Protein Ligation

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Diels–Alder Ligation and Surface Immobilization of Proteins**

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The site-specific equipment of proteins and other biomolecules with additional functional groups for subsequent biochemical and biological investigations, for example, fluorophores, spin probes, photoactivatable groups, and affinity tags, is among the most frequently employed and important methods of research in the life sciences. A number of techniques for protein derivatization are now established.[1] Recently, in particular, aldehyde-assisted ligations,[2] native chemical ligation,[3] expressed protein ligation,[4] expressed enzymatic ligation,[5] Staudinger ligation,[6] and the application of the Huisgen azide cycloaddition[7] have been developed as powerful new methods, thereby advancing the field significantly. However, due to the multifunctionality of biomacromolecules in general and proteins in particular and the manifold applications of such techniques, there is a major and continuing demand for the development of new technology providing alternatives to the methods mentioned above. The chemistry required must be compatible with the functional groups found in proteins and proceed chemoselectively under mild conditions and in aqueous solution, preferably in

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the absence of any potentially denaturating cosolvent. The Diels–Alder reaction is a highly selective transformation and can proceed in water with a higher velocity and selectivity than in organic solvents.[8] Its compatibility with biomolecules has been explored elegantly in the bioconjugation and/or immobilization of oligonucleotides and other biomolecules.[9]

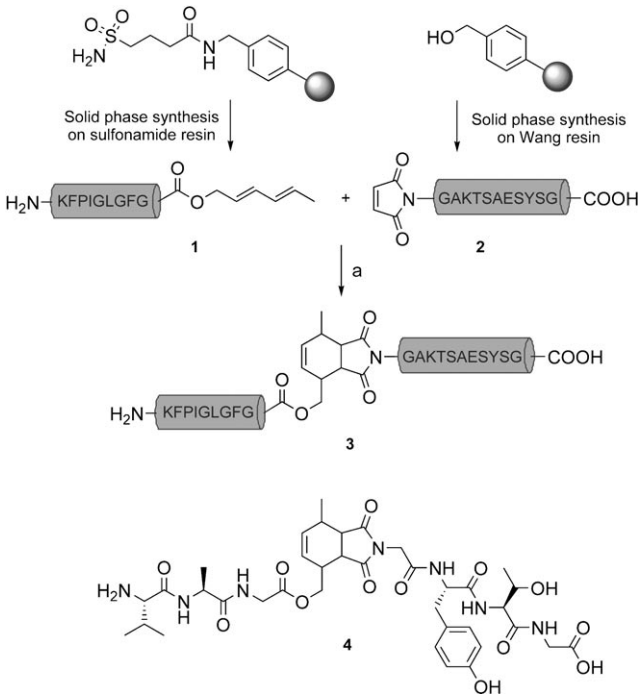
Herein, we demonstrate that the Diels–Alder reaction can be used for efficient ligation reactions of peptides and entire functional proteins.

In order to develop the underlying methodology, several peptides were initially equipped with a diene and dienophile, respectively. For instance, nonapeptide **1** (Scheme 1) was synthesized on the solid phase by employing a sulfonamide resin.[10] The *trans*,*trans*-2,4-hexadienyl ester was introduced by alkylation of the acylsulfonamide linker and subsequent treatment with the corresponding alcohol.[11] The maleimido peptide **2** was synthesized on Wang resin by applying the 9-fluorenylmethoxycarbonyl (Fmoc)/*t*Bu protocol.[11]

