Bioconjugate Chemistry

Article

pubs.acs.org/bc

Assembly of Protein Building Blocks Using a Short Synthetic Peptide

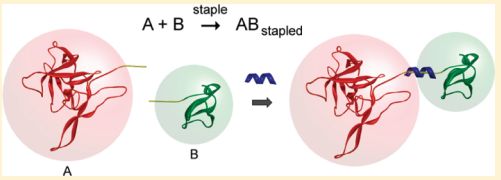
Enrico Ferrari,[†] Mikhail Soloviev,[‡] Dhevahi Niranjan,[†] Jason Arsenault,[†] Chunjing Gu,[†] Yvonne Vallis,[†] John O'Brien,[†] and Bazbek Davletov*[,†]

[†]MRC Laboratory of Molecular Biology, Hills Road, CB2 0QH Cambridge, United Kingdom

[‡]School of Biological Sciences, Royal Holloway University of London, Egham, TW20 0EX Surrey, United Kingdom

Supporting Information

ABSTRACT: Combining proteins or their defined domains offers new enhanced functions. Conventionally, two proteins are either fused into a single polypeptide chain by recombinant means or chemically cross-linked. However, these strategies can have drawbacks such as poor expression (recombinant fusions) or aggregation and inactivation (chemical cross-linking), especially in the case of large multifunctional proteins. We developed a new linking method which allows site-oriented, noncovalent, yet irreversible stapling of modified proteins at neutral pH and ambient temperature. This method is based on two distinct polypeptide linkers which self-assemble in the presence of a specific peptide staple allowing on-demand and irreversible combination of protein domains. Here we show that linkers can either be expressed or be chemically conjugated to proteins of interest, depending on the source of the proteins. We also show that the peptide staple can be shortened to 24 amino acids still permitting an irreversible combination of functional proteins. The versatility of this modular technique is demonstrated by stapling a variety of proteins either in solution or to surfaces.



INTRODUCTION

Combining proteins with different functions into a single entity can offer new opportunities to the field of protein engineering. Protein chimeras, made from protein building blocks that nature already optimized for a specific function by natural selection, could be especially attractive for multienzyme mediated catalysis,[1] nanobiotechnology,[2] and improved medicinal proteins.[3,4] Proteins carrying artificially combined domains are already widely used both in research and in medicine: recombinant proteins are often fused to affinity tags to facilitate their purification, fluorescent fusion proteins are used to track their localization in cells and throughout the body, biotin–protein conjugates are used for immobilization purposes, antibody conjugates are widely used for detection of antigens or for therapeutic use, and medicinal toxins can be targeted to new localities using various targeting domains.[3,4]

Despite the ever-growing interest in novel multimodular proteins, there are factors that limit development and production of such chimeras. A general versatile platform to develop multimodular functional proteins is still missing and successful production of protein chimeras still relies on an empirical approach. Recombinant DNA technology allows fusion of two or more modules in a single polypeptide chain; however, this strategy may not be convenient: for example, when production of the engineered chimera (i) leads to low expression levels or yields insoluble protein, (ii) results in orientation of fused parts not optimal for biological function, (iii) raises safety issues like in the case of modified botulinum neurotoxin,[5] or (iv) results in cytotoxicity.[6] Chemical cross-linking, on the other hand, utilizes physically separate proteins and relies on the reactivity of either natural (cysteine or lysine) or non-natural amino acids.[7] Site-specific chemical conjugation is, in practice, possible only in a limited number of cases, and chemical-cross-linking often leads to partially inactive chimeras or aggregated conjugates. Recently, several techniques utilized self-assembling linkers which can be fused to proteins prior to their assembly. 'Protein trans-splicing' technique utilized a split intein—mixing of two tagged proteins allowed binding, incision, and trans-thioesterification resulting in a covalent bondage of the two proteins.[8] In contrast, the 'expressed protein ligation' method[8] exploited the self-splicing capability of an intein, recombinantly fused to the C-terminal of a precursor protein, to trigger formation of α-thioester followed by trans-thioesterification to another protein carrying N-terminal cysteine. Both intein-based approaches require a number of critical steps and conditions to successfully orient and bind two precursor proteins and the conditions often cannot be practically fulfilled. Heterodimeric leucine zippers fused to proteins were proposed for protein linkage[9,10] but the affinity of natural leucine zippers is not strong enough to compete with recombinant fusion or chemical cross-linking. Another recently introduced platform, so-called dock-and-lock platform, relies on association of a sequence from Protein Kinase A (PKA) with A-Kinase Anchoring Protein (AKAP).[11] Recombinant fusion of two separate proteins of interest to the PKA and AKAP-derived peptides allows site-oriented protein assembly. However, the flexibility of this system is limited due to the obligatory

