Published in final edited form as:

Bioconjug Chem. 2012 June 20; 23(6): 1119–1126. doi:10.1021/bc200577u.

Development of an *Influenza virus* protein array using Sortagging technology

Antonia Sinisi^{†,‡,a}, Maximilian Wei-Lin Popp^{†,b}, John M. Antos[†], Werner Pansegrau[†], Silvana Savino[‡], Mikkel Nissum[‡], Rino Rappuoli[‡], Hidde L. Ploegh^{†,*}, and Ludovico Buti^{†,‡,c}

[†]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA

[‡]Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

Abstract

Protein array technology is an emerging tool that enables high throughput screening of protein-protein or protein-lipid interactions and identification of immunodominant antigens during the course of a bacterial or viral infection. In this work we developed an *Influenza virus* protein array using the sortase-mediated transpeptidation reaction known as “Sortagging”. LPETG-tagged *Influenza virus* proteins from bacterial and eukaryotic cellular extracts were immobilized at their carboxyl-termini onto a pre-activated amine-glass slide coated with a Gly₃ linker. Immobilized proteins were revealed by specific antibodies and the newly generated Sortag-protein chip can be used as a device for antigen and/or antibody screening. The specificity of the Sortase A (SrtA) reaction avoids purification steps in array building and allows immobilization of proteins in an oriented fashion. Previously, this versatile technology has been successfully employed for protein labeling and protein conjugation. Here, the tool is implemented to covalently link proteins of a viral genome onto a solid support. The system could readily be scaled up to proteins of larger genomes in order to develop protein arrays for high throughput screening.

INTRODUCTION

In the last decade, applications of microarray technology have grown exponentially, but a simple and fast technique to immobilize functional proteins onto solid supports still represents a major challenge¹. Various protein tags and several different strategies have been employed to attach proteins to solid supports. Examples include hexahistidine-tagged fusion proteins, and *in vivo/in vitro* biotinylation procedures, which confer a specific orientation to the arrayed proteins. However, these approaches often give rise to a high level of background which may compromise the quality of the protein chip². Chemical procedures or chemoselective reactions between protein α-thioesters and a cysteine-coated glass slide are examples of a more general but even less specific reaction to immobilize proteins onto a solid support due to the random crosslinking of lysines to carboxylic acid coated slides^{3–4}.

*Corresponding author: ploegh@wi.mit.edu.

^aCurrent address: Istituto Nazionale di Genetica Molecolare, Via F. Sforza 35, 20122 Milano, Italy.

^bCurrent address: University of Rochester School of Medicine and Dentistry 601 Elmwood Avenue Rochester, NY 14642, USA.

^cCurrent address: Ludwig Institute for Cancer Research, Nuffield Department of Clinical Medicine, University of Oxford, Old Road Campus Research Building, Headington, Oxford OX3 7DQ, UK

CONFLICT OF INTEREST

The authors declare no competing financial interest.

Supporting Information Available: Supporting information contains experimental procedures describing the ELISA assay and deglycosylation reaction, tables of primers used in this study and additional figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

To overcome these issues, we made use of the specificity of the Sortase-catalyzed transpeptidation reaction to develop a method to covalently anchor proteins to a solid support. The enzyme Sortase A (SrtA) from *Staphylococcus aureus* recognizes and cleaves the peptide bond between the threonine and glycine residue of an LPXTG motif, incorporated close to the C-terminus of a substrate⁵⁻⁷. Following sortase-mediated cleavage, SrtA and the substrate form a thioester intermediate, which is subsequently resolved by an oligoglycine nucleophile, resulting in the final transpeptidation product. Depending on the nature of the glycine derivative, this reaction may yield fluorescently labeled proteins, circularized proteins or proteins covalently linked by an intermolecular isopeptide bond⁸⁻¹³. Here, we used the transpeptidase reaction (also known as Sortagging) to covalently attach LPETGG-tagged antigens from *Influenza A* virus to a solid support, thereby creating a Sortagged-influenza protein array from unpurified cellular lysates. *Influenza virus* is the cause of major respiratory illness in human populations, where the viral hemagglutinin (HA) and neuraminidase (NA) proteins are the two most prominent antigens recognized by the host immune system¹⁴. Early studies have shown that neutralizing antibodies are mainly directed against the receptor binding domain of hemagglutinin¹⁵ (fragment HA1).

