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Development of a Chemoenzymatic-like and Photoswitchable Method for the Ordered Attachment of Proteins to Surfaces

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Development of a Chemoenzymatic-like and Photoswitchable Method for the Ordered Attachment of Proteins to Surfaces

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Abstract

Protein arrays are the best tool for the rapid analysis of a whole proteome thus helping to identify all the protein/protein interactions in a living cell and they can also be used as powerful biosensors. The objective of this proposal is to develop a new entropically activated ligation method based in the naturally occurring protein trans-splicing process . This method will be used for the generation of spatially addressable arrays of multiple protein components by standard photolithographic techniques. Key to our approach is the use of the protein trans-splicing process. This naturally occurring process will allow us to create a truly generic and highly efficient method for the covalent attachment of proteins through its C-terminus to any solid support.

Introduction/Background

Many experimental techniques in biology and biophysics, and applications in diagnosis and drug discovery, require proteins immobilized on solid substrates [1-3]. In fact, the concept of arrays of proteins attached to a solid support has attracted increasing attention over the last three years due to the sequencing of several genomes, including the human genome. When a genome has been deciphered, the daunting task of determining the function of each protein encoded in the genome still remains. Protein arrays can be used easily for such analysis in a parallel fashion [2, 4]. Another powerful application employs ordered nanometric arrays of proteins as nucleation templates for protein crystallization or for structural studies. Recent advances in nanoprinting techniques have allowed the creation of sub-micrometer arrays of proteins [5, 6]. All these applications demonstrate the use of protein arrays and also highlight the need for methods able to attach proteins in a well defined and ordered way onto a solid supports.

Various methods are available for attaching proteins to solid surfaces. Most rely on non-specific adsorption, [6, 7] or on the reaction of chemical groups within proteins (mainly, amino and carboxylic acid groups) with surfaces containing complementary reactive groups [8, 9]. In both cases the protein is attached to the surface in random orientations. The use of recombinant affinity tags addresses the orientation issue. However, in most cases the interactions of the tags are reversible and therefore not stable over the course of subsequent assays or require large mediator proteins [10-13].

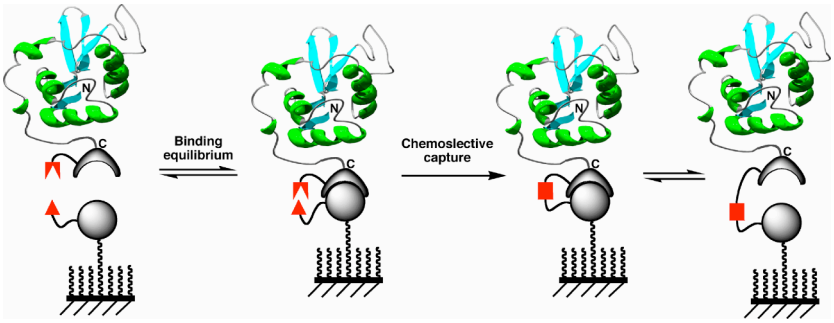
Methods for the chemoselective attachment of proteins to surfaces has been also developed recently by our group[14, 15]. These methods rely in the introduction of two unique and mutually reactive groups on the protein and the support surface. The reaction between these two groups usually gives rise to the selective attachment of the protein to the surface with total control over the orientation [15]. However, these methods, although highly selective, rely on uncatalyzed pseudo-bimolecular reactions with little or not entropic activation at all. This lack of entropic activation means that the efficiency of these bimolecular-like reactions will depend strongly on the concentration of the

reagents (i.e. the protein to be attached). A way to overcome this intrinsic entropic barrier and make attachment reactions even more efficient and selective, even under high dilution conditions, is through the use of a highly selective molecular recognition event to bring together the two reactive species. This event will increase dramatically the local effective concentration of both reacting species thus accelerating the corresponding attachment reaction even under unfavorable conditions (i.e. low concentration and even in the presence of other proteins).

The objective of this proposal is to develop a new entropically activated ligation method based in the naturally occurring protein trans-splicing process [16, 17]. The use of this process will allow us to create a truly generic and highly efficient method for the covalent attachment of proteins through its C-terminus to any solid support. Furthermore this method will be totally traceless (i.e. on the protein of interest will be attached to the surface) and it could be used for the generation of spatially addressable arrays of multiple protein components by using standard photolithographic techniques.