Received: September 20, 2011
Revised: January 25, 2012
Published: February 2, 2012

   dx.doi.org/10.1021/bc2005208 | *Bioconjugate Chem.* 2012, 23, 479–484

dimerizing nature of the PKA sequence and the necessity of introducing cysteines to stabilize the final assembled product.[11]

We recently described a new approach to assemble proteins in solution using a 52 aa synthetic peptide.[5] This approach relies on two polypeptide linkers which can be fused to proteins of interest and then 'stapled' together (Figure 1) resulting in an oriented, noncovalent, and yet irreversible complex. The linkers and the staple are derived from the uniquely stable SNARE coiled-coil complex.[12] Since each module can be developed and processed separately, we performed an optimization of this new, promising technique. Here we report that the staple can be shortened to 24 aa still allowing firm linking of proteins. In addition, we extend the technique to proteins which cannot be routinely obtained by bacterial expression.

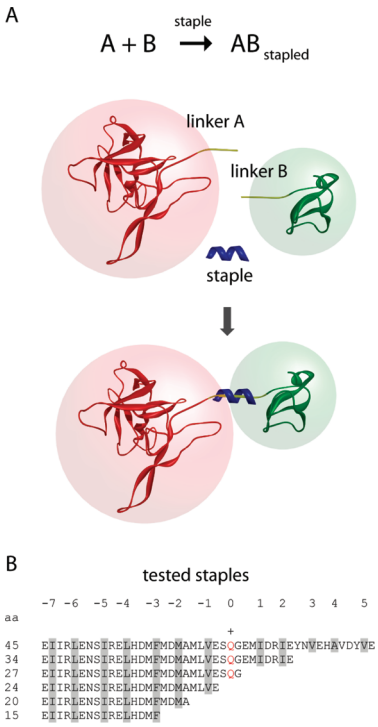


Figure 1. Schematic of protein stapling and design of staples. (A) Two protein modules are fused to separate linkers (yellow). In the presence of a peptide staple (blue), they assemble into an irreversible complex. (B) Tested staples range from 15 to 45 aa, encompassing 5 to 13 layers, characteristic of the coiled-coil SNARE complex structure.[12] Both the hydrophobic layers (boxed in gray) and so-called 'ionic' layer (glutamine in red) take part in the SNARE assembly.

EXPERIMENTAL PROCEDURES

Linkers/Staple Design and Synthesis. All recombinant proteins were expressed in BL21 strain of *E. coli* as glutathione-S-transferase (GST) C-terminal fusions cleavable by thrombin. The plasmid for expression was the pGEX-KG vector and the design, expression, and purification of all of them, except for the linker A-mCherry construct (see below), has been already reported.[13,14] Briefly, the linker A corresponds to the rat SNAP25 full-length sequence (GenBank: AAH87699.1), with all four cysteine residues mutated to alanine; the linker B corresponds to the rat synaptobrevin2 (NCBI Reference Sequence: NP_036795.1), amino acids 25 to 84. GST fusion constructs were purified by glutathione affinity chromatography and either cleaved using thrombin or eluted with excess of glutathione. The linker A-mCherry plasmid has been made by introducing the mCherry sequence from pmCherry-C1 vector (Clontech) into the *Eco*RI restriction site of pGEX-KG, following SNAP25 sequence (22−206 aa; four cysteines mutated to alanines). The botulinum neurotoxin serotype A (BoNT/A) light chain-translocation domain (LcTd) fusion to linker A and the linker B fusion to BoNT/A receptor binding domain (Rbd) have been previously described in detail.[13] The horseradish peroxidase (HRP)-linker B construct was made by cross-linking maleimide activated HRP (ThermoScientific) to a modified linker B peptide. The N-terminal acetylated and C-terminal amidated modified linker B peptide, with the sequence CGSGS RLQQT QAQVD EVVDI MRVNV DKVLE RDQKL SELDD RADAL QAGAS, was synthesized by Peptide Synthetics, UK, and stored in DMSO. See Supporting Information Table 1 for the list of all the recombinant proteins and the cross-linked conjugate used in this study.

Stapling peptides are based on rat syntaxin 1 (GenBank: EDM13396.1) and were synthesized by Peptide Synthetics, UK, and stored in DMSO. The native lysine was substituted with arginine to remove primary amines; individual sequences are shown in Figure 1B. All peptides have been modified with an acetyl group and FITC at the N-terminus and two additional lysine residues at the C-terminus were separated from the syntaxin motif by a flexible aminohexanoic acid linker. This design allows site-oriented attachment of the staples to bromocyanide (BrCN)-activated Sepharose 4B resin (Amersham Biosciences) via reactive lysines.[15] Both linkers and staples migrate as single bands on SDS-PAGE gels (NuPAGE 10% Bis-Tris Gel, Invitrogen) (Supporting Information Figure 1A). Among linkers A and B, only the linker A migrates sharply on native gels (12% Tris-HCL, Bio-Rad) (Supporting Information Figure 1B).