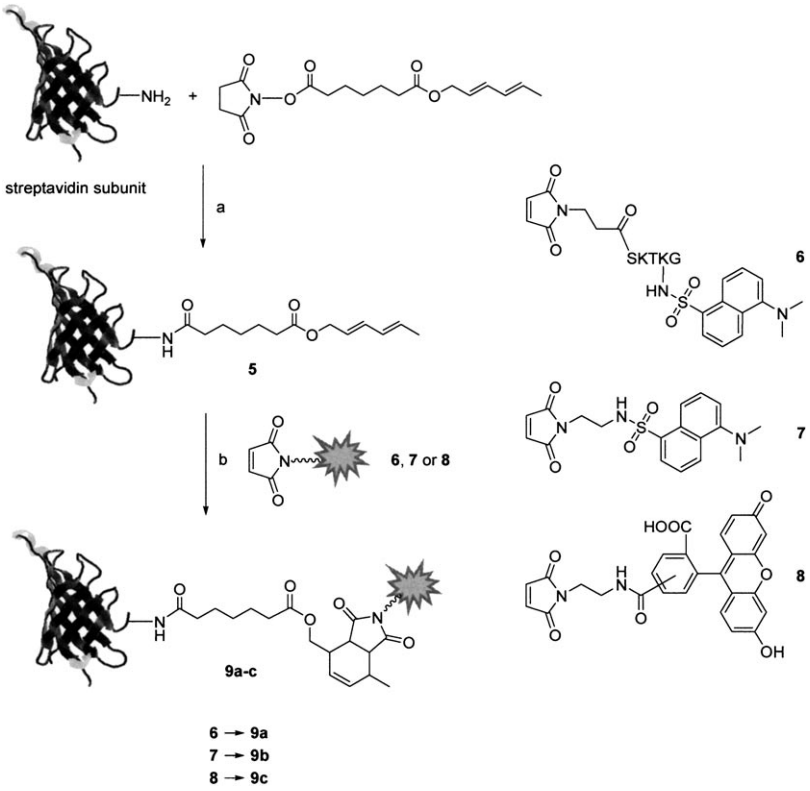
Upon exposure of the peptides to each other at 25 °C in water, the desired cycloaddition proceeded smoothly with a conversion of 92 % after 47 h. Peptide **3** was isolated in 67 % yield and identified by NMR spectroscopy and ESI-MS (calculated mass: *m*/*z* 2153.1; observed mass: *m*/*z* 2153.0). Spectroscopic and chromatographic analysis of Diels–Alder adduct **3** did not allow the stereoselectivity of the cycloaddition to be conclusively determined. However, peptide **4**, which was synthesized by analogy, was obtained as a single *endo* stereoisomer.[12] This observation indicates that in this transformation the peptides function as efficient stereodirecting groups to yield a cycloadduct with at least 95 % diastereomeric excess.

These results demonstrate that the Diels–Alder process is fully compatible with the nucleophilic side chains incorporated into the amino acids lysine, serine, threonine, and tyrosine. We note, however, that free SH groups of cysteine residues undergo conjugate addition to the maleimide and, therefore, they require protection (data not shown; see also below).

The applicability of the method and the reaction conditions described above to protein labeling was initially investigated by employing streptavidin as a model protein. Streptavidin is composed of four biotin-binding subunits containing lysine but not cysteine residues.[13] The protein was acylated with a hexadienyl bifunctional linker on the lysine side chains (Scheme 2). MALDI-TOF analysis indicated that, on average, each subunit was acylated once if a linker/protein ratio of 6:1 was chosen (see the Supporting Information). After purification by ultrafiltration, the modified protein **5** was treated with maleimide-functionalized fluorescent peptide **6** or maleimide-functionalized fluorophores **7** or **8** at 25 °C in water. After removal of unligated peptide dienophile by ultrafiltration, analysis of the reaction products by sodium dodecylsulfate (SDS) PAGE (Figure 1) and MALDI-MS (see the Supporting Information) showed that the desired cycloadducts had been formed. If nonfunctionalized streptavidin was combined with dienophile **6** at pH values of 5.5–



Scheme 1. Peptide ligation by means of a Diels–Alder cycloaddition. a) 10 mM Peptide, H_2O, 25 °C, 47 h.



Scheme 2. Diels–Alder ligation of the streptavidin-derived diene **5** with dienophiles **6**–**8**. a) 2 mg mL^{-1} Streptavidin (1 equiv) in H_2O, hexadienyl cross-linker (6 equiv), 2 h, 25 °C; b) 100 μM **5** (1 equiv) in H_2O or 10 mM sodium phosphate buffer (pH 5.5–6.5), **6**, **7**, or **8** (30 equiv), 24 h, 25 °C.

