Published in final edited form as:

Tetrahedron Lett. 2008 ; 49(16): 2510–2513. doi:10.1016/j.tetlet.2008.02.111.

Chemoselective immobilization of biomolecules through aqueous *Diels–Alder* and PEG chemistry

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Abstract

Aqueous Diels–Alder chemistry combined with a poly(ethylene glycol) (PEG) spacer was used to immobilize a diverse group of biomolecules onto a solid surface. Briefly, α , ω linear PEG conjugates were synthesized containing cyclopentadiene in the α position and either biotin, lactose, or protein A in the ω position. Linkers were coupled to *N*-maleimide (EMC)-functionalized glass substrates, and surface immobilization of biomolecules was confirmed by confocal fluorescence imaging.

Keywords

Diels–Alder reaction; Chemical selective immobilization; Poly(ethylene glycol); Biomolecules

Chemical selective immobilization of biomolecules onto solid surface has been a target of numerous synthetic endeavors because this process facilitates many potential applications of biomolecules. For example, microarrays, microbeads, nanoparticles, biosensor chips, and surface functionalization of medical devices play increasingly important roles in basic biological research and biomedical applications. A number of techniques for biomolecule immobilization have been established. However, conventional methods for surface bioconjugation are limited by low efficiency, selectivity, and harsh reaction conditions. Therefore, there remains a demand for the development of alternative approaches, in which the chemistry is compatible with the functional groups found in biomolecules and proceeds chemoselectively under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturing cosolvents and catalysts.

Diels–Alder cycloaddition has been recognized as a promising procedure for the bioconjugation of biomolecules, since it is fast and efficient in aqueous media¹ in addition to being chemoselective. It has been used for the modification of peptides² and proteins,³ for the labeling of DNA and RNA fragments with biotin or fluoresceine derivatives,⁴ and, most recently, for the immobilization of oligonucleotides^{5a} on glass surfaces, as well as a carbohydrate^{5b} and RGD peptides^{5c} on self-assembled alkanethiol monolayers on gold. The *Diels–Alder* approach, which involves a diene and a dienophile not present in any biomolecule, allows for a chemoselective reaction without the need for protecting groups, and water has an extraordinary rate-accelerating effect on the reaction process. It has been reported that the

Diels–Alder reaction in water turned out to be accelerated by a factor up to 10^4 when compared to that in organic solvents.⁶

Poly(ethylene glycol) (PEG) is widely used to functionalize solid surfaces and to modify proteins and peptides, as well as liposomes for drug delivery applications.⁷ For example, monofunctional PEG molecules coupled to proteins are known to prolong the protein circulation time in blood and reduce immunogenicity.⁸ Research in drug targeting has also utilized PEG molecules to modify liposomes surfaces.⁹ While functionalized carboxyl or amine PEGs are available, they remain expensive and require further chemical modification. Furthermore, these functional PEGs are available mainly in high molecular weights, which may limit the formation of a close packed PEG monolayer. Herein, we report the applicability of a *Diels–Alder* reaction combined with PEG chemistry for a chemical selective and biocompatible immobilization of biomolecules onto solid surfaces (Fig. 1). Specifically, we synthesized a cyclopentadiene-containing PEG for the immobilization of biotin, lactose, and protein A, as model ligands, onto EMC-derivatized glass.¹⁰ Notably, to an *s-trans* conjugated diene, cyclopentadiene adopts the *s-cis* conformation, which is exceptionally reactive to EMC.^{11,12}

As illustrated in Scheme 1, the key heterofunctionalized PEG, cyclopentadiene tetra(ethylene glycol) acetic acid **2** was prepared in four steps from tetra(ethylene glycol) **1** in 20% overall yield.^{5b} Next, the biotin–PEG–diene **6** was prepared by the coupling of carboxylic acid of **2** with biotinyl ethylamine (**3**, Sigma) with the activation of isobutylchloroformate in 81% yield.¹³ Similarly, lactose–PEG–diene **7**¹⁴ was synthesized from lactosyl ethylamine (**4**) in 57% yield.¹⁵ Protein A–PEG–diene **8** was synthesized through the acylation of lysine amine in protein A (Sigma) in the presence of EDC and NHS coupling reagents.

Next, the chemical selective immobilization of biomolecules onto EMC-functionalized glass slides (XENOPORE Corp.) was investigated. Incubation of EMC-slide with cyclopentadiene–PEG₄–biotin **6** in water at room temperature for 12 h, followed by washing with deionized (di) water three times gave a biotin–PEG functionalized surface. The successful immobilization was confirmed by streptavidin binding. As shown in Figure 2, the specific binding of FITC-labeled streptavidin to the biotin surface was confirmed by confocal microscopy with uniform binding (Fig. 2A), while no FITC-labeled streptavidin binding was observed in the presence of free biotin (Fig. 2B) or untreated EMC glass (Fig. 2C).

Likewise, lactose immobilization was performed by the incubation of EMC-glass slides in an aqueous solution containing cyclopentadiene–PEG–lactose at room temperature. A FITC-labeled lectin (from *Archis hypogaea*), which binds to β -galactose, confirmed the presence of surface bound lactose by confocal microscopy (Fig. 3), which gave a very uniform lectin binding image (Fig. 3A), while untreated EMC-glass slide did not show lectin binding (Fig. 3B). Meanwhile, nonspecific lectin Con A-FITC did not show lectin binding to this lactose–PEG functionalized glass slide either (Fig. 3C).