Proteins Stapling and Pull-Down Assays. The stapling reactions (Figures 2C, 4, and 5A) were performed in 20 mM Hepes, 100 mM NaCl, 0.8% *n*-octylglucoside (OG), pH 7.4 (buffer A). Linkers and protein modules fused to the linkers, were at 5 μM, and were incubated 1 h at 24 °C with a 1.5-fold excess of relevant staple. Electrophoresis in both the native gel in Figure 2C and the SDS-PAGE gel in Figure 5A was performed at 4 °C to avoid overheating of the protein assembly during the gel run. 300 μL samples of the 5 μM stapled proteins used in Figure 4 have been analyzed by size-exclusion chromatography to attest purity and integrity of the complexes using an AKTA purifier 10 system (GE Healthcare). The readouts at 280 and 490 nm have been acquired and 0.5 mL fractions have been collected and analyzed by Coomassie stained SDS-PAGE gels (Supporting Information Figure 2).

Pull-downs have been performed using BrCN-activated Sepharose beads after cross-linking of the staples as previously reported[15] (Figure 2A) or glutathione Sepharose beads after 1 h preincubation with 5 μM GST-linker fusion proteins (Figure 3). Incubations were performed in buffer A for 1 h at 24 °C with shaking, followed by extensive washes in buffer A to remove the unbound reagents. Fluorescence and absorbance readings in Figure 3 were performed using Saphire 2 plate reader (Tecan, Switzerland). Densitometry analysis of the Coomassie stained gels (Figure 2B) has been done using the software *Quantity One* v 4.5.1 (Bio-Rad). Stapled conjugates used in this work are listed in Supporting Information Table 1.

Biacore Assay. Analysis of binding and stapling was conducted using a Biacore 2000 system (Figures 2D, 5B, and SI Figure 4). CM5 chip (Biacore) was used to covalently

dx.doi.org/10.1021/bc2005208 | *Bioconjugate Chem.* 2012, 23, 479−484

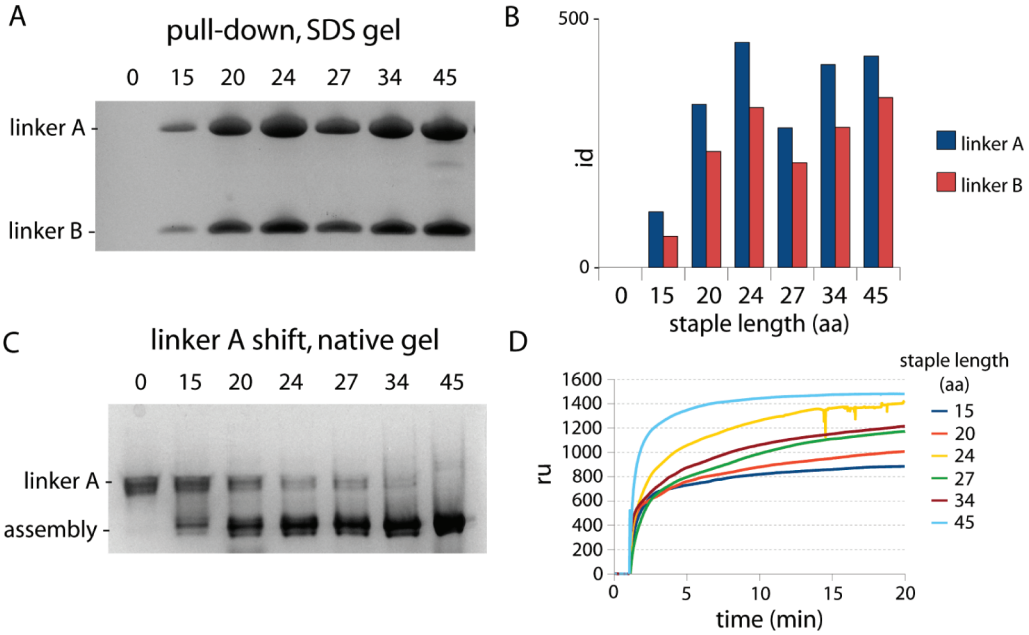


Figure 2. 24 aa and 45 aa staples exhibit the best linker binding properties. (A) Coomassie-stained SDS-PAGE gel showing the amounts of linkers A and B bound to staples immobilized on beads. The length of peptide staples is indicated above the gel. (B) Bar chart showing integrated density (id) values of Coomassie-stained protein bands in the SDS-PAGE gel from panel A. Note that the higher values for linker A compared to linker B are because larger proteins bind more Coomassie stain. (C) Coomassie-stained native gel showing a shift of linker A migration after 30 min incubation in the presence of linker B and indicated staples. (D) Biacore traces showing the stapling reaction on the chip surface carrying immobilized linker A. The traces were obtained following the injection of the linker B mixed at equimolar ratios with different staples. Values are in resonance units (ru) after subtraction of signals due to the loading buffer.

immobilize GST-linker A. For each staple, binding of linker B combined in equimolar ratio to the staple was recorded. All proteins/peptides were at 5 μM in buffer A and were applied to the chip at a rate of 5 μL/min for 20 min. For stability measurements (Figure 5B), the assembled complex was exposed to 1% SDS for 1 min.

