Research Article

Journal of PeptideScience

Received: 12 January 2010 Accepted: 11 February 2010 Published online in Wiley Online Library: 6 April 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1227

Protein immobilization on liposomes and lipid-coated nanoparticles by protein *trans*-splicing‡

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A plethora of methods exist to link proteins to surfaces in order to generate functionalized materials. However, general tools that lead to functional immobilization of recombinantly expressed proteins on membranes such as liposomes or lipid-coated nanoparticles are rare. Here we present an approach that takes advantage of a double-palmitoylated peptide that mediates stable membrane anchoring in combination with protein *trans*-splicing for efficient immobilization of recombinant proteins fused to split intein segments. Two different DnaE split inteins from *Synechocystis* and *Nostoc punctiforme* are tested and compared to immobilization via direct native chemical ligation using a protein thioester. Protein *trans*-splicing proceeds at low protein concentrations and leads to functionalized vesicles and membrane-coated silica nanoparticles. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: protein modification; membranes; nanoparticles; protein immobilization; split inteins

Introduction

A substantial number of eukaryotic and eubacterial proteins are associated with cellular membranes via covalently attached lipids. These posttranslational modifications provide organisms with a highly effective way to control protein targeting to cell organelles, compartments, and protein clusters. Furthermore, such modifications allow cells to regulate biophysical properties of the carrier proteins, their interaction with other proteins, and to relocate proteins by cycling between lipidated and nonlipidated states [1]. These options do not require a change in expression patterns or cellular protein transport.

Typical examples for protein lipidation are *C*-terminal glycosylphosphatidylinositol (GPI) anchors, palmitoyl thioesters as well as geranylgeranyl and farnesyl thioethers or *N*-terminal myristoylation. The number of lipid moieties per protein is not limited to one, but two to three such modifications have been observed. Generally, posttranslational modification of proteins with two lipid groups leads to tight association with membranes, as has been shown for members of the Ras GTPase superfamily [2,3]. Similarly double-palmitoylated GPI anchor mimics lead to stable membrane association of target proteins [4,5]. In order to study biophysical and biochemical properties of membrane-attached proteins *in vivo* and *in vitro*, strategies are required to generate posttranslationally modified proteins by chemical means. Two recently described approaches for *C*-terminal attachment of lipid groups to proteins [4,6] use native chemical ligation (NCL) reactions [7]. In one of these approaches, detergent-solubilized cysteine lipids are reacted with a protein thioester and the resulting lipidated protein is subsequently incorporated into supported bilayers [4]. In the other approach, cysteine-modified lipids were incorporated into liposomes prior to the ligation reaction providing a tool for specific functionalizing liposomes [6]. Such liposomes are not only of interest for biophysical studies of proteins in a more native-like environment but also because of their use as drug delivery vehicles addressing specific tissues via antibodies or peptide–protein interactions [8]. These methods provide practical tools to control protein lipidation and lipid bilayer or micelle modification. However, some methodological restrictions limit their general application. *C*-terminal thioesters of peptides or proteins have to be generated by chemical peptide synthesis or intein chemistry, a task not easily achieved in all cases. A second problem concerns the ligation reaction itself, which is quite sensitive toward the presence of detergents or lipids. Membranes or micelles might conceal the ligation site making it inaccessible for the reaction partner.

A route to circumvent these difficulties can potentially be found in the split intein (or *trans*-splicing) technology [9,10]. This technology is based on two naturally occurring protein domains that

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‡ *Special issue devoted to contributions presented at the Symposium "Probing Protein Function through Chemistry", 20–23 September 2009, Ringberg Castle, Germany.*

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Journal of PeptideScience

spontaneously associate with each other and form a functional intein complex. This newly formed intein complex can link the flanking *N*- and *C*-terminal extein domains via a native amide bond. This method has several advantages over the commonly used NCL. First, recombinantly expressed proteins can be used directly without the need for additional purification steps since only split intein fusion constructs are forming the reactive complex. Second, because of the small dissociation constant of split intein segments, the concentrations needed for efficient *trans*-splicing are rather low. In contrast, chemoselective reactions strongly depend on high reactant concentrations. A recent comparison of selective immobilization of proteins on surfaces on the one hand via expressed protein ligation and on the other hand via a split intein approach showed that the latter approach is more effective in terms of immobilization yield and the concentration range that can be used [11,12].