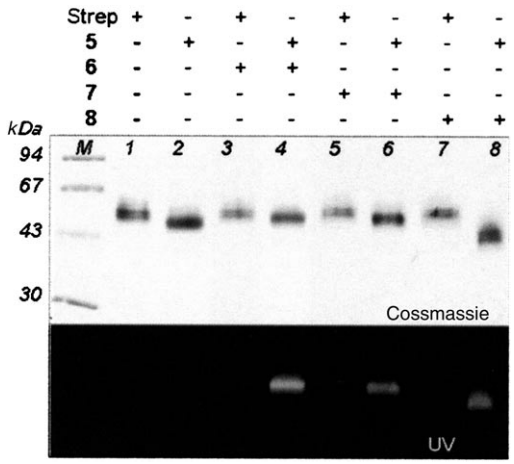


Figure 1. SDS PAGE analysis of the streptavidin conjugates **9a–c** obtained by Diels–Alder ligation of streptavidin-diene **5** (lane 2) with maleimides **6** (lane 4), **7** (lane 6), and **8** (lane 8), respectively. Control experiments showed that no binding occurs when wild-type streptavidin (lane 1) is combined with maleimides **6** (lane 3), **7** (lane 5), or **8** (lane 7) in water under slightly acidic conditions (pH 5.5–6.5). The 15% SDS PAGE gel was photographed while exposed to UV light (lower part) prior to Coomassie Blue staining (upper part). Strep: streptavidin, M: molecular-weight marker.

6.5, coupling to the protein through nucleophilic attack to the α,β-unsaturated imide could not be detected, whereas this side reaction occurs at pH 7. Thus, under slightly acidic conditions, the Diels–Alder ligation employing maleimido-modified peptides and fluorescent probes is chemoselective. The labeling of streptavidin by the cycloaddition method did not impair the biotin-binding activity of the protein,[14] a fact indicating that it remains fully functional after the Diels–Alder ligation process.

The stability of the diene unit incorporated into proteins in aqueous solutions and its compatibility with the functional groups found in these biopolymers opens up an opportunity to combine the Diels–Alder ligation method with other ligation techniques. In such a combined strategy, the protein of interest is initially functionalized by a general strategy with a diene unit and then the resulting protein-derived diene can be further functionalized site specifically, that is, at the C terminus, by Diels–Alder reactions with different dienophiles under very mild conditions.

This approach was successfully implemented by employing the Rab7 protein as a representative biologically relevant example. The Rab proteins are key regulators of vesicular transport that control budding, transport, and fusion of intracellular vesicles.[15] Many aspects of these multistep processes including, for instance, the timing of the cellular events and the localization of the proteins involved are only partly understood; differently and site-specifically labeled Rab proteins are considered to be versatile probes for the study of such biological events.[16–18]