Hippocampal Neurons Culture and Western Immunoblotting. Primary cultures of hippocampal neurons were prepared as previously described[5] and used after 7−10 days in culture. For imaging of the uptake of the reassembled toxins (Figure 4B), neurons were exposed to 10 nM of the indicated compound for 2 h, DAPI-stained, fixed with 4% PFA and the fluorescence was observed on a Radiance Confocal system (Zeiss/Bio-Rad) linked to a Nikon Eclipse fluorescence microscope equipped with an oil immersion objective (100×, 1.4 N.A.). The fluorescent staples alone have been used as a negative control (SI Figure 3). For the functionality assay (Figure 4C), neurons were incubated for 20 h with 10 nM of the indicated compound, lysed in 60 mM Tris (pH 6.8), 2 mM $MgCl_2$, 2% SDS, and benzonase (250 U/mL, Novagen). SNAP25 cleavage was then analyzed by immunobloting using an anti-SNAP25 antibody (clone SMI81, Covance) which recognizes both the cleaved and uncleaved proteins.

RESULTS

Optimization of the Staple. Syntaxin-derived SNARE motif used previously for stapling[5] is 52 aa in length, presenting a challenge for routine, mass-scale linking of proteins in solution or to surfaces. To determine the minimal length of staple (Figure 1B), we investigated the ability of chemically synthesized peptides, covalently attached to Sepharose beads, to pull-down linkers A and B (Figure 2A). The staples were immobilized on BrCN Sepharose beads via two lysines and then incubated with an excess of both linkers. As expected, binding of both linkers to the immobilized staples occurred in parallel and in equimolar ratio (Figure 2A). The Coomassie-stained SDS-PAGE gel and its densitometry quantitation (Figure 2B) show that efficient binding of linkers occurs only when the length of staples exceeds 20 aa with the 24 aa staple already reaching maximum. Interestingly, extending the staple to 27 aa compromises binding of the two linkers before the interaction recovers at the 45 aa length. Next, we exploited the ability of linker A and stapled assemblies to migrate in native gel electrophoresis as separate distinct bands (Figure 2C). We incubated the staples in the presence of the two linkers in solution for 60 min and then analyzed protein migration by Coomassie-staining of the nondenaturing gel. Figure 2C shows that staples as short as 20 aa can be effective in triggering linkers association. Indeed, linker A shifts into a faster migrating entity, most likely due to the addition of negative charges from linker B and the staples, and/or the tight structure of the resulting SNARE bundle.[12]

To further test the efficiency of the stapling reaction we employed the surface plasmon resonance (SPR) approach. Linker A was immobilized on the surface of the Biacore chip and an equimolar mixture of linker B and staple was injected into the microfluidic chamber. Figure 2D shows the traces acquired for each staple during the 20 min incubation. The end-point values show that larger staples yielded faster assembly; however, the 24 aa peptide clearly showed an increased binding compared to its neighbors. This confirms that the 24 aa peptide is uniquely effective in stapling reaction. Since dissociation of the linker A/linker B/staple complex was not observed with any of the staples used (see SI Figure 4), a dissociation constant K_D cannot be calculated. How different staples yield different