In this work we present results that show the general applicability of protein *trans*-splicing to covalently modify target proteins with lipid anchor peptides. We compare two different split intein systems and direct NCL for immobilizing proteins. This approach leads to lipidated target protein that is efficiently anchored on liposomes but also on silica nanoparticles covered with a lipid bilayer.

Materials and Methods

Boc-protected and Fmoc-protected amino acids, 2-(1H-benzotriazol-1-yl)−1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) and Boc-Leu-PAM resin were purchased from Novabiochem (Nottingham, UK) and Orpegen (Heidelberg, Germany). Trifluoroacetic acid was from Roth (Karlsruhe, Germany). Hydrogen fluoride was purchased from Merck (Nottingham, UK). DMF was obtained from Biosolve (Valkenswaard, The Netherlands). All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany) at the highest purity available.

Peptide Synthesis

Solid-phase peptide synthesis was performed on a custom-modified 433A peptide synthesizer from Applied Biosystems (Darmstadt, Germany), using Boc-chemistry, *in situ* neutralization, and HBTU activation protocols [13] or manually using Fmoc-chemistry [14]. Cleavage of Mmt-protecting groups was achieved by treatment with acetic acid, trifluorethanol, and DCM (1 : 2 : 7); after neutralizing the resin with 10% DIEA in DMF, palmitoylation was done with 20 equivalents of palmitoylchloride, 20 equivalents of HOBT, and 22 equivalents of triethylamine in DCM/DMF (3 : 1) for 4 h. Peptides synthesized by Boc-chemistry were deprotected and simultaneously cleaved from the resin using anhydrous hydrogen fluoride, whereas those synthesized by Fmoc-chemistry were cleaved by 2.5% H_2O and 5% TIS in TFA. Crude products were subsequently precipitated with diethyl ether, dissolved in 50% aqueous acetonitrile (0.1% TFA), and lyophilized. Peptides were purified by RP-HPLC on C4-columns from Vydac (Hesperia, CA, USA) using linear gradients of buffer B (acetonitrile with 0.08% trifluoroacetic acid) in buffer A (water with 0.1% trifluoroacetic acid).

HPLC and Mass Spectrometry

HPLC analysis of peptides was performed on an analytical RP-C4-column (Vydac) at a flow rate of 1 ml/min for 30 min with a gradient from 5 to 80% (v/v) buffer B in A. Peptide and protein masses were determined by electrospray ionization mass spectrometry on an LCQ Advantage Max (Thermo Finnigan, Bremen, Germany) operating in positive ion mode. The molecular masses were deconvoluted from the charged ion spectra.

Native Chemical Ligation

NCLs of synthetic peptides were carried out in 6 M GdnHCl, 300 mM NaP_i, and 1% (v/v) thiophenol [optional addition of 17 mg/ml dodecylphosphocholine (DPC)] at pH 7.4 – 7.7 using concentrations of 5 – 6 mM of *C*- and *N*-terminal peptides, respectively [7]. NCL reactions of enhanced green fluorescent protein (eGFP) thioester with peptide **1** were carried out in 100 mM Tris – HCl in the presence of 17 mg/ml DPC and 1% (v/v) thiophenol or 50 mM MESNA at pH 7.8 and concentrations of 1 mM eGFP thioester and 1.5 – 2 equivalents of peptide **1** unless other stated in the text.

Reactions were quenched by addition of three volume equivalents of ligation buffer and 20% (v/v) of β-mercaptoethanol for 20 min. The ligation mixtures were purified by RP-HPLC on a C4-column from Vydac using linear gradients.

