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Multivalent Peptide and Protein Dendrimers Using Native Chemical Ligation**

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Multiple, simultaneous interactions are often used in biology to enhance the affinity and specificity of binding, an effect that is known as multivalency.[1] The principle of multivalency has been recognized as an important strategy for the development of (semi)synthetic ligands with high affinity and specificity for biological targets.[1–6] Dendrimers are well-defined, hyperbranched polymers with a high density of functional groups and are therefore attractive scaffolds for multivalent display of natural ligands.[7–9] Dendrimers have been successfully applied in the design of multivalent sugar ligands,[10–12] but much less work has been reported on multivalent peptide and protein dendrimers. Solid-phase peptide synthesis has been used to construct dendritic peptide wedges by condensation of successive generations of lysine residues followed by functionalization of the α and ε amine groups with peptides.[13,14] These so-called multiple antigen peptides (MAPs) typically contain two to eight peptide chains and have been found to be more immunogenic than single

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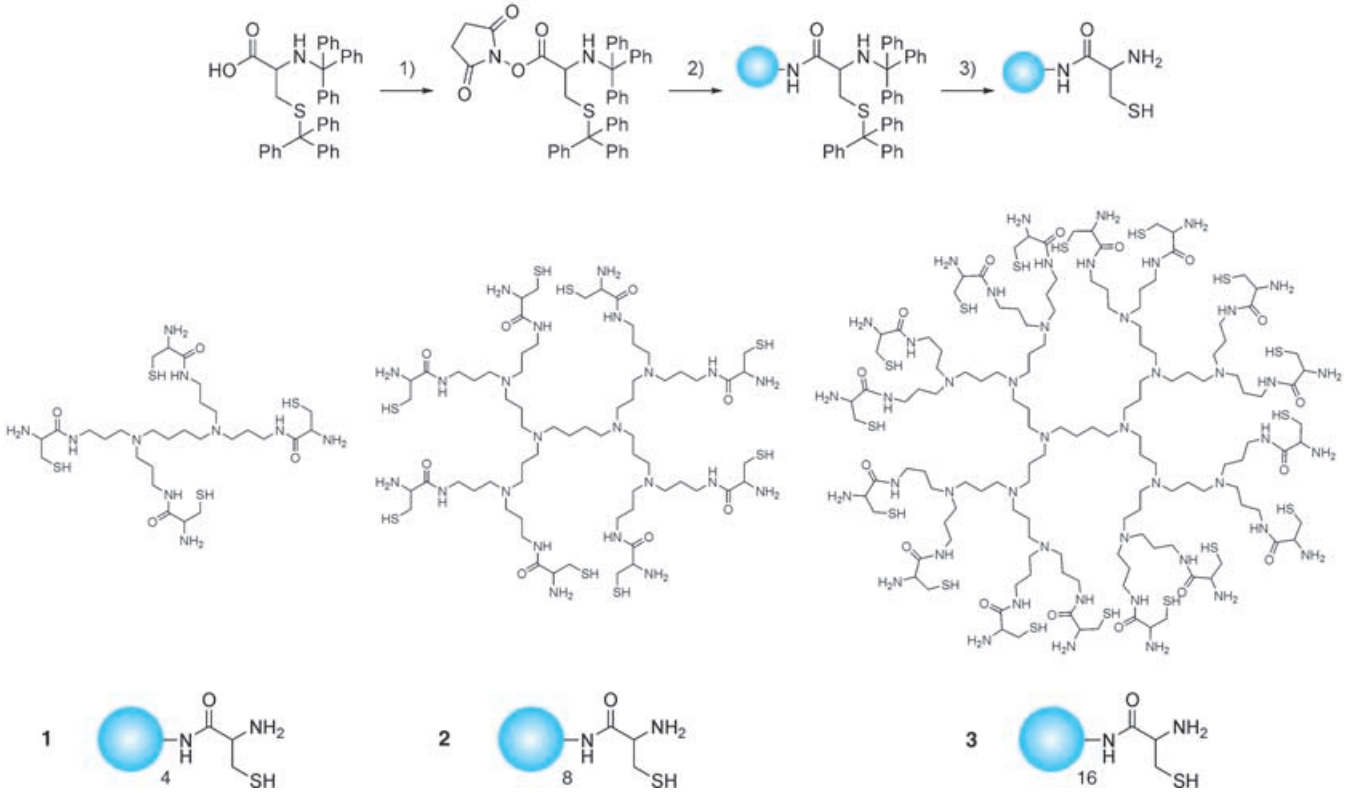
Supporting information (synthesis and characterization of the dendrimers, including mass spectra for all the peptide and protein dendrimers) for this article is available on the WWW under http://www.angewandte.org or from the author.