dx.doi.org/10.1021/bc2005208 | Bioconjugate Chem. 2012, 23, 479−484

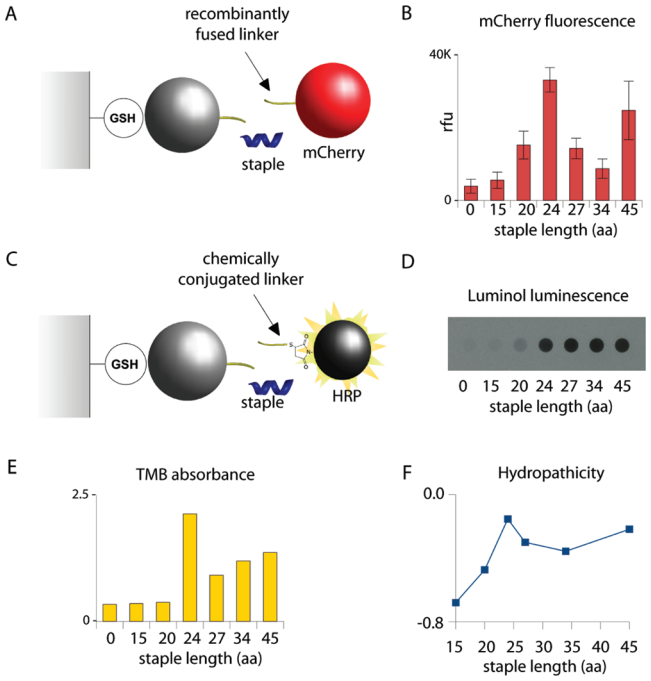


Figure 3. Peptide-dependent stapling of the mCherry fluorescent protein or horseradish peroxidase (HRP) to glutathione-S-transferase (GST). (A) Schematic showing GST-linker (gray) immobilized on glutathione beads which were used to pull-down mCherry (red). (B) Histogram showing relative fluorescence units (rfu) bound to GST-linker beads in presence of different staples. Error bars represent standard deviation (n = 3). (C) Schematic showing GST-linker (gray) immobilized on glutathione beads which were used to pull-down HRP. (D) X-ray film showing relative amount of HRP bound to beads in a 96-well plate in the presence of the different staples, revealed using a luminol reaction. (E) Histogram showing relative amount of HRP bound to GST-linker beads in the presence of the indicated staples as revealed by a TMB-based colorimetric reaction. (F) Plot showing grand average of hydropathicity (GRAVY) index of the stapling peptides as a function of their sequence and length.

resonance units in irreversible binding reactions after 20 min is currently unclear.

Stapling of Functional Proteins. Next we investigated the efficiency of stapling proteins with diverse functions. We fused one of the linkers to GST, functionality of which can be tested by binding to glutathione−Sepharose beads, whereas the other linker was fused to mCherry fluorescent protein. mCherry provides easy readout in pull-down experiments. Figure 3B shows that the amount of stapled mCherry has a similar trend to that in Figure 2, confirming that the 24 and 45 aa staples exhibit the strongest binding.

It was also important to assess the suitability of our stapling system to protein which cannot be routinely expressed, e.g., HRP. We designed a modified version of linker B specifically for the chemical cross-linking by adding a cysteine residue which can be reacted via maleimide reaction with proteins of interest. The modified linker B was conjugated to maleimide-activated HRP enzyme which can be easily detected via luminescent or colorimetric reactions. We then tested the ability of staples to attach the HRP enzyme to GST-linker A-containing beads in a 96-well plate. Following extensive washing of individual wells, the amount of HRP bound to Sepharose beads was estimated by the luminol reaction which emits light. Exposure of an X-ray film to the 96-well plate

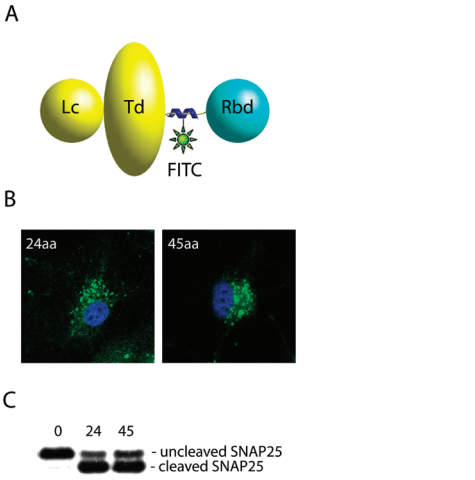


Figure 4. 24 aa peptide is sufficient for stapling functional parts of botulinum neurotoxin. (A) Schematic showing design of the botulinum construct: the Rbd part (light blue) is stapled to the enzymatic part (yellow), that consists of the light chain (Lc) and translocation domain (Td). LcTd-linker A is produced as a single protein. (B) Confocal fluorescence images showing the uptake by hippocampal neurons of the reassembled toxins stapled with the indicated length peptides labeled with FITC (green). Blue represents the DAPI-stained nucleus. (C) Western immunoblot showing the intact (upper bands) and cleaved (lower bands) endogenous SNAP25 upon incubation of hippocampal neurons with reassembled botulinum toxin stapled with the indicated length peptides.

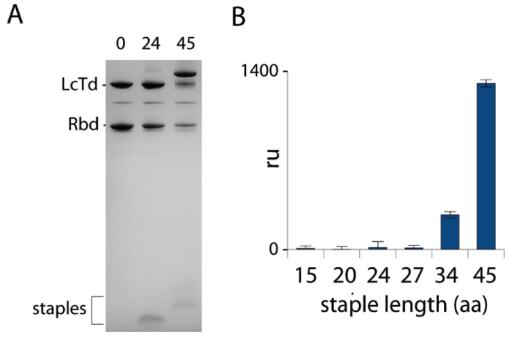


Figure 5. Resistance of stapled molecules to SDS. (A) A Coomassie stained SDS-PAGE gel showing the migration of BoNT/A LcTd-Rbd complexes stapled with the indicated length peptide. The individual components migrate as a 120 kDa (LcTd-linker A), a 55 kDa (linker B- Rbd) and less than 10 kDa bands (staples). Only when the staple is 45 aa long the assembly of the two modules is clearly visible in the gel as an increase in the molecular weight. (B) Stability of stapling by the indicated peptides under denaturing conditions. Bars indicate the amount in resonance units (ru) of linker B remaining on linker A-functionalized Biacore chip after incubation with 1% SDS. Error bars represent standard deviation (n = 4).