Liposomes Preparation

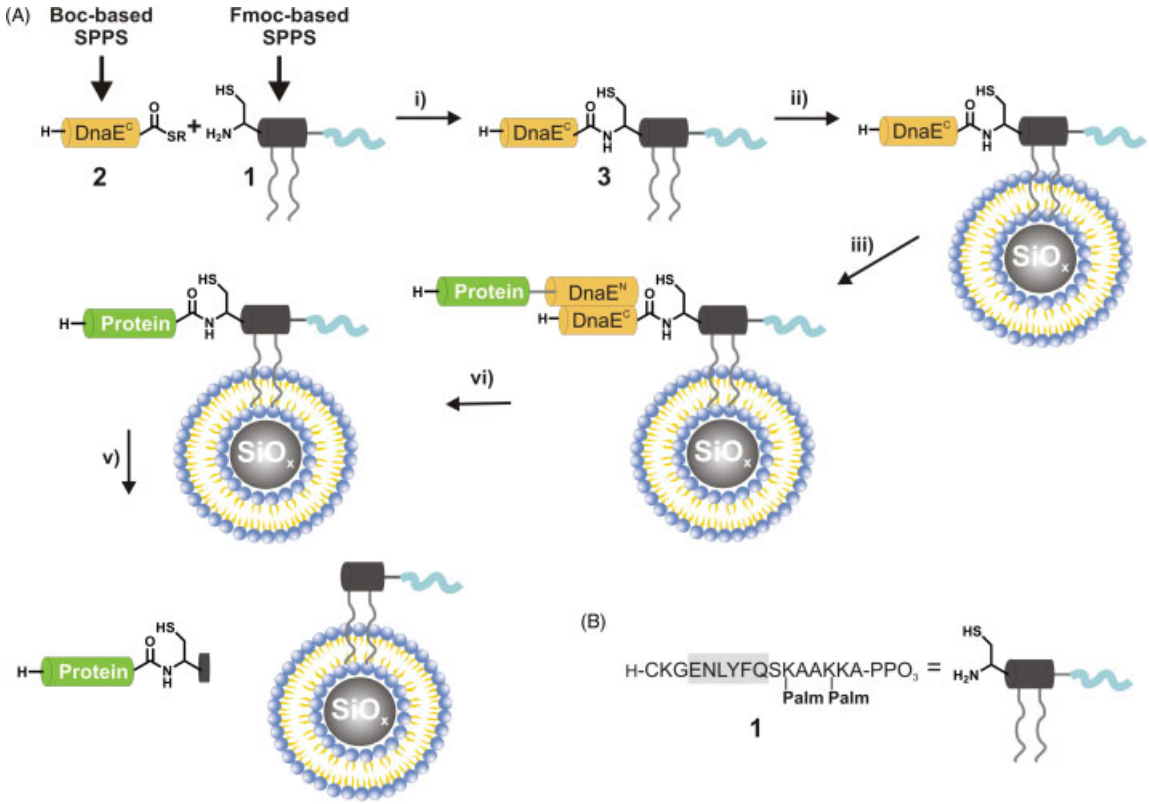
Two milligrams of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was dissolved in 1 ml chloroform and transferred under argon into a 25-ml round-bottom flask. The solvent was evaporated under rotation in a stream of helium gas and the resulting lipid layer was dried in high vacuum for at least 2 h. The DOPC layer was resuspended in 1 ml of splicing buffer (100 mM Tris – HCl, 500 mM NaCl, 2 mM EDTA, 1 mM, pH 7.5). The suspension was transferred into a 15-ml tube and kept under argon. The lipid suspension was sonicated until the solution became opalescent. After centrifugation at 100 000 *g* (43 000 rpm in Beckmann Ti 100.4 rotor), the clear supernatant was used for splicing experiments on liposomes.

Lipid-Coated Nanoparticles

The lipidation of silica nanoparticles (Evident Technologies; average particle diameter: 800 nm) was carried out with a 16-fold excess of lipids to nanoparticles. The necessary volume of nanoparticles stock solution was centrifuged at 3000 rpm for 3 min. The particles were resuspended in 1 ml of methanol, sonicated for 5 min, and centrifuged as described above. Afterwards the nanoparticles were suspended in 1 ml of 1 M KOH followed by extensive washing (at least five times) with 1 ml of splicing buffer. The small unilamellar vesicles (SUVs) (prepared as described above) and the nanoparticles were mixed and incubated at room temperature under gentle agitation for 2 h.

Expression and Purification of eGFP Fusion Constructs

Recombinant eGFP was cloned into a modified pTXB3 vector (New England Biolabs, Frankfurt a.M., Germany) containing the GyrA mini intein and a chitin-binding domain with an additional His tag in between them. Expression was carried out in *Escherichia coli* BL21(DE3), and cells were harvested and lysed as described below. Initial purification was achieved via affinity chromatography using Ni-NTA (Qiagen, Hilden, Germany) and elution with 250 mM imidazole. After dialysis against a buffer containing 100 mM Tris – HCl at pH 8.0, the fusion construct was loaded onto chitin beads and eGFP thioester was released after addition of 250 mM MESNA. eGFP thioester was further processed by another Ni-NTA affinity purification after fast dilution to decrease MESNA concentrations.