peptides. Dendrimers functionalized with cysteine-reactive chloroacetyl[15] and maleimide[16] groups have been used to conjugate cysteine-containing peptides, but this strategy cannot be applied to peptides and proteins containing functionally important native cysteine residues. Convergent synthesis of peptide dendrimers has also been reported by condensation of ketone-functionalized polyamidoamine (PAMAM) dendrimers with aminooxy-functionalized peptides, but this method is unsuitable for native proteins.[9] The few reports of protein ligation to dendrimers typically involve the coupling of a single protein (for example, an antibody) using nonspecific conjugation chemistry that results in conjugation at multiple sites on the protein. Recently, the preparation of multivalent hemoglobin dendrimers was reported that involved specific conjugation of a fourth generation PAMAM dendrimer to hemoglobin using a hemoglobin-specific cross-linking agent.[17] However, no general synthetic strategy is currently available that allows conjugation of dendrimers with both oligopeptides and recombinant proteins in a chemoselective manner.

Native chemical ligation was first reported by Dawson et al. as a unique method to ligate two unprotected peptide fragments to form a native peptide bond.[18] This chemoselective reaction occurs spontaneously between a peptide with a C-terminal thioester and a peptide with an N-terminal cysteine residue under aqueous conditions at neutral pH. Native chemical ligation has allowed the chemical synthesis of large proteins (by multistep ligation of several peptide fragments), the synthesis of proteins with synthetic moieties such as fluorescent dyes and biotin tags, and the immobilization of peptides and proteins on surfaces.[19–23] The application of native chemical ligation was recently extended to recombinantly expressed proteins by the development of expression systems based on self-cleavable intein domains that generate proteins containing N-terminal cysteine or C-terminal thioester groups.[24,25] Herein we report the application of native chemical ligation as an attractive and general synthetic strategy to conjugate both oligopeptides and recombinantly expressed proteins to dendrimers, thus resulting in multivalent peptide and protein dendrimers.

Dendrimers can either be functionalized with thioester groups or with cysteine residues to allow native chemical ligation. We chose the latter strategy because the synthesis of these cysteine dendrimers is more straightforward. Cysteine dendrimers generations 1–3 (Scheme 1)were synthesized by reaction of the amine end groups of poly(propyleneimine) dendrimers with succinimide-activated and trityl-protected cysteine residues (Scheme 1). Deprotection of the trityl-protected amine and thiol groups using trifluoroacetic acid (TFA) yielded the cysteine-functionalized dendrimers **1**–**3** in good yields. Analysis of **1**–**3** by electrospray ionization mass spectrometry (ESI-MS) showed that the cysteine residues were reduced in the presence of the mild reductant β-mercaptoethanol. Removal of the reductant and addition of



Scheme 1. Top: synthesis of cysteine-functionalized poly(propyleneimine) dendrimers: activation of trityl-protected cysteine with disuccinimidylcarbonate in acetonitrile (1), coupling of activated cysteine to poly(propyleneimine) dendrimer using triethylamine in dichloromethane (2), and deprotection of cysteine end groups using trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water (3). Bottom: schematic structures of cysteine dendrimers **1**, **2**, and **3**.