Background

Most of the available methods for the chemoselective attachment of proteins to surfaces are based on uncatalyzed bimolecular-like reactions with little or not entropic activation at all. This lack of entropic activation means that the efficiency of these bimolecular-like reactions will depend strongly on the concentration of the reagents (i.e. on the concentration of the protein to be attached to the corresponding surface). This is basically due to the loss of entropy associated by the two reacting groups having to be brought together in the right orientation for the reaction to happen. A way to overcome this intrinsic entropic barrier and make ligation reactions more efficient even under high



***Scheme 1**. Principle for the entropically activated chemoselective reaction. Application to the selective attachment of proteins to surcaces.*

dilution conditions is using specific non-covalent interactions for bringing both reactive groups in close proximity (Scheme 1). Under these conditions the rate of bimolecular protein chemical ligation reactions should be very efficient even under high dilution conditions. This effect was first reported by Sheppard and co-workers [18] who noticed that pancreatic trypsin inhibitor fragments obtained by CNBr cleavage were able to spontaneously religate forming the original native peptide bond between them. The same effect was also observed with cytochrome C [19]. In both cases, the two protein fragments produced after the CNBr cleavage, were able to cooperatively refold thus bringing the homoserine lactone at the C-terminus of one of the fragments in close proximity of the α-amino group of the other fragment. As before, the high local concentration effect of

these groups and the mild activation of the homoserine lactone functionality resulted in spontaneous amide bond formation. More recently, Mrksich and co-workers [20] have used this same principle for the selective attachment of protein onto surfaces with total control over the orientation. In their approach, they used the protein calmodulin fused with the enzyme cutinase as a capture protein. Cutinase is a 22 kDa serine esterase that is able to form a site-specific covalent adducts with chorophosphonate ligands [21]. In this case the ligand (a chlorophosphonate group) mimics the tetrahedral transition state of an ester hydrolysis. When it binds specifically to the active site of the enzyme, the hydroxyl group of the catalytic serine residue reacts covalently with the chlorophosphonate to yield a stable covalent adduct that is resistant to hydrolysis The final result of the process is the selective attachment of the protein to the surface through the capture protein that acts like a linker. The attachment is so selective that even the whole crude *E. coli* periplasmic lysate containing the cutinase can be used for the specific attachment of the enzyme thus demonstrating that the protein does not need to be purified before the immobilization step.

Technical Plan

Selective attachment of proteins by protein trans-splicing. The main limitation of the previous method, however, is that the linker between the protein of interest and the surface is always another protein. The presence of such a big linker could potentially give rise to some problems, specially in those applications where the attached protein will be involved in studying protein/protein interactions with complex protein mixtures [2, 4], mainly due to potential cross-reactivity issues.

In order to solve this problem, our group is developing a new method for the chemoenzymatic-like attachment of proteins to surfaces based on the protein trans-

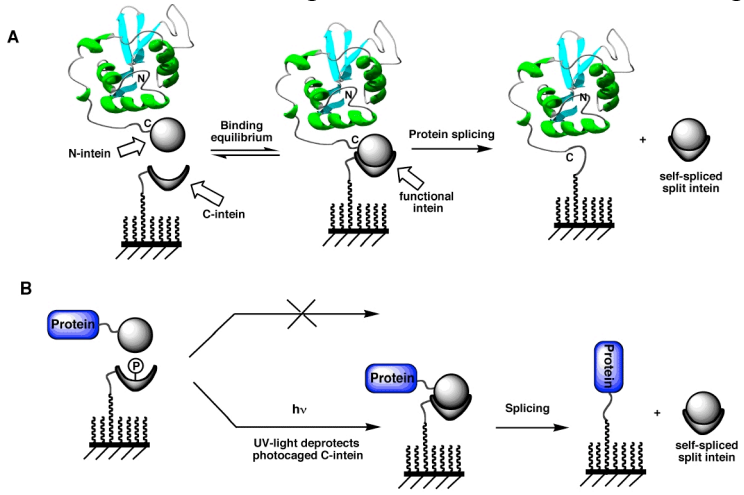


Figure 1. *Attaching proteins to solid surfaces by using a protein trans-splicing technique.* ***A.*** *Principle of the protein trans-splicing approach for the immobilization of proteins. The C-intein fragment is attached to the surface and the N-intein fragment is fused to the C-terminus of the protein to be attached. When this fusion protein is exposed to C-intein-containing surface, the two intein fragments associate yielding a fully operational intein domain that then splices out attaching at the same time the protein to the surface.* ***B.*** *The attachment of proteins using this technique can be easily controlled by photo-caging the C-intein fragment. Under this conditions the intein complex can not be formed and consequently the protein is not attached. Only when the photo-labile group is removed by action of UV light the C-intein can yield the active intein domain which then attaches the protein to the surface*

splicing process (Fig. 1). This process is similar to the protein splicing with the only difference that in this case the intein self-processing domain is split in two fragments (called N-intein and C-intein, respectively) [16, 22-25].