Scheme 1. Schematic representation of protein *trans*-splicing as a tool for immobilization of proteins on lipid-coated nanoparticles. (A) NCL scheme of peptide **1** and the *C*-terminal split intein segment $DnaE^C$ thioester (**2**) (i). Incorporation of **3** into lipid-coated nanoparticles (ii). Formation of an active *trans*-splicing complex between a recombinantly expressed fusion protein with the *N*-terminal split intein segment $DnaE^N$ (iii). Protein immobilization on lipid-coated nanoparticles (iv). Tev-mediated proteolytic removal of protein from lipid-coated nanoparticles (v). (B) Amino acid sequence of peptide **1** and its schematic representation.

Typical yields were 8 mg of eGFP-GyrA fusion construct from 1 l of *E. coli* culture that resulted in 2 mg of purified eGFP thioester.

Recombinant eGFP was also cloned into the pTXB3 (New England Biolabs) vector and expressed in *E. coli* BL21(DE3)RIL as a fusion protein with the $DnaE^N$ split intein from *Synechocystis sp. (Ssp)* as well as with the $DnaE^N$ split intein from *Nostoc punctiforme* (*Npu*). A His-Tag was included to facilitate purification. Protein expression was induced with IPTG and gave *ca* 5 mg of eGFP-$DnaE^N$ fusion protein per 1 l of *E. coli* culture. The cells were harvested by centrifugation and lysed in a buffer containing 50 mM NaPi, 500 mM NaCl, 20 mM imidazole, and pH 8.0. The lysate was loaded on a Ni-NTA column and the fusion constructs eluted with 250 mM imidazole as described above. Typical yields were 1 mg of eGFP-$_{Ssp}$$DnaE^N$ and 5 mg of eGFP-$_{Npu}$$DnaE^N$ from 1 l of *E. coli* culture.

Confocal Fluorescence Microscopy

The fluorescence images were recorded with a Leica confocal microscope. Excitation of eGFP was carried out at 475 nm. Fluorescence emission was detected above 500 nm.

ITC Measurements

ITC measurements were performed using an AVP-ITC MicroCalorimeter (MicroCal, Piscataway, USA).

In order to determine the affinity of the two split intein segments in PBS buffer, eGFP-$DnaE^N$ was provided in the reservoir at a concentration of 50 μM and $DnaE^C$-**3** (at a concentration of 800 μM) was titrated into the reservoir in increments of 8 μl.

Results and Discussion

The DnaE *trans*-splicing systems from *Ssp* and *Npu* used here consist of two major building blocks [15,16]; the lipidated membrane anchor peptide **1** attached to the *C*-terminus of the *C*-terminal split intein segment $DnaE^C$ **3** or **3a** from either variant and recombinantly prepared target protein *N*-terminally fused to the *N*-terminal split intein segment $DnaE^N$. The $DnaE^C$-membrane anchor constructs **3** and **3a** can be incorporated into lipid bilayers or detergent micelles. Upon addition of a protein-$DnaE^N$ fusion construct to this preparation, a tight complex between $DnaE^N$ and $DnaE^C$ is formed by which the ligation reaction between peptide and protein is induced (Scheme 1A).

In order to generate an efficient membrane anchor, a short peptide (**1**) with three functionalities was designed. It consists of 17 amino acids and comprises an *N*-terminal cysteine residue, two lysine residues for lipid attachment, and a protease cleavage site (Scheme 1B). In order to increase the solubility of the double-palmitoylated peptide, a solubilization tag at the *C*-terminus was introduced. The polyethylene glycol-like polymer was chosen because it is compatible with solid-phase peptide synthesis [17], chemically inert, and efficiently solubilizes hydrophobic peptides [18].

Peptide (**1**) was synthesized by Fmoc-chemistry and was, after cleavage from the resin, purified by RP-HPLC (Figure 1A). Synthesis of the $DnaE^C$ segments from *Ssp* (**2**) and *Npu* (**2a**) equipped with a *C*-terminal thioester was achieved by Boc-based SPPS. Peptide (**1**) and $DnaE^C$ segments were fused by NCL [19,20] with yields of