In addition, antibodies directed against the membrane-proximal and more conserved stem region of HA have also been described¹⁶⁻¹⁷. These antibodies have been shown to neutralize several subtypes among the virus clades which, in principle, could allow for a wide coverage.

Recombinant epitopes could be used for early identification of genetic variations among virus clades, and may therefore provide the basis for a universal flu vaccine¹⁷. From this perspective, it would be desirable to develop tools for generating protein arrays to screen and identify new viral epitopes. Moreover, a useful antigen chip with influenza virus proteins and/or fragments of HA and NA major antigens could be updated seasonally and used for yearly screening. At the moment, inactivated viruses, pseudoparticles, or proteins purified from virus isolates are used for testing immunogenic response¹⁸ and for vaccine development purposes¹⁸⁻²¹. Here, we developed a simple process to Sortag proteins to a glass slide derivatized with oligoglycine groups. We show an efficient alternative to existing methods that avoids protein purification steps and allows an high efficient immobilization reaction between the proteins of interest and the substrate.

EXPERIMENTAL PROCEDURES

Generation of an *Influenza virus* library in *E.coli* and cloning HA constructs for mammalian expression

cDNA fragments from *Influenza A virus* (A/WSN/1933/H1N1) genome were amplified and inserted into a bacterial expression vector pET28 b(+) LPETG (Novagen). PCR primers containing ± 20 bp of gene specific regions and adapter sequences were used (Table S1). The adapter sequence, which becomes incorporated into the termini flanking the amplified gene, are homologous to the outer ends of the linearized vector and ensure annealing and ligation of the PCR products into the expression vector, which is subsequently transformed in chemically competent TOP 10 cells (Invitrogen). Cloned genes contain at the C-terminus the following adapter sequence for ligation and sortagging: 5'-GAA GGA GAT ATA CAT ATG-3' and 5'-CGG TAG GCC GCC GCT GCC GCC GCC GCC-3'. cDNA fragments from *Influenza A virus* HA A/Vietnam/1194/2004 (H5N1); HA A/Panama/2007/1999 (H3N2); HA A/NewYork/396/2005 (H3N2); HA A/California/7/2009 (H1N1) were amplified and inserted into the eukaryotic expression vector pcDNA 3.1(+)-LPETG (Geneart) (Figure 1 SI). The constructs contain an in frame C-terminal Flag tag, an LPETG motif, followed by an HA tag and a His₆ tag. All cloned plasmids are sequence-verified (Table S2).

Expression and cell cultures

Sequence-confirmed plasmids were expressed in BL21 Codon Plus competent cells (Stratagene), grown in auto-induction media (15g/L glycerol, 30 g/L Yeast extract, 0,5 g/L MgSO₄, 20 g/L K₂HPO₄, 5 g/L KH₂PO₄) for 12–18 h, and harvested after overnight (o/n) growth²². SrtA and mCherry proteins were expressed and purified as previously reported¹⁰.

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (fetal bovine serum) and vitamins at 37 °C under humidified air containing 5% CO₂. All transfections were carried out using Polymer and Xfect (Clontech) using the instructions provided by the manufacturer.

SDS-PAGE and Western Blot analysis

SDS-PAGE analysis was performed using Nu-PAGE™ 4–12 % Bis-Tris gradient gel (Invitrogen) according to the manufacturer's instructions. Gels were stained with Colloidal Coomassie Blu G-250 (Invitrogen) or processed for Western Blot analysis using standard protocols. Polyclonal antibody specific for Influenza A – recognizing H1N1 and H3N2 and -Flu A strains (Millipore) and polyclonal antibody specific for PB1 and NS1 were used at a dilution of 1/1000. Secondary rabbit anti-goat IgG-HRP conjugated antibodies were used at a dilution of 1/10 000.