revealed that the HRP can be efficiently stapled to the GST-linker A beads and that the minimal staple must be at least 24 aa long (Figure 3D). A quantitative measurement of the bound HRP was obtained by the HRP-catalyzed colorimetric reaction in presence of the 3,3′,5,5′-tetramethylbenzidine (TMB), confirming the optimal length for the staple being 24 aa (Figure 3E).

To better understand the reason for the unique efficiency of the 24 aa staple, we analyzed the hydropathicity as a function of peptide length (analysis performed using ProtParam tool available at http://expasy.org/tools/protparam.html). The

dx.doi.org/10.1021/bc2005208 | Bioconjugate Chem. 2012, 23, 479−484

plot in Figure 3F revealed that the 24 aa staple is the most hydrophobic of all the stapling peptides, which may explain its enhanced ability to drive SNARE assembly which depends on N-to-C zippering of hydrophobic layers.[12] Extending the syntaxin peptides beyond 24 aa incorporates the ionic layer (see Figure 1B), bringing to the consequent drop in hydropathicity, likely explaining why 27 aa and 34 aa have a reduced ability to staple proteins. The hydropathicity increases again at 45 aa, with the incorporation of more hydrophobic layers, and the stapling efficacy increases accordingly.

Functional Testing of the 24 aa Staple. Next we tested whether the shortened peptides can allow a stable assembly as judged by a biologically relevant assay. We chose to staple two functional parts of botulinum neurotoxin type A (BoNT/A), widely known as BOTOX,[16,17] which needs to penetrate neuronal interior to proteolyze its molecular target, the SNAP25 protein. The two BoNT/A modules used in this work (see the Experimental Procedures section and Supporting Information Table 1) are the linker B - Rbd, which accomplish the function of binding to the specific neuronal receptors, and LcTd - linker A, whose functions are (i) translocation of the bound and endocytosed neurotoxin from the endocytic vesicles to the cytoplasm (Td part) and (ii) proteolysis of the target (Lc part) that eventually impairs the fusion of the synaptic vesicles and therefore the neurotransmitter release. Binding to neurons, entry into the cytosol, and cleavage of the molecular target occur only when the two modules are physically connected, and thus the BoNT/A offers a useful paradigm to study the functionality of the stapling method. We stapled the two neurotoxin parts using 24 aa and 45 aa peptides, both tagged with FITC, and applied the stapled fluorescent products to cultured mouse hippocampal neurons. Functionality of the stapled toxins was assessed by penetration of the fluorescently labeled toxin into neurons and proteolytic cleavage of intraneuronal SNAP25. Figure 4B shows that both stapled botulinum products efficiently entered the neuronal interior combining binding and translocation functions of the neurotoxin. Both the 24 and 45 aa stapled products triggered extensive cleavage of intraneuronal SNAP25 at 10 nM concentration, with no proteolytic cleavage detected in the absence of staples (Figure 4C). Clearly, the translocation module, LcTd, must be stapled to the binding part, Rbd, to be taken up by the endocytotic machinery. This proves that a staple as short as 24 aa is able to efficiently link the functional modules together allowing the neurotoxin to accomplish its sophisticated functions: binding to neuronal membranes, entry of the botulinum enzyme into cytosol, and a highly specific proteolysis of its intraneuronal target.

One feature of the stapling assembly is its resistance to SDS which could be useful in verifying the success of the stapling reaction simply by SDS-PAGE. We analyzed whether the two stapled botulinum products, which migrate identically in a size-exclusion chromatography (SI Figure 2), exhibit such SDS resistance. Figure 5A shows that, only in the presence of the 45 aa staple, the botulinum product becomes SDS-resistant, whereas SDS breaks the 24 aa stapled product into its constituent parts. To better understand the boundary where SDS resistance is acquired, we analyzed the resistance of linker/staple associations to 1% SDS solution using the surface plasmon resonance method. Figure 5B shows that only 45 aa staple was able to confer the SDS resistance to the stapling system, confirming possible utility of the longer linker in aiding visualization of stapled products in SDS-PAGE gels (Figure 5A).