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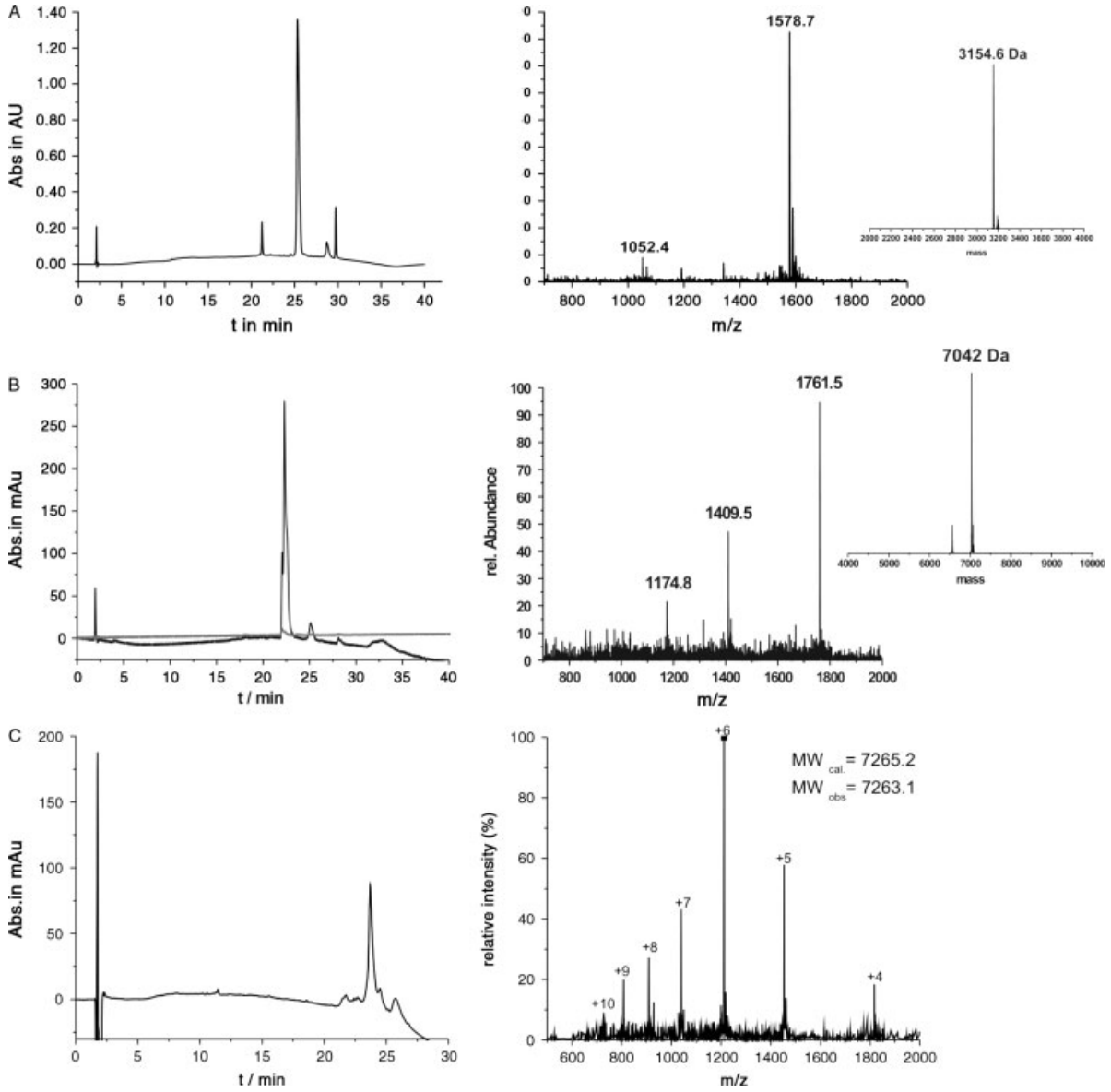

Figure 1. RP-HPCL and MS analysis of membrane anchor peptide **1** and NCL products with *C*-terminal DnaE split intein segments from *Ssp* and *Npu* (**3**). (A) RP-HPLC chromatogram of membrane anchor peptide **1** after purification (left) and the corresponding ESI mass spectrum of this compound together with a deconvoluted spectrum (calculated mass: 3155 Da; right). (B) RP-HPLC chromatogram of **3** with the *Ssp* DnaE^{C} segment after purification (left) and the corresponding ESI mass spectrum of this compound together with the deconvoluted spectrum (calculated mass: 7041.8 Da; right). (C) RP-HPLC chromatogram of **3a** containing the *Npu* DnaE^{C} segment instead of the *Ssp* DnaE^{C} after purification (left) and the corresponding ESI mass spectrum of this compound (right).