hydrogen peroxide resulted in the immediate formation of dendrimers with two, four, or eight intramolecular disulfide bonds, respectively (see Supporting Information). To assess the reactivity of the cysteine dendrimers in native chemical ligation reactions we performed ligation reactions with several peptides functionalized with an MPAL thioester at their C-terminus.[19] Ligation of **1** with four equivalents of LYRAG, a peptide previously used in studies on native chemical ligation,[19] resulted in the formation of the peptide tetramer **1**-(LYRAG)$_4$ (Figure 1 a). Analysis by liquid chromatography/mass spectrometry (LC-MS) showed that the reaction went to completion as no **1**-(LYRAG)$_{1-3}$ were detected (not shown). Ligation reactions were also performed between LYRAG and dendrimers **2** and **3** to test the efficiency of the ligation reaction for higher generations of cysteine dendrimers. Ligation of **2** with LYRAG again went to completion and yielded the peptide octamer **2**-(LYRAG)$_8$ exclusively (Figure 1 b). The MALDI-TOF spectrum obtained after ligation of **3** with LYRAG showed a signal consistent with the formation of **3**-(LYRAG)$_{16}$, but also contained signals attributable to **3**-(LYRAG)$_{12-15}$. Similar results were obtained for other oligopeptides such as GRGDSGG (Table 1). The RGD sequence present in this peptide is known to bind to various extracellular integrin receptors and can thus be used to attach dendrimers to cell surfaces.

Table 1: Overview of peptide and protein dendrimers obtained by native chemical ligation.

Dendrimer	Observed mass [Da]	Calculated average mass [Da][a]
1-(LYRAG)$_4$	3139.7	3139.8
2-(LYRAG)$_8$	6419.7	6419.9
3-(LYRAG)$_{16}$	12262[b]	12307.6
1-(GRGDSGG)$_4$	3243.2	3243.5
2-(GRGDSGG)$_8$	6627.1	6627.1
1-(GFP)$_1$	28288.6 (0.5)[c]	28291
1-(GFP)$_1$(GRGDSGG)$_3$	30172.5 (0.7)[c]	30177
1-(GFP)$_4$	110971.1 (4.7)[c]	110976

[a] Average masses were calculated assuming the complete reduction of the cysteine groups. [b] In addition to the signal of **3**-(LYRAG)$_{16}$, the MALDI-TOF spectrum showed signals consistent with **3**-(LYRAG)$_{12-15}$. [c] The number between brackets is the experimental error as determined using the program MaxEnt 1.