Chemically derivatized amine glass slides

Super amine slides were purchased from Arrayit (Arrayit, CA). BSA coated slides were fabricated following the literature procedure²³. Briefly, amine slides were treated with 100 mM *N,N*-Disuccinimidyl carbonate (DSC) and 100 mM *N,N*-Diisopropylethylamine (DIPEA) in a final volume of 400 ml anhydrous *N,N*-Dimethylformamide (DMF). Slides were agitated in this solution for 3h at room temperature. The slides were rinsed twice with 95% ethanol and then incubated in 400 ml of PBS pH 7.5 containing 1% BSA for 12 h at room temperature. The slides were rinsed twice with H₂O, twice with 95% ethanol and dried using a Microarray High-Speed Centrifuge (Arrayit, CA). The slides were then placed in a 2×8 multiwell cassette to provide discrete wells for further chemical derivatization. Attachment of Gly₃ to the slide surface was achieved by adding 100 µl of Gly₃ conjugation solution (100 mM Fmoc-Gly₃, 100 mM PyBOP, 100 mM HOBt, and 300 mM DIPEA in NMP) to each well. The reaction was quenched by replacing the Gly₃ conjugation solution with a solution of 100 mM ethanolamine and 300 mM DIPEA in NMP. Slides were then washed for 10 min in NMP and then rinsed in 20% piperidine NMP for 30 min. Following washing steps in NMP, ddH₂O and sortase buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1 mM CaCl₂), slides were dried by centrifugation and stored.

Transpeptidation reaction conditions

Bacterial pellets were solubilized in lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM CaCl₂, 0.5% NP40, 1 mM benzamidine HCl) and/or denaturing buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM imidazole, 0.5% Triton X-100, 0.3% SDS, 10% glycerol) and then sonicated. Protein concentrations were normalized by SDS PAGE analysis. HEK 293 cells were lysed by sonication in Nonidet P-40 containing buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM CaCl₂, 0.5% NP40, 1 mM benzamidine HCl) and cleared by centrifugation.

Sortase reaction on glass slides

To perform the sortase reaction on glass slides 100 µl of a solution containing sortase buffer (final concentrations: 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM CaCl₂, 5% milk) was supplemented with 0.5–3 µM of various mammalian and bacterial lysates and 50 µM

Sortase A. The mixture was applied to appropriate wells on the glass slides and the sortagging reaction was carried out at room temperature. Slides were washed three times in sortase buffer in the presence of detergents (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM CaCl₂, 0.5% NP40) and dried by centrifugation.

Serum screening probing and scanner analysis

Slides were blocked with 5% milk, 2% BSA, 1% Tween in PBS at 4°C for 4 h or o/n, and washed 3 times in 0.1% Tween PBS (PBST). The slide was incubated with a dilution of commercial *Influenza A virus* H1N1 serum (Millipore) and Influenza anti-A/New York/55/2004 HA, anti A/Vietnam/1194/04 HA, anti-A/California/7/2009(H1N1) (Nibsc) in blocking buffer for 1 h at 4°C. Slides were washed 3 times in PBST and probed for 1 h at 4°C with 1 µg/ml of Alexa-647-labelled anti-mouse or -goat secondary antibody (Invitrogen) in PBST. Washing steps in PBST and PBS were followed by a final step in ddH₂O. The slides were air dried after brief centrifugation and analyzed using a Tecan Power scannerTM. Intensities were quantified using Image J software. All signal intensities were corrected for spot-specific background. Protein concentrations in bacterial lysates in each well were normalized with respect to internal protein standards.