15–20% based on the crude starting peptides (Figure 1B and C). Incorporation of the resulting DnaE^{C}-membrane anchor construct (**3** or **3a)** into DOPC liposomes was achieved by addition of 4 mole% of **3/3a** to fresh liposome preparations for 1 h at room temperature (see 'Materials and Methods'). Subsequent high-speed centrifugation efficiently separated nonimmobilized **3/3a** from modified liposomes.

The second building block consisted of DnaE^{N} segments from *Ssp* and *Npu* fused to eGFP, **4** and **4a**, which were readily expressed in *E. coli*. The eGFP-DnaE^{N} fusion constructs were purified by Ni-NTA affinity chromatography prior to their use in *trans*-splicing experiments (see supporting information). eGFP was chosen for these experiments because of its fluorescent properties, which allow direct detection of membrane association on lipid-coated beads as well as on liposomes and cells. Initial *trans*-splicing experiments were performed with liposomes containing **3.** Mixing 6.7 μM liposomes containing 0.3 μM **3** with a fivefold excess of eGFP-DnaE^{N} for 12 h at room temperature lead to immobilization of 0.1–0.2 μM of eGFP on liposomes. eGFP-modified liposomes were separated from starting materials by ultracentrifugation in a sucrose gradient from 5 to 20% and analyzed by western blotting with a commercially available eGFP-specific antibody (Rabbit Anti-eGFP, Torrey Pines Biolabs), which provides quantitative readout on *trans*-splicing and immobilization yields (Figure 2A). Typical yields were in the range of 30–50%. As a side reaction, cleavage of the eGFP-DnaE^{N} fusion construct to give nonmodified eGFP was also observed (10–15%). However, unmodified eGFP did not hamper further experiments since it did not attach to liposomes or

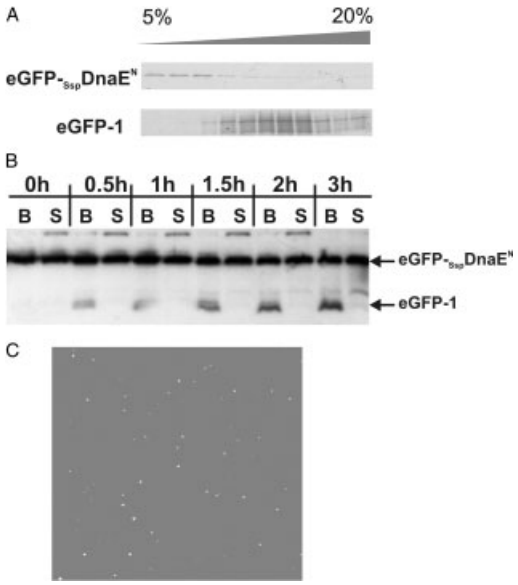

Figure 2. (A) Western blot analysis of liposome fractions after ultracentrifugation in a sucrose gradient from 5 to 20%. Early fractions contain eGFP-DnaE^N fusion protein not attached to liposomes and fractions between 10 and 15% sucrose contain liposome-attached eGFP-**1**. (B) Time course of immobilization of eGFP-$_{Ssp}$DnaE^N on lipid-coated nanoparticles loaded with **3** analyzed by western blotting. The reaction is significantly decelerated after 3 h and reaches its maximum yield after 5 h at room temperature. (C) Confocal microscopy picture of lipid-coated nanoparticles loaded with eGFP-**1** via *trans*-splicing. The fluorescent nanoparticles are depicted as white objects in order to improve visibility.

lipid-coated nanoparticles and can easily be removed by centrifugation. This side reaction can be diminished by the addition of 5 mM MESNA, which also accelerated the splicing reaction and lead to an increase in *trans*-splicing efficiency by approximately 15%.

To exclude unspecific adsorption to or insertion into liposomes of eGFP-DnaE^N or eGFP, control reactions were carried out with eGFP-DnaE^N and eGFP in the absence of **3**. These reactions clearly indicated that liposome attachment only occurs when eGFP was linked to **1**.