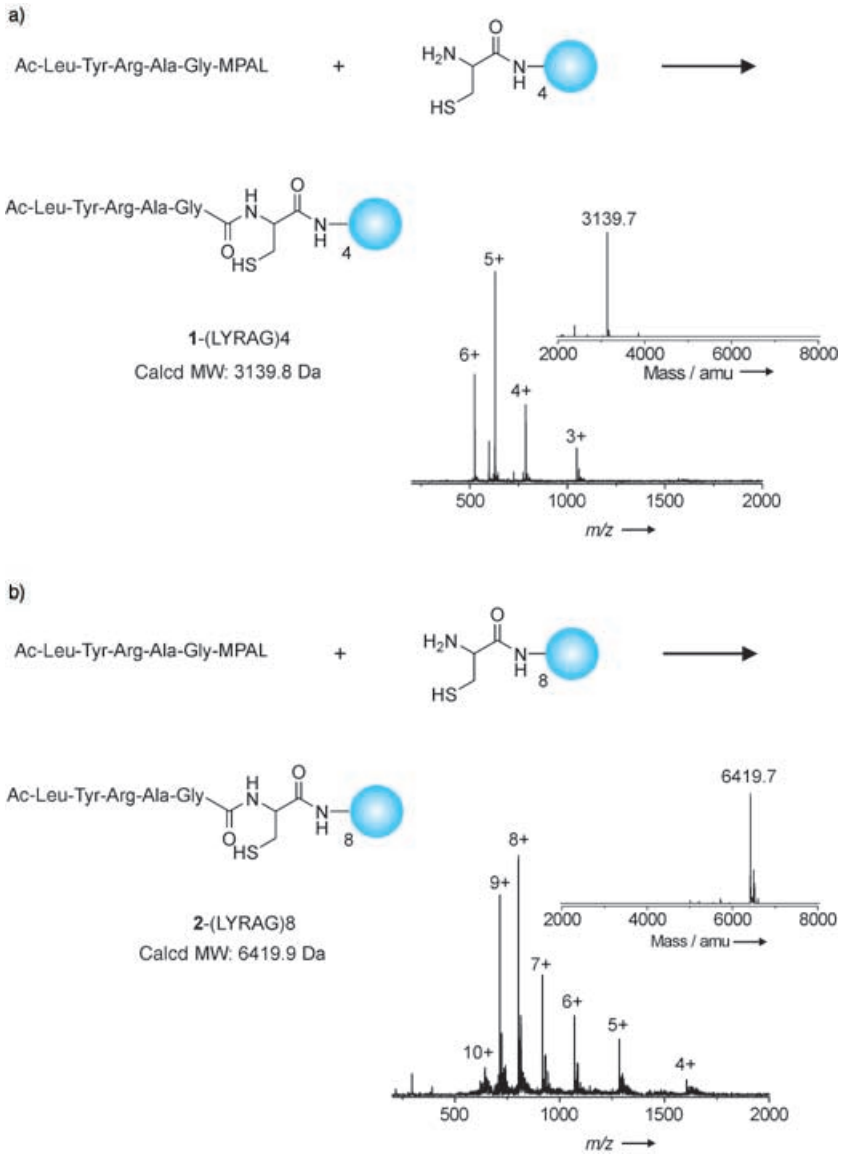


Figure 1. Native chemical ligation of the MPAL-thioester of LYRAG to first (a) and second (b) generation cysteine dendrimers **1** and **2**. Dendrimer was dissolved in 0.1 M Tris, 6 M guanidine, 2% (v/v) thiophenol, and 2% (v/v) benzyl mercaptan pH 7.0–7.5 and treated with four (**1**) or eight equivalents (**2**) of MPAL-peptide for 1 h at 37 °C. The mass spectra were obtained by ESI-MS after removal of the buffer components by RP-HPLC.

Green fluorescent protein (GFP) was chosen as a model protein to study the ligation of folded proteins to cysteine dendrimers. GFP was cloned into the IMPACT vector pTXB1 to yield an *E. coli* expression vector for a fusion protein of GFP with an intein domain and a chitin-binding domain (CBD). The cloning strategy used here meant that the GFP contained eight additional residues at the C-terminus of GFP, that is, NEFLEGSS. Overnight incubation of the GFP-intein-CBD protein bound to the chitin column with MESNA resulted in cleavage of the peptide bond to the GFP and the formation of GFP thioester. ESI-MS analysis showed the presence of a single protein signal with a mass of 27705 Da that is consistent with the MESNA thioester of GFP (theoretical mass: 27703.9 Da). The GFP thioester was first ligated to dendrimer **1** using a large excess of the dendrimer. ESI-MS of the reaction mixture after incubation for 20 h at room temperature showed the clean conversion of GFP thioester to **1**-(GFP)$_1$, with no indication of the presence of the initial GFP thioester or dendrimer with more than one GFP (Figure 2). The large difference in molecular weight between **1**-(GFP)$_1$ and **1** allowed the easy removal of excess of **1** from the ligation mixture by repeated concentration/dilution steps using a centrifugal filter with a 10-kDa molecular-weight cutoff. Subsequently, **1**-(GFP)$_1$ was treated with an