RESULTS

Design and expression of *Influenza A virus* protein antigens compatible with Sortagging

An *Influenza virus* cDNA library was cloned into a dedicated bacterial expression vector. Proteins were expressed in frame with the C-terminal LPETG motif, followed by a His₆ tag. The *Influenza A viral* genome contains eight single (non-paired) RNA strands encoding ten proteins with high antigenic variability that is relevant for applicative studies in vaccine research. The library represents each of ten genes present in the viral genome. In the case of Hemagglutinin (HA), two fragments were obtained, hereafter called HA chain A (1 to 342 aa) and HA chain B (343 to 566 aa). For Neuraminidase (NA), a fragment called NA/C₂ (141 to 273 aa), was cloned and expressed with a C-terminal Flag tag. Additional fragments were generated for HA and NA, however, because of their poor expression these were excluded from follow-up experiments (data not shown). The library was cloned and successfully expressed in BL21-Codon Plus cells using auto-induction medium (Figure 1). As further discussed below, the small LPETG motif at the C-terminus does not influence the expression or solubility of the recombinant proteins.

Chemical derivation of the solid support and Conjugation of Gly₃ peptide to solid support

Different types of chemically derivatized glass slides were tested to determine the optimal solid support for use as a Sortagging platform. Aldehyde, epoxy, and amine glass slides were treated (data not shown) and the latter was found to be best suited for Sortagging experiments.

Scheme 1 illustrates the steps used to build the Sortag-protein array. First, BSA coated slides were fabricated via DSC (*N,N'*-Disuccinimidyl carbonate) crosslinking following the reported procedure²³. An amide linkage was then formed between free BSA lysine residues and the C-terminus of a Fmoc-protected triglycine peptide (Gly₃) using PyBOP mediated coupling. Finally, Fmoc removal with piperidine unmasked the triglycine N-terminus, allowing it to function as a nucleophile in the sortase-mediated transpeptidation reaction.

As an initial demonstration of the efficiency of the slide modification procedure described above, a Gly₃ peptide with TMR attached at the N-terminus was conjugated to the slide to allow direct visualization of covalent linkage onto the solid support (Figure 2A). Next, the optimal concentration of Gly₃ nucleophile loaded onto the slide was determined for the

Sortagging reaction. Purified mCherry-LPETGG-His₆ protein was used as a model protein. The fluorescence of immobilized mCherry-LPETGG His₆ tag protein was observed to correlate with the initial concentration of Gly₃ used in the slide derivatization procedure in a linear fashion (Figure 2B). 100 mM of Gly₃ was chosen as the optimal concentration to use in the following experiments. Thus we were able to demonstrate that appropriately modified solid supports allow covalent attachment of proteins to a surface using Sortagging, and by altering the concentration of Gly₃, we can control and maximize the final transpeptidation yield on the activated slide. It should be also noted that sortase mediated transpeptidation has been used previously to attach proteins to other solid supports including beads and Biacore sensor chips^{24–26}.

Different experimental conditions do not affect the enzymatic activity of Sortase A

The purification of expressed proteins often represents a limiting step when building a protein array. Therefore, we sought to define the conditions that would allow us to avoid such steps. In previous studies, Sortagging was performed with high efficiency using purified starting materials, but sortase-mediated transpeptidation was also obtained in the presence of crude mammalian or bacterial cell extracts. The NP protein extracted from *E.coli* cells in both mild (A) NP40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.5% NP40) and harsh (B) SDS/Triton lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.5% SDS, 1% Triton) was briefly studied for its ability to participate in sortase mediated transpeptidation reactions. Crude bacterial extract was produced by sonication using (A) or (B) conditions (Figure 3). Lysates containing ~ 15 μM of LPETG-tagged substrate were incubated with 2 mM of the fluorescent TMR- Gly₃ peptide in the presence of 150 μM SrtA. Sortagging was detectable at 3 h and appeared complete after 7 hours. The presence of SDS in the reaction mixture did not affect the overall reaction conversion. The sortase reaction was additionally monitored by anti-His₆ immunoblotting. In the absence of SrtA, or at the initial time point of the reaction (T₀), the C-terminal His₆ tag of the LPETG-tagged substrate remained intact. At later time points, the His₆ tag was cleaved and replaced by the fluorescent probe, indicating that Sortagging had occurred (Figure 3C).