The dissociation constant characterizing the interaction of *N*- and *C*-terminal DnaE domains was previously determined by a FRET assay (with both reaction partners in solution) to be 42 nM and a similar value was obtained by equilibrium titrations [21,22]. However, the palmitoylation of DnaE^C-terminal segment **3** might influence solubility and possibly binding to eGFP-DnaE^N and as consequence could alter K_d of this interaction. Indeed isothermal titration calorimetry with **3** and eGFP-DnaE^N (**4**) indicated a significantly weaker interaction between the two split intein domains with a K_d of 3.5 μM (see supporting information). At first glance this effect might reflect the influence of lipid attachment. However, it must also be noted that the DnaE^C peptide used in these experiments is slightly different from those used in the previous experiments [21,22]. In our experiments, a *C*-terminally truncated analog of DnaE^C was used, which does not include three amino acids (CFN) from the natural extein of the *Ssp* DnaE split intein. These amino acids have been included in DnaE^C peptides for previously described binding studies and resulted in three additional amino acids at the splice junction. Such a change in amino acid composition could also influence the K_d value but obviously had no effect on the *trans*-splicing efficiency when comparing *Ssp* DnaE^C with and without these three *C*-terminal amino acids (see supporting information).

Having shown that this approach was applicable for the modification of lipid vesicles, it was also applied to modify lipid-coated nanoparticles because of their importance in cellular biology or for specific drug delivery and targeting. Two samples of silica beads with an average diameter of 200 and 800 nm, respectively, were coated with a lipid bilayer of DOPC and loaded with **3**. Incubation of these nanoparticles with eGFP-$_{Ssp}$DnaE^N initiated *trans*-splicing and generated eGFP-coated beads. Successful immobilization of eGFP on nanoparticles was proven by western blotting after stripping the lipid and protein coat from the nanoparticles by treatment with SDS-containing buffer (Figure 2B). Maximum yields were obtained after 5 h at room temperature. After removal of excess eGFP-$_{Ssp}$DnaE^N, the nanoparticles were also analyzed by fluorescence microscopy and intensely fluorescent nanoparticles clearly demonstrated efficient immobilization of eGFP on these particles (Figure 2C). The protein can be stripped off the membrane by treatment of the nanoparticles with TEV protease (Scheme 1A). After 6 h incubation, a significant decrease of eGFP linked to nanoparticles (*ca* 50%) was observed (see supporting information). This observation clearly points to an attachment of eGFP-**1** to the lipid bilayer via its two palmitoyl groups.

As mentioned above, NCL between protein thioesters and cysteine-modified lipids can be used for linking proteins to vesicles [4,6]. In order to test if protein *trans*-splicing is more efficient at low concentrations, we carried out direct NCL reactions with SUVs doted with **1** and eGFP thioester prepared by cleavage of an eGFP-GyrA intein construct expressed in *E. coli* (supporting information). Ligation product eGFP-**1** was only observed at concentrations starting at 100 μM of eGFP thioester and in the presence of 50 mM MESNA as ligation mediator (Figure 3A and B). At lower concentrations no ligation product can be observed in western blots. These observations are in good agreement with previously reported comparisons of *trans*-splicing and NCL reactions for surface immobilization of proteins [11,12], which showed that *trans*-splicing contrary to NCL is successful even at concentrations in the nanomolar range.

It was recently reported that the DnaE split intein system from *Npu* is a more efficient *trans*-splicing system in terms of reaction rate when compared with the *Ssp* system [23,24]. To this end we also synthesized the Npu DnaE^C segment and linked it to peptide **1** in order to generate the membrane anchor **3a** suitable for *trans*-splicing reactions with eGFP-$_{Npu}$DnaE^N (Figure 1C), which was purified from *E. coli* lysate (see supporting information). Test reactions were performed in solution without vesicles at concentrations of 5 μM eGFP-$_{Npu}$DnaE^N and 5 μM **3a** (Figure 3C). Two different MESNA concentrations were tested to analyze the impact of this reducing agent on *trans*-splicing and to suppress hydrolysis reactions as described above for the *Ssp* system. Reaction timescales were comparable to what has been observed by us for *trans*-splicing with the *Ssp* DnaE split intein in the presence of 5 mM MESNA. However, at 50 mM MESNA a faster reaction rate was observed and lead to almost quantitative conversion of eGFP-$_{Npu}$DnaE^N into eGFP-1 after 24 h. The *Npu* split intein system shows a slightly superior performance to the *Ssp* system in our experiments and will therefore help to improve efficiency and applicability of this approach in the future.