These two intein fragments alone are inactive, however, when they are put together under the appropriate conditions they bind specifically each other yielding a totally functional splicing domain, which it splices itself at the same that ligates both extein sequences. In our approach, one of the fragments (C-intein) will be covalently attached to the surface through a small peptide-linker meanwhile the other fragment (N-intein) will be fused to the C-terminus of the protein to be attached to the surface. When both intein fragments interact, they will form the active intein which will then ligate the protein of interest to the surface at the same that the split intein is spliced out thus remaining in solution (see Fig. 1).

Key to our approach is the use of the split DnaE intein from Synechocystis sp. PCC6803. This naturally occurring split intein was first discovered by Liu and co-workers [16] and also predicted through sequence analysis in a independent study by Gorbalenya [24]. In contrast with other inteins engineered to act as trans-splicing elements [22, 23], which only work after a refolding step (Note that this require first unfold the precursors proteins), the C- and N-intein fragments of the DnaE intein are able to self-assemble spontaneously not requiring any refolding step [17, 26]. The DnaE split intein consists of a N-intein fragment comprising 123 residues and a C-intein fragment of only 37 residues in length. Our approach consists expressing a recombinant fusion proteins where the DnaE C-intein fragment will be fused to the C-terminus of the protein to be attached to the surface (Fig. 2). On the other hand, the C-intein fragment will be synthesized as synthetic peptide by using a Solid-Phase Peptide Synthesis (SPPS) approach. This synthetic approach will allow us to introduce an alkylthiol moiety to the C-terminus of the C-intein peptide which will be used for attachment to a gold or Si-based surface (Fig. 2).

In our approach, one of the fragments (C-intein) will be covalently attached to the surface through a small peptide-linker meanwhile the other fragment (N-intein) will be fused to the C-terminus of the protein to be attached to surface. When both intein fragments interact, they will form the active intein which will then ligate the protein of interest to the surface at the same that

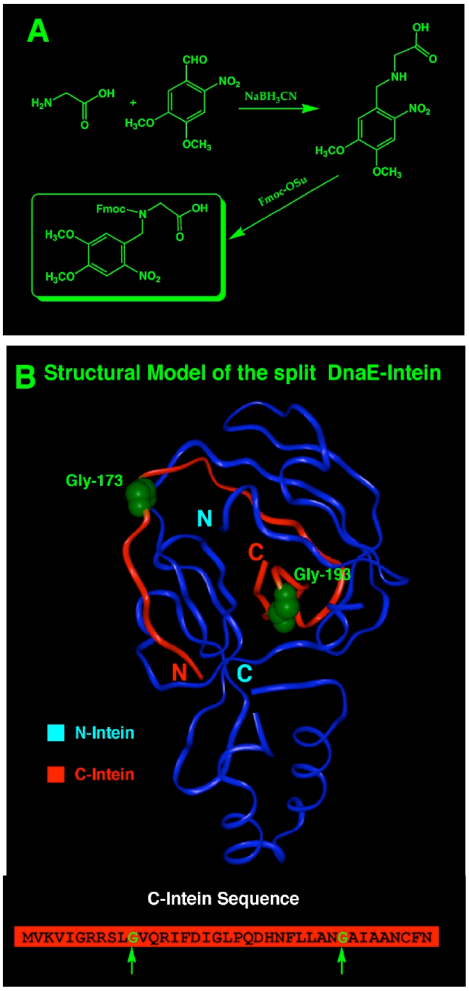


Figure 2. *Preparing a photocaged C-intein polypeptide.* ***A***. *Synthesis of a backbone photocaged Gly residue for the solid-phase peptide synthesis of the photocaged C-intein.* ***B***. *Structural model of the split DnaE-intein showing the Gly residues that will be photocaged in order to prevent the association of the C-intein and N-intein fragments*

the split intein is spliced out thus remaining in solution (see Fig. 2A).