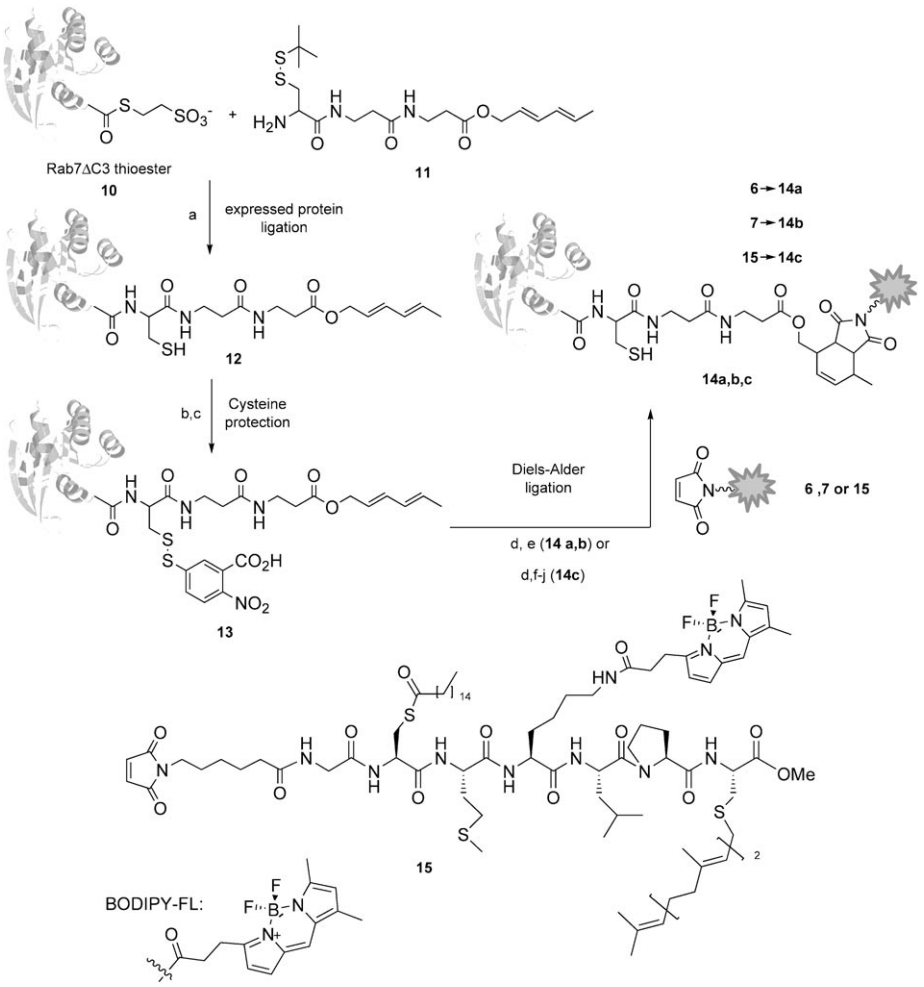
Initial functionalization of Rab7 was achieved by means of expressed protein ligation.[4] To this end, recombinant truncated Rab7 thioester **10** was expressed as described[17] and then ligated with peptide hexadienyl ester **11** under reducing conditions[16] to yield Rab hexadienyl ester **12** (Scheme 3). The ligation process generates a nucleophilic cysteine side chain in addition to the three cysteines that are buried in the core of the Rab protein. To avoid undesired modification of these mercapto groups in the subsequent reaction with the maleimide, the accessible cysteine side chains were protected as disulfides by treatment with Ellmann's reagent[19] immediately after the ligation reaction to yield masked protein ester **13**.[20]

Protein hexadienyl ester **13** was then dialyzed against phosphate buffer (pH 6) to remove all small molecules (MESNA, Ellmann's reagent, dienyl peptide **11**) and subjected to a Diels–Alder reaction with peptide-derived dienophile **6** or dansyl derivative **7** for 24 h. The coupling reactions were terminated by addition of excess dithiothreitol which traps the dienophile and simultaneously converts the disulfides into unmasked thiols.

The ligation products **14a** and **14b** were analyzed by SDS PAGE (Figure 2A), and all intermediates and final products were identified by ESI-MS (see the Supporting Information). In the presence of a 100-fold excess of dienophile the hexadienyl protein is converted into the expected fluorescent conjugates with high efficiency (approximately 90%). In the presence of 30 equivalents of dienophile the ligation proceeds as well; however, in this case a longer reaction time is required to reach complete transformation. Undesired multiple labeling of Rab7 could not be detected. The purification of the ligated Rab proteins was carried out as previously described.[16a]

Upon UV illumination a distinct fluorescence band is observed that cannot be detected for wild-type Rab7 prefunctionalized with Ellman's reagent prior to ligation. Treatment of dienophile **6** with wild-type Rab that was not protected at the cysteine SH groups leads to the incorporation of the fluorescent label. Likewise treatment of dienophile **6** with wild-type Rab that was protected at the cysteine SH groups does not lead to incorporation of the fluorescent label (see the Supporting Information).