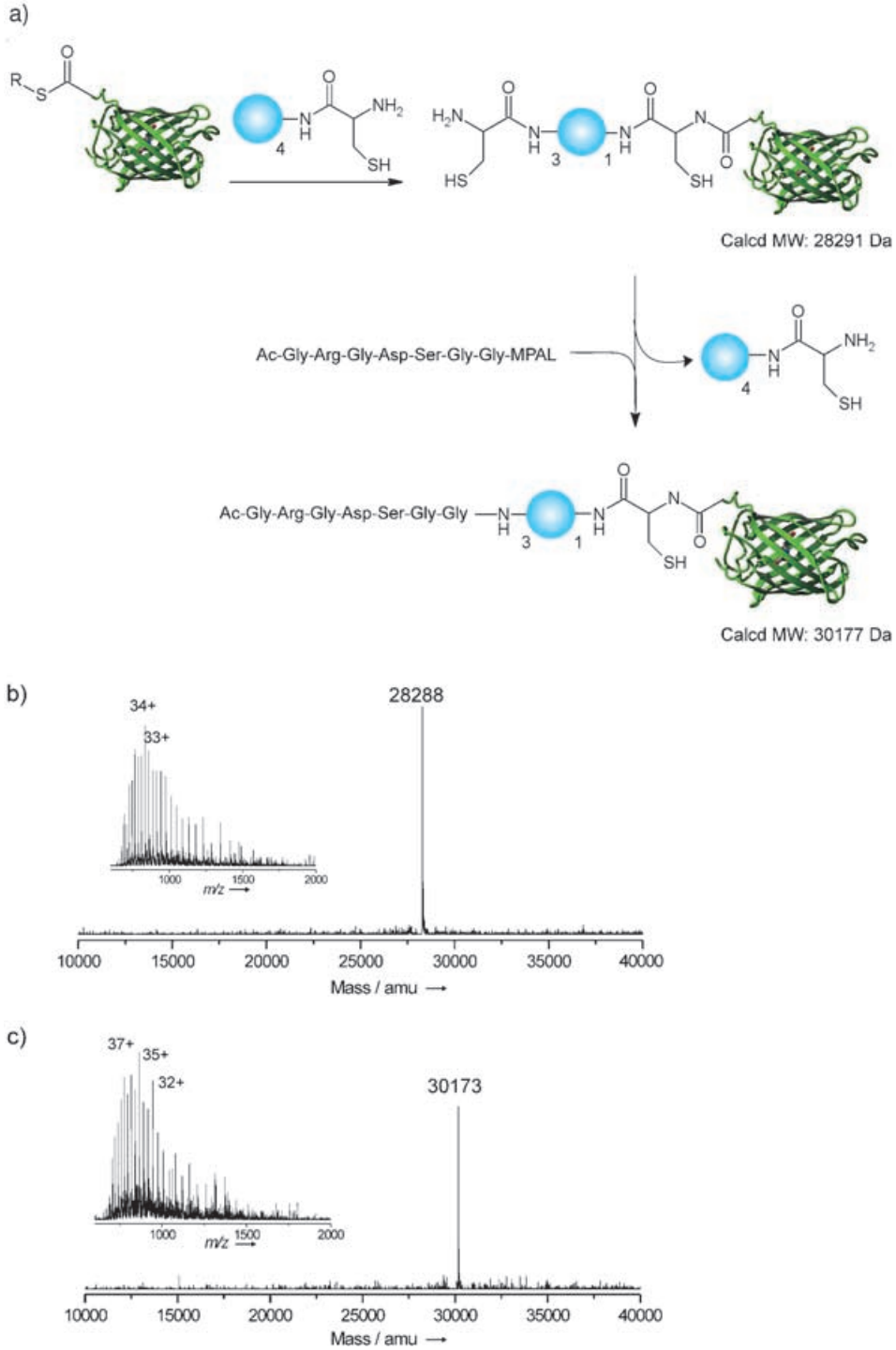


Figure 2. a) Synthesis of a dendrimer containing both protein and peptide end groups. GFP thioester (75 μM) was treated with a large excess of dendrimer **1** (2 mM) for 20 h at 20 °C to yield the monofunctionalized **1**-$(GFP)_1$ exclusively. After removal of remaining dendrimer **1**, **1**-$(GFP)_1$ was treated with excess GRGDSGG-MPAL peptide (375 μM) for 4 h at 20 °C to yield **1**-$(GFP)_1(GRGDSGG)_3$ exclusively. b) Experimental (inset) and deconvoluted ESI-MS of **1**-$(GFP)_1$. c) Experimental (inset) and deconvoluted ESI-MS of **1**-$(GFP)_1(GRGDSGG)_3$.

excess of the MPAL thioester of GRGDSGG. ESI-MS showed the presence of a single, high-molecular-weight signal at 30173 Da, which is exactly the mass expected for **1**-$(GFP)_1(GRGDSGG)_3$ assuming formation of two intramolecular disulfide bonds. This synthetic methodology should be generally applicable to prepare dendrimers with (exactly) one copy of any recombinant protein and multiple copies of any peptide or other synthetic ligand with a thioester functionality.