Another key aspect of our approach is the potential to create spatial addressable protein arrays with multiple protein components (i.e. a real protein chip). This can be easily accomplished by creating a C-intein fragment where some of the key residues for the interaction with the N-intein will be caged with a protecting group removable by UV-light (i.e. photocaged, see Fig. 3). This blocked C-intein fragment will be unable to assemble with the N-intein fused protein thus resulting totally inactive, however, when this protecting photo-labile group is removed by the action of UV-light, the two intein fragments will assemble thus allowing the attachment of the corresponding protein to the surface through protein splicing (see Fig. 2B). Hence this approach will open the possibility to immobilize different proteins sequentially onto a surface.

Results/Technical Outcome

Chemical synthesis of Dna-E C-Int. The DnaE C-Int (residues 1-40 from the DnaE C-intein) was synthesized by SPPS using a Fmoc-based protocol on a commercially available Rink-amide resin. In each case 30 min coupling and 20 min deprotection times were used, after which clevage and global side-chain deprotection was accomplished by treatment with trifluoroacetic acid (TFA) for 2 h. The crude material was purified by preparative High Pressure Liquid Chromatography (HPLC) and fully characterized by analytical HPLC and Electro-Spray Mass Spectrometry (ES-MS) (see Fig. 3A and B).

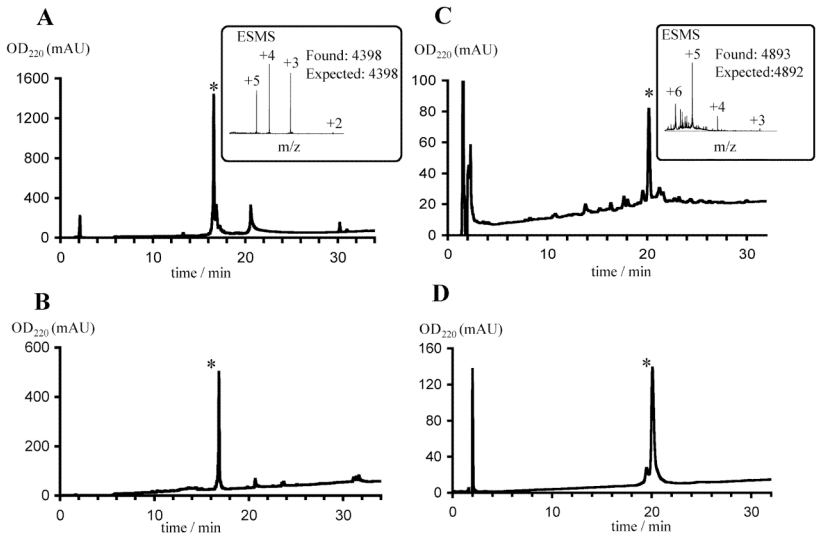


Figure 3. Characterization of synthetic C-Int polypeptides. A. HPLC analysis of C-Int crude material after TFA cleavage (Inset ES-MS analysis). B. HPLC analysis of the C-Int polypeptide after purification. C. HPLC analysis of C-Int PEGylated crude material after TFA cleavage. D. HPLC analysis of C-Int PEGylated after purification (Inset ES-MS analysis).

In order to allow the attachment of the C-Int polypeptide onto a chemically-modified surface, the C-Int polypeptide was also synthesized by SPPS with C-terminal linker containing a poly-(ethylene glycol) (PEG) moiety and a thiol group as depicted in Figure 4A. PEG linkers are well-known for their ability to prevent non-specific interactions and also act as hydrophilic spacers minimizing any detrimental interaction between the

attached polypeptide and the solid surface. The addition of a thiol group to the linker allows the chemoselective attachment of the polypeptide through its C-terminus to an acryloxy-containg surface (Fig. 4B). Note that in this case the Cys residue presented in the C-int natural sequence has to be protected as Cys(S-StBu). The synthesis of the PEGylated C-int was carried out using Fmoc-based SPPS as before but using a modified trityl-chloride resin as shown in Figure 4A. The desired peptide was cleaved, purified and fully characterized by HPLC and ES-MS (Fig. 3C and D).