The use of the *Diels–Alder* reaction for covalent immobilization of large biomolecules has not been well described. Protein A, a 42 kDa factor produced by *Staphylococcus aureus*, binds the constant region of a wide range of antibodies from various classes and has been utilized for both purification of immunoglobulins,¹⁶ and antibody immobilization with preserved antigen-binding capacity, sensitivity, and stability compared with covalently antibody coupling.¹⁷ In this study, a Protein A PEG functionalized surface was produced by the incubation of EMC-derivative glass in the presence of a cyclopentadiene–PEG–protein A conjugate in di-water at room temperature for 12 h, followed by washing three times in di-water. The successful immobilization of protein A was confirmed by specific binding of a FITC-labeled antibody (Sigma) (Fig. 4A), while untreated EMC-glass slide did not show FITC-labeled antibody

binding (Fig. 4B). It is understandable that the *Diels–Alder* reaction is fully compatible with the nucleophilic side chains incorporated into the amino acids lysine, serine, threonine, and tyrosine. However, the free thiol groups might undergo Michael addition reaction to the malimide if the protein contains cysteine residues. Nevertheless, the incorporated PEG-spaced cyclopentadienes are much more accessible than the residual thiol groups in the protein backbone. In the current experiment, protein A does not contain a cysteine residue. Therefore, the successful immobilization of protein A was through *Diels–Alder* reaction.

In conclusion, we have demonstrated the applicability of aqueous *Diels–Alder* chemistry for the immobilization of diverse biomolecules onto a solid surface. Specifically, versatile heterofunctionalized poly(ethylene glycol) (PEG) linkers carrying cyclopentadiene were tested for the immobilization of biotin, lactose, and protein A onto EMC-functionalized glass slides. The reported approach is compatible with most functional groups found in biomolecules and proceeds chemoselectively under mild conditions and in aqueous solution, preferably in the absence of any potentially denaturing cosolvents and catalysts. On the other hand, the diene unit incorporated into the biomolecules is stable in aqueous solution. Therefore, it will overcome the limitations of conventional methods for surface bioconjugation, which are low efficiency, selectivity, and harsh reaction conditions. The current approach will find wide application in the functionalization of solid surface such as microchip, sensor, and nanoparticle as well as diagnostic and therapeutic medical devices.

Acknowledgements

We acknowledge financial support under the grants from the AHA-0565287B and the AHAF-H2007027 (X.-L.S.).

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13. Compound **6**: ^1H NMR (400 MHz, CDCl_3) δ 7.60 (s, 1H), 7.17 (s, 1H), 6.43-6.36 (m, 1H), 6.23-6.21 (m, 1H), 6.17-6.03 (m, 1H), 4.52 (m, 1H), 4.32 (s, 1H), 4.10 (s, 2H), 3.75-3.60 (m, 14H), 3.40 (m, 4H), 3.16 (m, 1H), 3.00-2.90 (m, 3H), 2.78-2.61 (m, 3H), 2.20 (m, 2H), 1.80-1.60 (m, 5H), 1.40 (m, 3H), 0.99 (t, $J = 10.2$ Hz, 2H).
14. Compound **7** (^1H NMR, 400 MHz, D_2O) δ 6.43-6.36 (m, 1H), 6.23-6.21 (m, 1H), 6.17-6.03 (m, 1H), 4.60 (s, 1H), 4.36 (d, $J = 7.8$ Hz, 1H), 4.30 (d, $J = 7.8$ Hz, 1H), 4.00 (m, 2H), 3.91-3.40 (m, 26H), 3.38-3.24 (m, 10H), 2.92 (m, 2H), 2.66-2.61 (m, 2H).
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Fig. 1.
Immobilization of biomolecules through an aqueous *Diels–Alder* reaction and PEG chemistry.

Fig. 2. Streptavidin-FITC binding to cyclopentadiene-PEG₄-biotin immobilized onto EMC-glass surfaces. (A) *Diels-Alder* immobilized biotin; (B) *Diels-Alder* immobilized biotin with free biotin; (C) untreated EMC-glass slide. Bar size: 100 µm.

Fig. 3.

Lectin-FITC binding to cyclopentadiene-PEG₄-lactose immobilized onto EMC-glass surfaces. (A) *Diels-Alder* immobilized lactose incubated with lectin-FITC; (B): untreated EMC-glass slide incubated with lectin-FITC; (C) *Diels-Alder* immobilized lactose incubated with lectin Con A-FITC. Bar size: 100 μm.

Fig. 4. IgG binding to cyclodiene-PEG₄-Protein A bound to EMC-glass slides. (A) Protein A coated surfaces incubated with IgG-FITC; (B) untreated EMC-glass slide incubated with IgG-FITC. Bar size: 100 μ m.

Scheme 1.

Synthesis of biotin/lactose/protein A-PEG₄-cyclopentadiene conjugates from tetra(ethylene glycol) (**1**, PEG₄).