In order to establish whether the generated proteins remain natively folded and functionally active, we analyzed the interaction of semisynthetic protein **14a** with Rab escort protein 1 (REP-1). REP-1 is an accessory factor that facilitates prenylation of Rab guanosine triphosphatases (GTPases) by presenting them to Rab geranylgeranyltransferase and subsequently by delivering them to the target membranes. Recognition of Rab proteins by REP-1 occurs through a large protein–protein interface and requires integrity of the Rab GTPase ternary structure.[17] To analyze the interaction of Rab7 with REP-1 we took advantage of the dansyl label attached to the flexible C terminus of Rab7; this label enables the use of fluorescence spectroscopy to monitor the interaction of both proteins. When the solution of semisynthetic Rab7 **14a** fluorescently excited at 333 nm was titrated with increasing concentrations of REP-1a, a dose-dependent and saturable increase of the fluorescence emission at 440 nm was observed (Figure 3). Titration data could be fitted by using a quadratic equation describing the binding curve and were consistent with a 1:1 stoichiometry and a K_d value of 2 nM. This K_d value is very close to the one determined earlier with mant-GDP-bound Rab7.[21] The



Scheme 3. Combination of expressed protein ligation and Diels–Alder ligation for site-specific modification of the Rab7 protein. a) 0.3 mM Thioester-tagged Rab7ΔC3 **10** in 5 mM sodium phosphate buffer (pH 7.5), 20 mM NaCl, 20 mM MESNA, 0.4% CHAPS, 10 μM GDP, 0.2 mM $MgCl_2$, and 6 mM peptide **11**, overnight, 16°C; b) DTNB, 30 min, 25°C; c) dialysis against DA buffer (5 mM sodium phosphate buffer (pH 6.0), 20 mM NaCl, 0.2 mM $MgCl_2$, 20 μM GDP); d) 40 μM Rab7 **13** (1 equiv) in DA buffer, maleimide **6** or **7** (100 equiv), 24 h, 25°C; e) DTT, 2 h, 25°C: f) 40 μM Rab7 **13** (1 equiv) in DA buffer, maleimide **15** (100 equiv) in $MeOH/CH_2Cl_2$ (3:1), 24 h, 25°C (ligated product precipitates); g) centrifugation and washing of the pellet with MeOH (×3) to remove excess peptide; h) dissolution of the precipitate in denaturation buffer (100 mM Tris-HCl (pH 8.0), 6 M guanidinium-HCl, 100 mM DTE, 1% CHAPS, 1 mM EDTA) overnight at 4°C; i) protein renaturation by 25-fold dilution dropwise into refolding buffer (50 mM HEPES (pH 7.5), 2.5 mM DTE, 2 mM $MgCl_2$, 10 μM GDP, 1% CHAPS, 400 mM arginine-HCl, 400 mM trehalose, 0.5 mM PMSF, 1 mM EDTA) with stirring, 25°C, 3 h; j) protein concentration to approximately 40 μM by ultracentrifugation (Amicon 10 kDa cut-off). MESNA = sodium 2-mercaptoethane sulfonate, CHAPS = 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate, GDP = guanosine 5′-diphosphate, DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid), DA = Diels–Alder, DTT = dithiothreitol, Tris = tris(hydroxymethyl)aminomethane, DTE = dithioerythritol, EDTA = ethylenediaminetetraacetate, HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, PMSF = phenylmethylsulfonyl fluoride.

obtained data prove that the developed procedure yields fully active functionalized Rab7 protein.

Our results demonstrate that the Diels–Alder ligation offers an advantageous new opportunity for the site-selective functionalization and labeling of proteins and peptides. It proceeds under very mild conditions with high selectivity and is compatible with most functional groups found in proteins. Its combination with other ligation methods, particularly expressed protein ligation, is feasible and allows a given protein to be rapidly equipped with different functional groups in a site-specific manner, that is, at the C terminus.