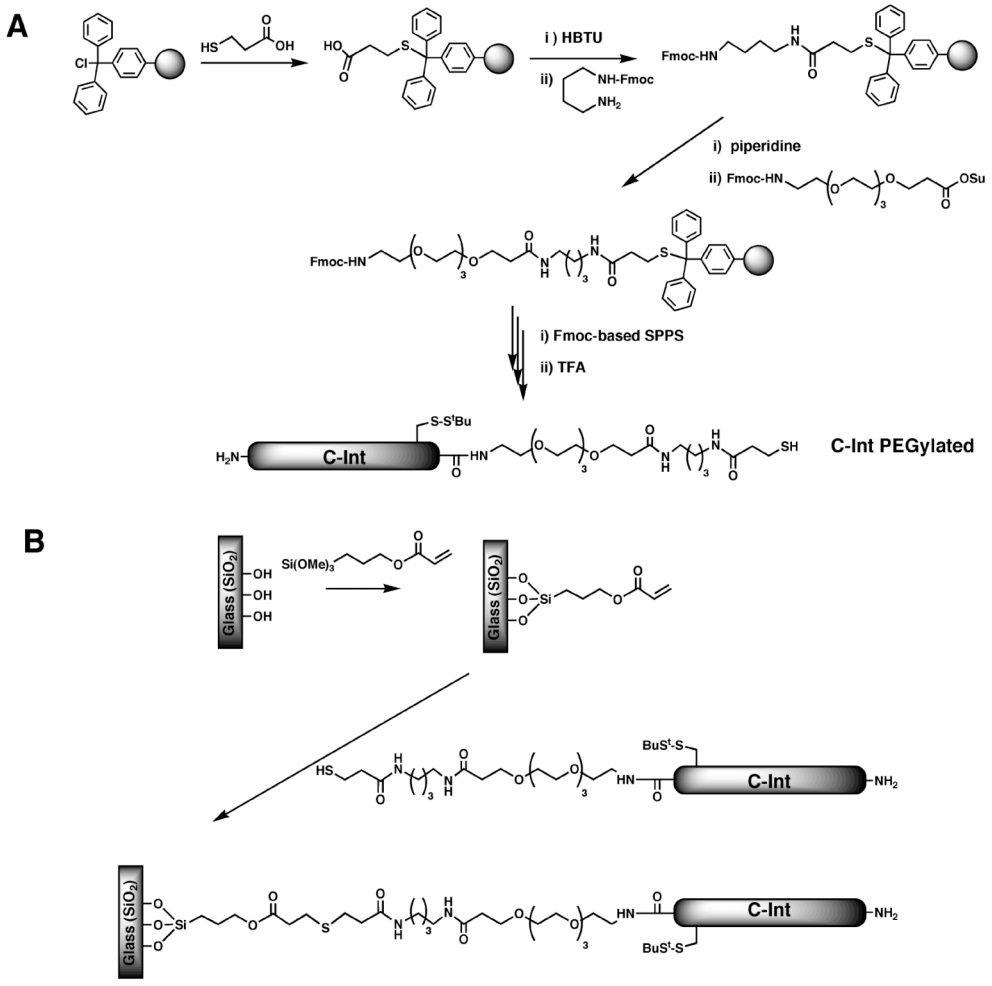


Figure 4. *A. Synthetic scheme for the synthesis of the C-Int PEGylated polypeptide. B. Chemoselective attachment of the C-Int PEGylated polypeptide through its C-terminus to an acryloxy-conatining glass surface.*

Cloning, expression and purification of MBP (maltose binding protein)-N-Int fusion protein. A bacterial expression was prepared in which the gene corresponding to the DnaE N-Int (residues 771-897 of DnaE N-intein) was cloned into the commercially available pMAL-p2X expression vector. The DnaE N-Int DNA coding sequence was inserted between the EcoRI and BamHI sites of the multi-cloning site of the pMAL-p2X vector. The recombinant MBP-N-Int fusion protein was expressed in *E. coli* to give, after affinity purification on an amylose column, a major component with the correct molecular weight, which was later confirmed by ES-MS analysis (Fig. 5).

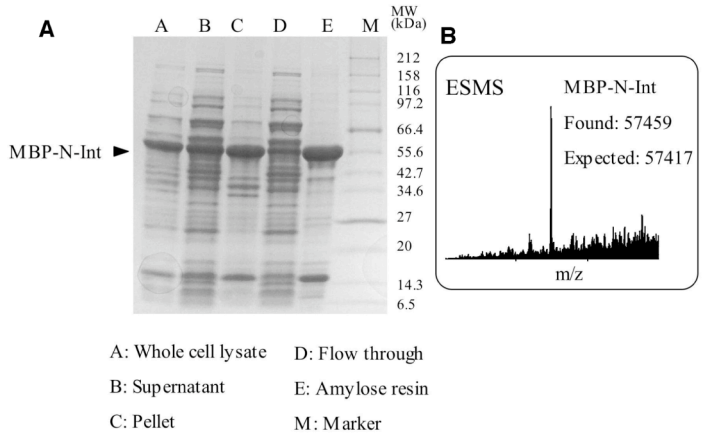


Figure 5. *Expression, purification and characterization of recombinantly expressed MBP-N-Int fusion protein. A. SDS-PAGE analysis of the expression and purification of MBP-N-Int fusion protein in E. coli. B. ES-MS analysis of purified MBP-N-Int fusion protein.*