DISCUSSION

Here we described optimization of the new protein stapling technique which allows linking of two modules using a synthetic peptide. We demonstrated that the staple as short as 24 amino acids in length can efficiently link proteins either in solution or to a surface. The shortening of the staple allows an easy and straightforward synthesis and also opens room for incorporation of other functionalities (for example, imaging reagents). We also demonstrated that the linkers can be chemically cross-linked to protein building blocks. The possibility to use the linking system for nonrecombinant proteins opens many possibilities for conjugation of widely used proteins such as naturally derived enzymes, lectins, growth factors, and so forth. When recombinantly fused, the linkers caused neither a reduction in the expression levels of proteins tested nor solubility issues. Conceivably, the unstructured nature of the SNARE-based linkers—prior to their assembly[12]—makes them easy to express with no deleterious effects on the function of the building blocks. In all cases, the obtained conjugates retained functions of individual modules.

Similarly to the intein-based protein trans-splicing technique and the dock-and-lock platform, protein stapling requires the expression of N-terminal and C-terminal linkers to bring together the two parts. However, there are important differences that can affect the choice of the ideal system for a specific use: SNARE-based protein stapling is robust in a very wide range of conditions while the intein mediated trans-esterification is highly affected by the chemical contest of the reaction or requires additional disulfide bridging in the case of the dock-and-lock platform. A sortase-driven protein conjugation, on the other hand, requires millimolar calcium and elevated pH and temperatures, limiting its utility.[18] SNARE-based protein stapling does not result in the formation of the peptide bond between the two parts, but allows parallel incorporation of chemical agents, e.g., fluorophores or other labeling compounds. Finally, while intein-based trans-splicing may allow reconstitution of a desired split-protein with fully preserved structure, the protein stapling evidently permits combinations of structurally unrelated proteins and thus could be suitable for production of multienzyme complexes, antibody−enzyme conjugates, heterofunctional targeting agents for binding two receptors, and so forth. The stapling mechanism between the two stapled domains can potentially give new beneficial features to the assembled product as was previously demonstrated for the stapled BoNT/A (preserved silencing of central neurons without muscle paralytic activity).[13]

In summary, we optimized the protein stapling method making it more attractive for protein engineering: (i) a functional peptide staple that is half the length of the previously reported, (ii) the possibility to cross-link a synthetic SNARE linker to a protein obtained by sources other than recombinant (e.g., HRP), and (iii) the possibility to immobilize a SNARE linker fusion protein to a surface through a staple (e.g., on a Biacore chip). Finally, we highlighted the possibility to follow supramolecular assembly by SDS-PAGE when using a 45 amino acid staple.

It is likely that future design and synthesis of supramolecular complexes and molecular machines made from heterologous protein domains[1,2,19,20] will be based on a modular approach. Clearly, production of individual building blocks carrying

SNARE linkers still relies on the recombinant fusion or on chemical cross-linking. However, once the expression of a SNARE linker-fusion protein is established, it will be straightforward to staple it to many protein or synthetic entities, fulfilling the step-by-step nature of the modular approach. On the contrary, the recombinant fusion of many different chimeras could be problematic in terms of time, different levels of expression, or even impossibility to synthesize the protein. Another specific advantage of the stapling method is the possibility to easily create protein−synthetic molecule conjugates. Pertinently, the fast SNARE-based assembly happens when the linkers and staple are at equimolar concentrations, while chemical cross-linking often requires a large excess of one of the reagents, leading to losses of valuable material and potential denaturation or aggregation. Moreover, the quick and robust self-assembling and the potential to visualize the assembled products in less than one hour together present a uniquely versatile platform for combinatorial assembly of multifunctional proteins.

■ ASSOCIATED CONTENT

Supporting Information

Table listing proteins and stapled assemblies used in this work; Coomassie stained SDS-PAGE and native gels showing purified linker A, linker B and staple; size-exclusion chromatography of the stapled BoNT/A LcTd-Rbd complexes used for the functional testing; confocal fluorescence images showing lack of uptake by hippocampal neurons of FITC-labeled staples in the absence of botulinum parts; Biacore traces showing the association of linker B on the chip surface carrying immobilized linker A in presence of different staples and indicating no complex dissociation for all individual staples. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: bazbek@mrc-lmb.cam.ac.uk. Telephone: +44(0) 1223402009. Fax: +44(0)1223402310.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BoNT/A, botulinum neurotoxin serotype A; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; HRP, horseradish peroxidase; OG, *n*-octyl-*β*-D-glucoside; SDS, sodium dodecyl sulfate; SNAP25, synaptosomal-associated protein 25; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TMB, 3,3′,5,5′ tetramethylbenzidine

■ REFERENCES

(1) Niemeyer, C. M., Koehler, J., and Wuerdemann, C. (2002) DNA-Directed Assembly of Bienzymic Complexes from In Vivo Biotinylated NAD(P)H:FMN Oxidoreductase and Luciferase. *ChemBioChem 3*, 242−245.

(2) Heddle, J. G. (2008) Protein cages, rings and tubes: useful components of future nanodevices? *Nanotechnol. Sci. Appl. 1*, 67−78.