To test whether our synthetic strategy can be used to obtain multivalent protein dendrimers, a 25 μM solution of dendrimer **1** was incubated with four or eight equivalents of

GFP thioester and the ligation reaction was monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3a). Analysis of the reaction after 20 h showed the presence of four distinct bands at 27, 55, about 120, and about 170 kDa that we attribute, respectively, to GFP thioester and **1**-$(GFP)_1$, **1**-$(GFP)_2$, **1**-$(GFP)_3$, and **1**-$(GFP)_4$. The bands of **1**-$(GFP)_3$ and **1**-$(GFP)_4$ appear at a higher apparent molecular weight than expected, which is probably a consequence of the branched structure of these multivalent proteins. A similar effect of branching on the apparent molecular weight has been reported for ubiquitinated proteins, which are a natural form of branched proteins.[26] The limited solubility of GFP under the conditions suitable for native chemical ligation (100–200 μM GFP thioester) probably makes the ligation of GFP slower than the peptide-ligation reactions that are typically performed at 2–10 mM.[27] A second factor that may prevent the full conversion into **1**-$(GFP)_4$ is increased steric crowding that occurs when attaching four 27-kDa proteins around a 0.7-kDa dendritic core.

The ligation product was further purified using size-exclusion chromatography and analyzed using electrospray ionization time-of-flight mass spectrometry under native conditions to provide definitive evidence for the formation of the GFP tetramer (Figure 3b, c). The spectrum of the first species that elutes from the column shows a limited number of signals that can be deconvoluted to a single mass of 110971 Da. This mass corresponds almost exactly to the average mass calculated for **1**-$(GFP)_4$ with two disulfide bonds (110972 Da). The other detected ions originated from GFP, **1**-$(GFP)_1$, **1**-$(GFP)_2$, and **1**-$(GFP)_3$ and clearly reveal that size-exclusion liquid chromatography coupled to mass spectrometry under native conditions is an ideal analytical tool to separate and analyze these protein mixtures. Moreover, the high m/z values and the narrow charge distribution indicate that the proteins may still be properly folded in **1**-$(GFP)_4$.[28,29]

In summary, we have demonstrated that native chemical ligation is an attractive ligation reaction to functionalize dendrimers with both oligopeptides and recombinantly expressed proteins. Furthermore, dendrimers can be derivatized with exactly one copy of a recombinant protein, and the remaining cysteine residues can then be further functionalized with oligopeptides. Our approach allows chemoselective dendrimer conjugation to the C-terminus of a protein, which is much less likely to interfere with protein function than other less-specific protein-ligation strategies. The modular approach presented here provides access to a wide variety of well-defined multivalent peptides and proteins that are attractive both for understanding the fundamental mecha-