Synthesis of Fmoc-(2-nitroveratryl)Gly-OH and its application to the synthesis of a model peptide. The synthesis of Fmoc-(2-nitroveratryl)Gly-OH was accomplished as depicted in Figure 2A. Briefly, 2-nitroveratraldehyde (211 mg, 1 mmol), H-Gly-OH•HCl (111.5 mg, 1 mmol) and $NaBH_3CN$ (126 mg, 1 mmol) were suspended in MeOH (15 mL) and stirred at 25°C for 90 min. The suspension was concentrated to dryness in vacuo, and the residual oil was resuspended in dioxane-H_2O (1:1, 10 mL). Solid $NaHCO_3$ (0.26 g, 3 mmol) was added, the suspension was cooledin an ice bath, and Fmoc-OSu (0.5 g, 1.5 mmol) in dioxane (4 mL) was added. Stirring was continued for 90 min while cooling in an ice-bath and at 25°C for another 90 min. The pH was adjusted to 9 by addition of $NaHCO_3$. The suspension was diluted with H_2O (40 mL) and washed with Et_2O (2 x 50 mL). The aqueous layer was acidified to pH 3 and the product extracted with EtOAc (2 x 50 mL). The organic phases were pooled and ocncntrated to dryness in vacuo. The crude material was finally purified by preparative HPLC using a linear gradient o 15%-100% solvent B (solvent A: H_2O, +0.1% TFA and solvent B: H_2O/MeCN 1:1, +0.1% TFA) over 30 min to give the desired Fmoc(2-nitroveratryl)-Gly-OH (300 mg, 70% overall yield). The final product was fully characterized by analytical HPLC and ES-MS (Fig. 6) as well as ^{1}H- and ^{13}C-NMR (data not shown).

In order to test the suitability for SPPS of this protected amino acid for, we synthesized the model peptide H-LYKG*A-NH2 (* stands for 2-nitroveratryl group). The synthesis was carried out on a Rink amide resin using standard Fmoc protocols. Once the peptide assembly was completed, the protected peptide resin was deprotected and cleaved with 95% TFA in the presence of 2% tris-isopropylsilane (TIS) for 3 h at room temperature. As anticipated, the nitroveratryl group was totally stable to the TFA treatment.

The crude material was finally purified by preparative HPLC and characterized by analytical HPLC and ES-MS (Fig. 7A) as the desired product.
As shown in Figure 7B, the 2-nitroveratryl group can be selectively deprotected with UV light at 365 nm in less than 30 min to yield the unprotected peptide.

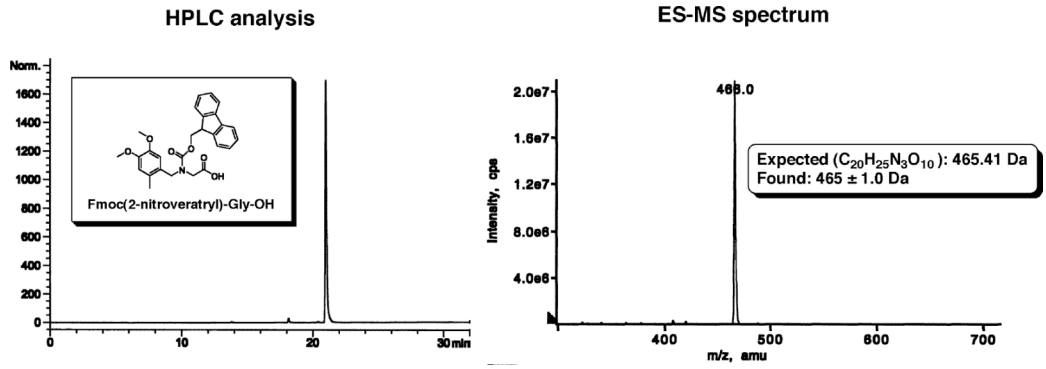


Figure 6. *Characterization of the photocaged amino acid Fmoc(2-nitroveratryl)-Gly-OH.*

