important to freely orientate the RGD-peptide on the surface towards the cell membrane.

Conclusion

The Diels–Alder reaction with inverse electron demand (DAR_{inv}) could be successfully employed to ligate peptides on a solid support. This method describes an efficient strategy to complex peptide conjugates without using catalysts or additive reagents and it is easily monitored. Low solubility as well as yield loss and potential fragmentation through cleaving tetrazine modified peptides from the resin are thereby circumvented. Furthermore, maleimide was found to be an easily accessible dienophile alternative for DAR_{inv} ligation on resin. Large DAR_{inv}-reagents could be applied to generate a spacer that might be useful for several biochemical applications. MD-simulations gave insights into the theoretical conformations of different DAR_{inv}-linkers, thereby providing crucial information for the choice of a distinct diene–dienophile pair. Thereby, tetrazine 2b in the DAR_{inv} product 8b resulted in the most enhanced cell adhesion because of its less flexible structure. With the here presented DAR_{inv} on resin the toolbox of orthogonal reactions for SPPS is broadened.

Acknowledgements

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References