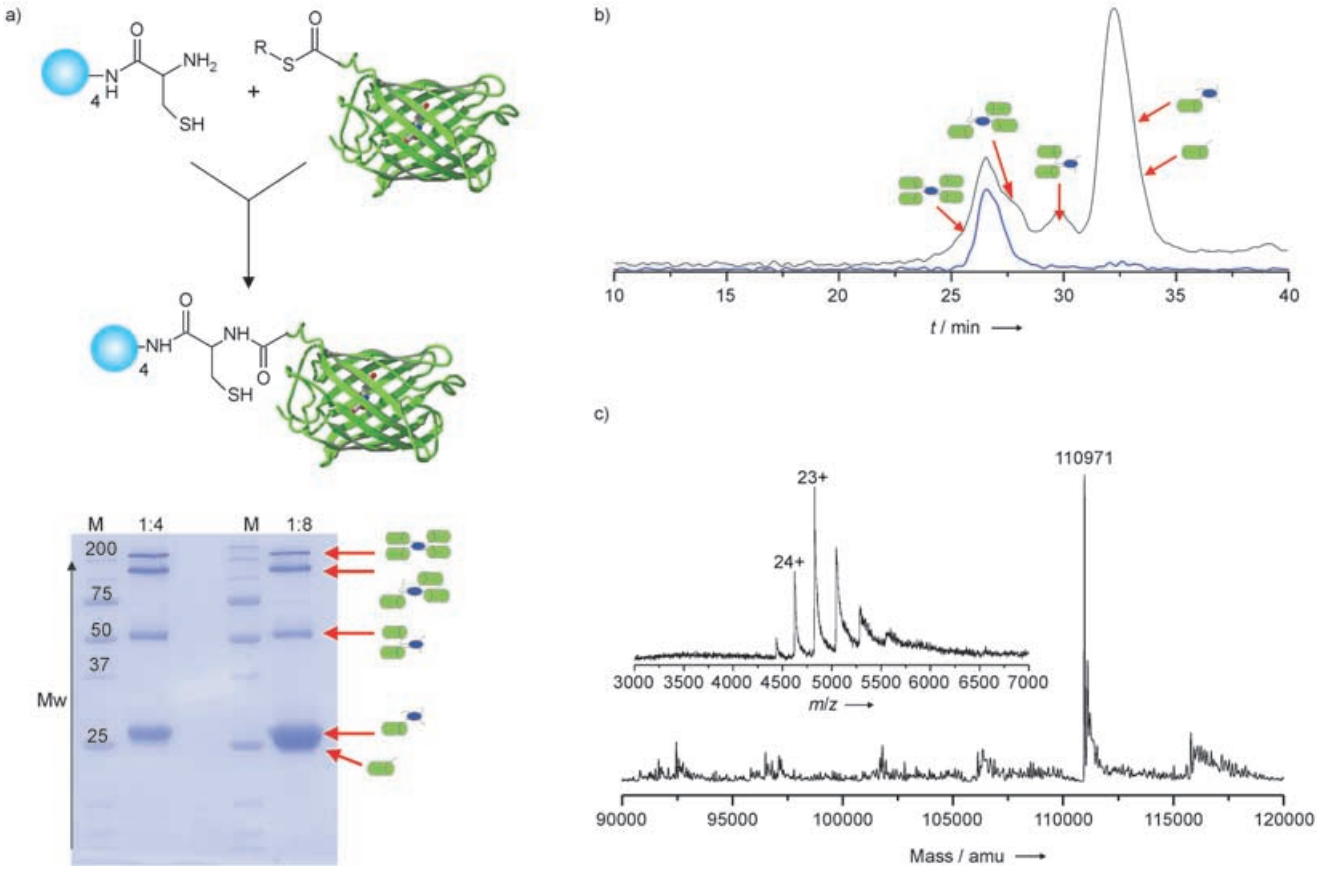


Figure 3. a) Native chemical ligation of dendrimer **1** (25 μM) with four (reaction 1) or eight equivalents (reaction 2) of GFP thioester in 0.1 M sodium phosphate, 2% (v/v) thiophenol, and 2% (v/v) benzyl mercaptan pH 7–7.5 for 20 h at 20°C. b) Size-exclusion chromatogram of the ligation reaction mixture on an analytical Superdex 200 PC 3.2/30 column showing the total ion current (black trace) and the selected ion current for **1**-$(GFP)_4$ (blue trace). c) Experimental (inset) and deconvoluted ESI-MS of **1**-$(GFP)_4$ (fraction that eluted between 25.7 and 27.6 min).

nisms of multivalency in biological interactions and for biomedical applications in targeted drug delivery, molecular imaging, and immunology.

Experimental Section

All peptide ligation experiments were performed in a buffer containing 0.1M tris(hydroxymethyl)aminomethane (tris), 6M guanidine, 2% (v/v) thiophenol, and 2% (v/v) α-toluenethiol (benzyl mercaptan) pH 7.0–7.5. One equivalent of dendrimers **1**, **2**, or **3** was treated with 4, 8, or 16 equivalents of peptide thioester (ca. 10 mg mL^{-1}), respectively, with stirring for 1 h at 37 °C. The ligation products were analyzed by LC-MS. All protein-ligation experiments were performed at 20 °C in a buffer containing 0.1M sodium phosphate, 2% (v/v) thiophenol, and 2% (v/v) benzyl mercaptan pH 7–7.5. Precipitates were removed after the reaction by centrifugation. **1**-$(GFP)_1$ was obtained by ligation of 75 μM GFP thioester with 2 mM dendrimer **1** for 20 h at 20 °C. Remaining dendrimer was removed by repeated concentration and dilution of the reaction mixture using an Amicon Ultra-4 centrifugal concentrator with a 10-kDa cut-off. **1**-$(GFP)_1(GRGDSGG)_3$ was obtained by subsequent ligation with 375 μM GRGDSGG-MPAL for 4 h at 20 °C, followed by analysis using LC-MS.

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