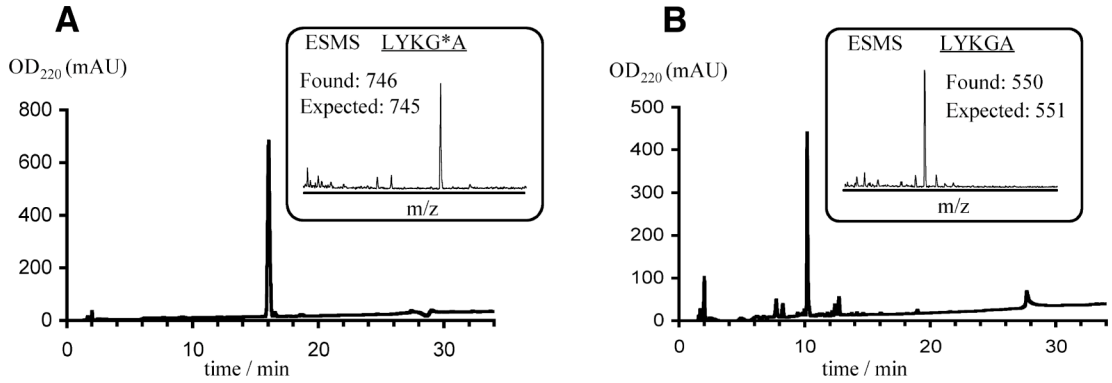


Figure 7. *Analytical HPLC and ES-MS analysis of protected peptide NH_2-LYKG*A-OH before (A) and after deprotection with a 4 W 365 nm UV-light source for 30 min at room temperature (B) (* denotes the 2-nitroveratryl group).*

Characterization of the trans-splicing reaction in solution. In order to evaluate the trans-splicing reaction in solution the recombinant MBP-N-Int fusion protein (200 μM) was incubated with the C-Int polypeptide (≈ 37 μM) in 1 mM EDTA, 1 mM TCEP, 20 mM NaPi and 500 mM NaCl buffer at pH 7.6. Small aliquots were taken at different times rapidly quenched and analyzed by SDS-PAGE (Poly-Acrylamide Gel Electrophoresis). Under these conditions, the trans-splicing reaction showed an efficiency of ≈ 60% and was considered completed in less than 6 h (Fig. 8A). The crude reaction was also analyzed by HPLC and ES-MS and confirmed the presence of the trans-splicing product, i.e. the MBP protein linked through its C-terminus to the peptide CFNK (Fig. 8B).

Encouraged by these results we decided to explore the minimum concentration required for the N-Int and C-Int polypeptides to observe efficient trans-splicing. Thus, several trans-plicing reactions were carried out where the concentrations of C-Int and MBP-N-Int ranged from 350 nM to 40 μM and from 50 nM to 2 μM, respectively. In all the cases the reactions were performed in 1 mM EDTA, 1 mM TCEP, 50 mM Tris•HCl, 400 mM NaCl buffer at pH 7.0 for 16 h. The extension of the reactions was analyzed

and quantified by SDS-PAGE. The results summarized in Figure 9, showed that in all the cases the extension of the trans-splicing reaction was between 65% and 100%. It is interesting to remark that the trans-splicing reaction is still efficient at concentrations in C-Int and N-Int as low as 350 nM and 50 nM, respectively, which suggests a strong binding constant between the C-Int and the N-Int domains.