One application of this method useful for cellular biology would be the delivery of proteins to specific cells. We therefore analyzed possibilities to directly modify the plasma membrane of an eukaryotic cell (COS-7). Treatment of these cells with

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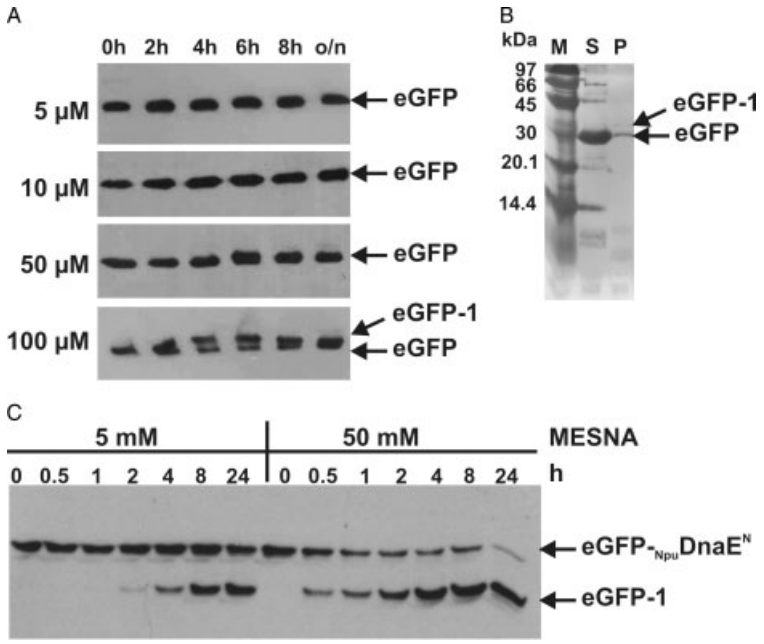

Figure 3. (A) Time course of NCL of eGFP thioester with **1** incorporated into DOPC vesicles at different concentrations analyzed by western blotting; 25 mM MESNA was present as a ligation mediator. (B) Silver-stained SDS gel showing the reaction of eGFP thioester (10 μM) with **1** as described above after ultracentrifugation to separate vesicles and supernatant after 5 h at room temperature. (C) Time course of the *trans*-splicing reaction of eGFP-$_{Npu}$DnaE$_N$ (5 μM) with **3** (containing the $_{Npu}$DnaE^C peptide instead of $_{Ssp}$DnaE^C as in previous experiments) in the presence of 5 and 50 mM MESNA, respectively.

3 indicated a direct labeling of the surface as determined by confocal fluorescence microscopy (see supporting information). However, under conditions the cells can still tolerate (MESNA concentration $<$5 mM, pH 7.5), no ligation reaction detectable with confocal fluorescence microscopy with eGFP-$_{Ss}$pDnaE^N did occur even after 24 h at 37 °C. To circumvent this problem, COS-7 cells were incubated with eGFP-**1**-loaded DOPC liposomes obtained as described above. An efficient transfer of this construct onto cells was observed under these conditions as demonstrated by fluorescence microscopy (supporting information). Even though no direct *trans*-splicing reaction on cell surfaces was achieved here, this approach facilitates the generation of protein functionalized vesicles via *trans*-splicing which in turn can easily be transferred onto live cells.

Conclusions

Protein *trans*-splicing represents a valuable instrument for the immobilization of proteins on liposomes and the generation of protein functionalized lipid-coated nanoparticles. A double-palmitoylated peptide (**1**) confers sufficient affinity to lipid bilayers to tightly attach proteins of interest to them and can be synthesized in high yields. NCL is used to link **1** to DnaE^C (**2**) and the resulting construct **3** readily inserts into lipid bilayers. Such bilayer preparations can then be used in *trans*-splicing experiments with different target proteins to obtain homogeneous preparations of proteins bound to either liposomes or lipid-coated nanoparticles. Even direct immobilization of DnaE^N fusion constructs out of complex mixtures such as cytoplasmic fractions should be possible due to the highly specific interaction between the split intein segments. The method described here is not restricted to *C*-terminally lipidated proteins but can be extended toward the synthesis of *N*-terminal lipid modifications by taking advantage of the recently described DnaB split intein system [25]. Lipidated proteins specifically attached to membranes are valuable tools, in combination with biophysical methods and cell biology, to provide new insights into this important protein family and the function of membrane association as well as targeting. Furthermore, applications in biotechnology can be envisioned when lipid-coated magnetic particles are used as carriers for relevant enzymes [26].

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

We thank T. Muir for providing the genes coding for the DnaE split intein segments, R. S. Goody for continuous support, and S. Gentz and K. Baeuml for help with peptide synthesis. We are grateful for financial support from the Max-Planck Society, Deutsche Forschungsgemeinschaft and Fonds der chemischen Industrie.

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