(3) Mantyh, P. W., Rogers, S. D., Honore, P., Allen, B. J., Ghilardi, J. R., Li, J., Daughters, R. S., Lappi, D. A., Wiley, R. G., and Simone, D. A. (1997) Inhibition of Hyperalgesia by Ablation of Lamina I Spinal Neurons Expressing the Substance P Receptor. *Science 278*, 275−279.

(4) Chaddock, J. A., Purkiss, J. R., Duggan, M. J., Quinn, C. P., Shone, C. C., and Foster, K. A. (2000) A conjugate composed of nerve growth factor coupled to a non-toxic derivative of *Clostridium botulinum* neurotoxin type A can inhibit neurotransmitter release in vitro. *Growth Factors 18*, 147−155.

(5) Darios, F., Niranjan, D., Ferrari, E., Zhang, F., Soloviev, M., Rummel, A., Bigalke, H., Suckling, J., Ushkaryov, Y., Naumenko, N., Shakirzyanova, A., Giniatullin, R., Maywood, E., Hastings, M., Binz, T., and Davletov, B. (2010) SNARE tagging allows stepwise assembly of a multimodular medicinal toxin. *Proc. Natl. Acad. Sci. U.S.A. 42*, 18197−18201.

(6) Wu, W., Wood, D. W., Belfort, G., Derbyshire, V., and Belfort, M. (2002) Intein-mediated purification of cytotoxic endonuclease I-TevI by insertional inactivation and pH-controllable splicing. *Nucl. Acid. Res. 30*, 4864−4871.

(7) Graaf, A. J., Kooijman, M., Hennink, W. E., and Mastrobattista, E. (2009) Nonnatural Amino Acids for Site-Specific Protein Conjugation. *Bioconjugate Chem. 20*, 1281−1295.

(8) Muralidharan, V., and Muir, T. W. (2006) Protein ligation: an enabling technology for the biophysical analysis of proteins. *Nat. Meth. 3*, 429−438.

(9) Kostelny, S. A., Cole, M. S., and Tso, Y. J. (1992) Formation of a bispecific antibody by the use of leucine zippers. *J. Immunol. 148*, 1547−1553.

(10) Chang, H. C., Bao, Z., Yao, Y., Tse, A. G., Goyarts, E. C., Madsen, M., Kawasaki, E., Brauer, P. P., Sacchettini, J. C., and Nathenson, S. G. (1994) A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments. *Proc. Natl. Acad. Sci. U.S.A. 91*, 11408−11412.

(11) Chang, C.-H., Rossi, E. A., and Goldenberg, D. M. (2007) The Dock and Lock Method: A Novel Platform Technology for Building Multivalent, Multifunctional Structures of Defined Composition with Retained Bioactivity. *Clin. Cancer Res. 13*, 5586s−5591s.

(12) Sutton, R. B., Fasshauer, D., Jahn, R., and Brunger, A. T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature 395*, 347−353.

(13) Ferrari, E., Maywood, E. S., Restani, L., Caleo, M., Pirazzini, M., Rossetto, O., Hastings, M. H., Niranjan, D., Schiavo, G., and Davletov, B. (2011) Re-Assembled Botulinum Neurotoxin Inhibits CNS Functions without Systemic Toxicity. *Toxins 3*, 345−355.

(14) Darios, F., Wasser, C., Shakirzyanova, A., Giniatullin, A., Goodman, K., Munoz-Bravo, J. L., Raingo, J., Jorgacevski, J., Kreft, M., Zorec, R., Rosa, J. M., Gandia, L., Gutierrez, M., Binz, T., Giniatullin, R., Kavalali, E. T., and Davletov, B. (2009) Sphingosine facilitates SNARE complex assembly and activates synaptic vesicle exocytosis. *Neuron 62*, 683−694.

(15) Ferrari, E., Darios, F., Zhang, F., Niranjan, D., Bailes, J., Soloviev, M., and Davletov, B. (2010) Binary polypeptide system for permanent and oriented protein immobilization. *J. Nanobiotechnol. 8*, 9.

(16) Montal, M. (2010) Botulinum Neurotoxin: A Marvel of Protein Design. *Annu. Rev. Biochem. 79*, 591−617.

(17) Davletov, B., Bajohrs, M., and Binz, T. (2005) Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. *Trends Neurosci. 28*, 446−452.

(18) Levary, D. A., Parthasarathy, R., Boder, E. T., and Ackerman, M. E. (2011) Protein-Protein Fusion Catalyzed by Sortase A. *PLOS One 6*, e18342.

(19) Stupp, S. I., LeBonheur, V., Walker, K., Li, L. S., Huggins, K. E., Keser, M., and Amstutz, A. (1997) Supramolecular Materials: Self-Organized Nanostructures. *Science 276*, 384−389.

(20) Yeates, T. O., and Padilla, J. E. (2002) Designing supramolecular protein assemblies. *Curr. Opin. Struct. Biol. 12*, 464−470.