The use of the two-step ligation strategy delineated above is particularly indicated if reporter groups or tags need to be introduced into proteins that are not stable under the conditions of expressed protein ligation. For instance, in the course of a different research program, we observed that the fluorescence of the BODIPY label (Scheme 3), one of the most advantageous and very often used fluorophores, is lost during attempted ligation of BODIPY-functionalized peptides to proteins by means of expressed protein ligation. However, fluorescently labeled Rab proteins are formed if diene-modified Rab protein **13** is subjected to Diels–Alder ligation with BODIPY-labeled peptides.

Thus, upon treatment of protein dienyl ester **13** with maleimide-tagged doubly lipidated peptide **15**, the desired lipid-modified protein **14c** was formed and isolated (Scheme 3 and Figure 2B). The ligated protein was identified by ESI-MS (calculated mass for $[M^+]$: 25496 Da; found: 25495 Da). Lipidated peptide **15** represents a fluorescently labeled analogue of the *S*-palmitoylated and *S*-farnesylated C terminus of the human Ras protein and was synthesized as described.[22] Notably, the synthesis of chimeric lipoprotein **14c** also demonstrates that the Diels–Alder ligation is applicable to the synthesis of sensitive protein conjugates like acid- and base-sensitive lipoproteins.[23]

In order to further demonstrate the scope of applications of the Diels–Alder reactions with dienyl-modified proteins, we investigated the possibility of employing this transformation for the immobilization of proteins on glass slides.[24] Streptavidin was chosen as a model protein and was first ligated to a cyclopentadiene derivative by analogy to the procedure described above to yield protein **16** (Scheme 4 A). On average, each streptavidin subunit was acylated with one dienyl linker, as indicated by the mass spectrum (data not shown). The protein–cyclopentadiene conjugate was then

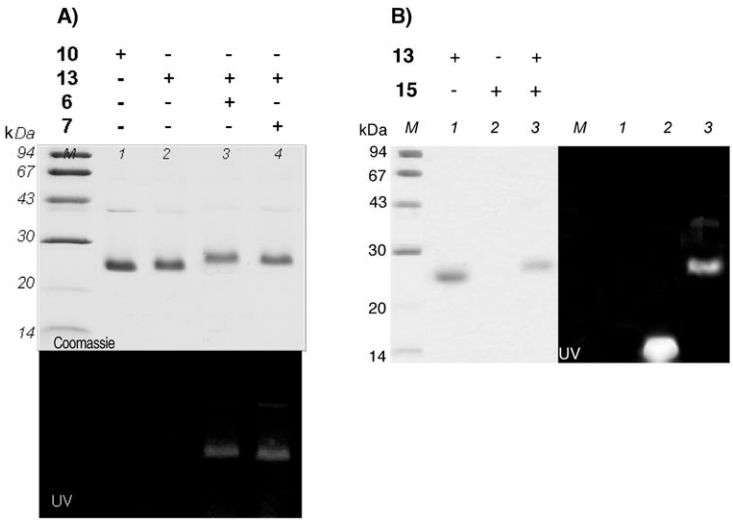


Figure 2. SDS PAGE analysis of the Diels–Alder ligation products. A) **14a** (lane 3) and **14b** (lane 4) obtained from masked Rab7 hexadienyl ester **13** (lane 2) with maleimide probes **6** and **7**, respectively. B) **14c** (lane 3) obtained from masked Rab7 hexadienyl ester **13** (lane 1) with BODIPY-labeled maleimide peptide **15** (lane 2).