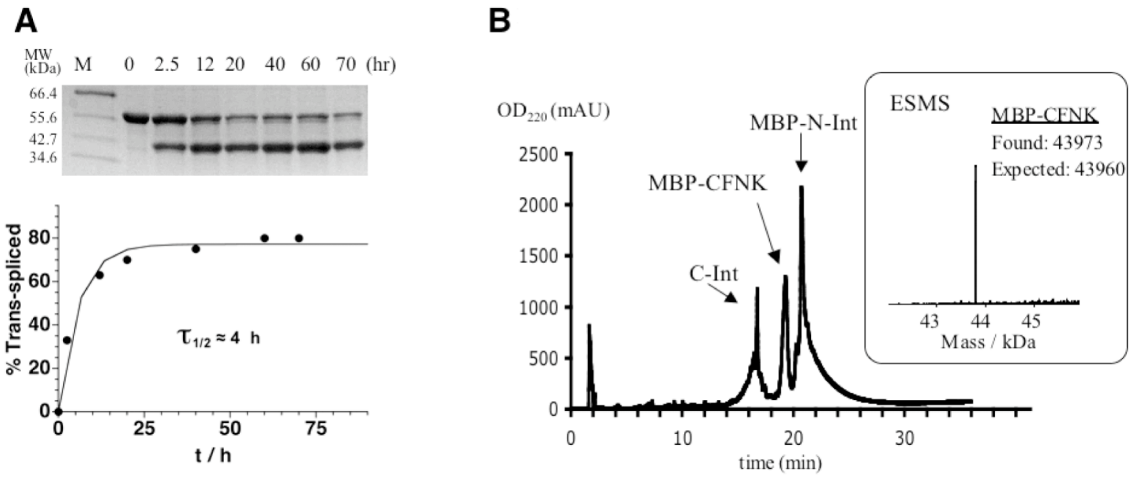
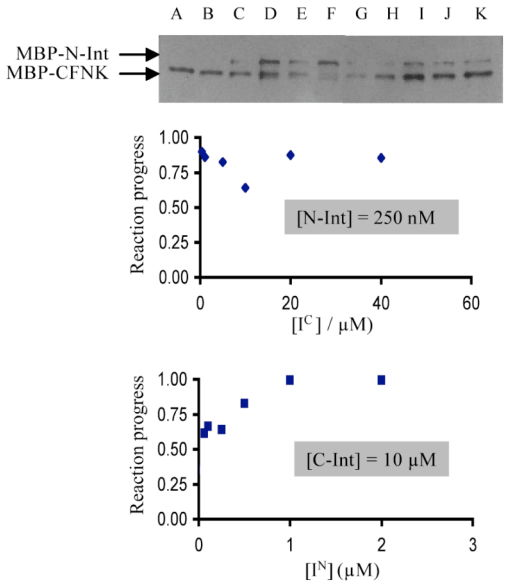


Figure 8. *Analysis and characterization of the trans-splicing reaction using the DnaE Intein system. A. Kinetics for the trans-splicing reaction were quantified by SDS-PAGE. B. After completion, the trans-splicing reaction was analyzed by HPLC and the trans-spliced product was characterized by ES-MS as the desired product (MBP-CFNK).*



Gel	[N-Int] (nM)	[C-Int] (µM)	Reaction progress (%)
A	250	40	85
B	250	20	88
C	250	10	64
D	250	5	82
E	250	1	86
F	250	0.35	90
G	2000	10	100
H	1000	10	100
I	500	10	83
C	250	10	64
J	100	10	66
K	50	10	65

Figure 9. *SDS-PAGE analysis and quantification of the extension of the of trans-splicing reaction at 16 h using different concentrations of MBP-N-Int and C-Int polyepeptides.*

Sumary

In summary, we have shown that at least in solution the trans-splicing reaction using the DnaE split-intein system works very efficiently at concentrations as low as low nM,

therefore showing great promise for its use on the chemoselctive attachment of proteins to surfaces. We have also shown that the 2-nitroveratryl group can be readily used for the synthesis of backbone-protected peptides using Fmoc-based SPPS. This group could be used in the future for the synthesis of photocaged versions of the C-Int polypeptide for the combination of selective protein attachment using protein trans-splicign and photolithographic techniques.
Future work will involve testing the trans-splicing reaction in solid phase using model proteins.

Exit Plan

The ability of attaching proteins to a solid support in an ordered fashion is expected to play a critical role in several key applications in the field of biology and biophysics. For example, the creation of functional protein microarrays is critical for the progress in proteomics research. Like DNA chips, protein chips will allow the analysis of thousand of proteins simultaneously, and rapidly screening them all for the discovery of new drug targets. Moreover, it will be also relatively easy to check a potential drug candidate and screen it using a protein chip for unwanted side-effects in all the metabolic pathways, for example. Protein chips can also be also used as a diagnosis tool for profiling protein expression in order to find potential potentially relevant biomarkers. Another potential application for the creation of ordered protein films on surfaces is the creation of optimized biosensors. An ordered protein film will have a higher activity density that a random protein film, where a significant percentage of the protein molecules will be bound to the surface in potentially inactive conformations. This will allow to miniaturize a biosensor without loosing sensitivity. Also, the combination of recent advances in nano-lithography techniques combined with the ability to bind proteins in extremely ordered fashion will allow the creation of protein nanopatterns which could be used as templates for the crystallization of biomolecules. This emerging technology is expected to have a huge impact in the structural biology field since one the main bottlenecks in the structure determination of biomolecule is its crystallization. In summary, all these potential applications make of protein biochips a very powerful tool that may provide an important role in the future of biology and biophysics.

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