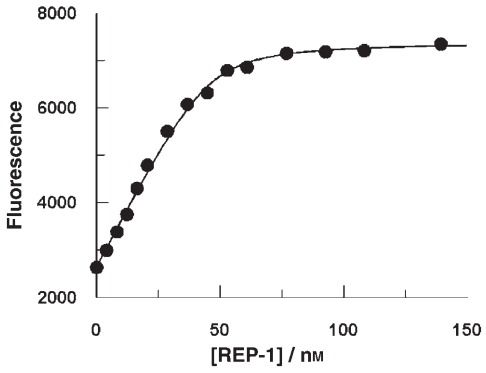
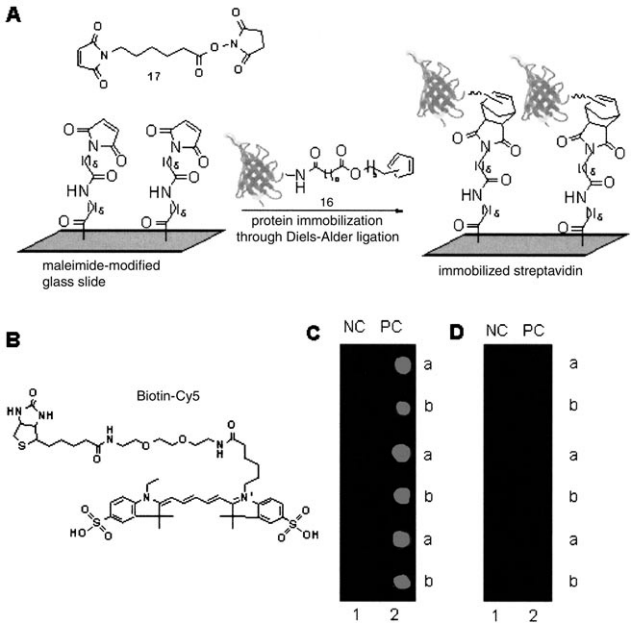


Figure 3. Spectrofluorometric titration of **14a** with REP-1. Conditions: 25 °C, 25 mM HEPES (pH 7.2), 40 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE, and 20 μM GDP. The concentration of **14a** complex was 50 nM. The excitation/emission wavelengths were fixed at 333/440 nm. Data were fitted to a quadratic equation as implemented in the program Grafit 5.1 (Erithacus software) leading to a dissociation constant (K_d) value of 1.9 ± 0.5 nM, which is consistent with a 1:1 stoichiometry.

subjected to immobilization on a maleimide-functionalized surface. To this end, glass slides with an amino-functionalized surface were decorated with the functionalized maleimide **17** (Scheme 4 A). For the preparation of the maleimide-functionalized surface, an aminocaproic acid linker was introduced to establish distance between the functional groups and the surface; this was followed by coupling of the maleimide through amide formation.[25]

For the immobilization experiments, the diene-modified streptavidin (positive control) and the nonmodified streptavidin as a negative control were dissolved in double-distilled water (pH ≈ 6) at 7 μM concentration and spotted to the maleimide-modified surface by using a piezo-driven spotting robot.[26] After 8 h the slides were washed with water (3 times for 5 min) and buffer (3 times for 5 min). The slides were then



Scheme 4. A) Immobilization of the diene-functionalized streptavidin **16** on maleimide-functionalized glass slides. B) Structure of biotin–Cy5. C) Binding of Cy5-labeled biotin to immobilized diene-functionalized streptavidin (lane 2) as a positive control (PC) and nonmodified streptevidin (lane 1) as a negative control (NC). a = without glycerol (15%), b = with glycerol. D) Same as (C) but with the maleimide-functionalized surface blocked by 5% mercaptoethanol.

treated with Cy5-labeled biotin (10 nM, 30 min at room temperature; Scheme 4 B), and the fluorescence signals were recorded and quantified after removal of excess reagent. The results for the immobilization of diene-modified streptavidin are shown in Scheme 4 C. The immobilization of diene-modified streptavidin, either without or in the presence of 15 vol% of glycerol in the spotting solution, gave clear fluorescence signals, whereas no signal was observable for the negative control. If the maleimide-modified surface was blocked by treatment with mercaptoethanol prior to application of the protein-derived diene no signal was observable (Scheme 4 D), a result showing that the immobilization was due to covalent-bond formation with the immobilized dienophile. These results clearly demonstrate that the Diels–Alder reaction provides a new method for the immobilization of proteins on glass slides.

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