Next, we determined the optimal concentration of SrtA necessary for optimal Sortagging when using cell extracts from stringent or mild lysis conditions. Although the Sortag-labeling still takes place in the presence of SDS at all enzyme concentrations tested, the reaction kinetics are more favorable at higher concentrations of SrtA. In contrast, SrtA concentration had little effect on reaction progress in the presence of milder NP40 lysis conditions (Figure 2 SI).

Altogether, the sortase remains active in the presence of crude cellular extracts or in the presence of detergents, indicating that the purification of recombinant proteins can be bypassed when fabricating the desired protein arrays.

Normalization and validation of system for bacterial extracts

To further define the rules of the immobilization reaction on the solid support, the NA/C₂ fragment from our *Influenza* proteins library was used. Increasing concentrations of NA/C₂ protein (from 1.5 to 9 μM in crude extract) were incubated overnight with SrtA on the Gly₃ derivatized slide.

The Flag tag upstream of the LPETGG motif allowed us to detect conjugated NA/C₂. Following the immobilization procedure, the slide was probed with a FLAG antibody coupled to Alexa-647. This allows the conjugation efficiency to be measured, allows an estimation of the reproducibility of the system, and allows normalization of the data against

the non-conjugated fraction. In the presence of increasing concentrations of NA/C₂ the mean fluorescence intensity (FI) of the FLAG signal increased, while background staining remained relatively low (Figure 4A). A graphical representation of the FI attributable to immobilized NA/C₂ protein shows the activity of sortase during the reaction on the solid support (Figure 4A). In the absence of SrtA the FI remained low, indicating that non-specific binding of the substrate to the slide is minimal. Moreover, three independent experiments show similar FI, further demonstrating the reproducibility of the conjugation of NA/C₂ to the solid support (Figure 4B). We conclude that the sortase activity, either in solution or on the solid support, is not influenced by the presence of unpurified starting material (Figure 3 and 4), nor by the presence of SDS (Figure 2 SI).

Screening the *Influenza virus* library

Given the good immobilization profile obtained with the NA/C₂ protein, we expanded the Sortag-ligation to include other flu proteins. To generate the protein array, proteins were manually spotted on the Gly₃ surface at a concentration of 3 μ M in bacterial lysates. The array was incubated overnight with SrtA and then washed extensively to remove unbound material. Each well was probed with a commercial H1N1 anti-serum and visualized by probing with an Alexa-647 coupled anti-goat secondary antibody. NA/C₂ was additionally visualized by probing the well with anti-FLAG-647. After extensive washing, the array was scanned with a GenePix microarray scanner. The fluorescence intensity of each protein on the slide was normalized for background FI. As expected, Flag staining only revealed immobilized NA/C₂ in the presence of sortase. In the absence of SrtA no such staining was detected, indicating that immobilization is strictly dependent on the activity of SrtA (Figure 5A–B). The H1N1 anti-serum recognizes most flu proteins immobilized on the array, however NS2, PA, PB1 and PB2 were not detected. To verify that these proteins were successfully immobilized on the surface of the slide, we probed the slide with antibodies specific for NS1, and PB1. The FI obtained with these antibodies indicates that indeed these recombinant proteins were successfully conjugated to the glass slide (Figure 5C–D) but not detected by the H1N1 serum. SDS-PAGE gel and immunoblotting of the same proteins confirms that only some of the overexpressed antigens were recognized by the H1N1 serum (Figure 5E), with a partial overlap with the results obtained with the glass slide (compare Figure 5A and 5E). In addition, using antibodies specific to PB1, we confirmed that PB1 is efficiently expressed (immunoblot analysis, Figure 3SI) and immobilized onto the slide (figure 5C). A possible explanation for this result is that the proteins spotted on the array were solubilized using mild lysis conditions. In this experimental setting, a partial folding can be maintained by the protein, thus allowing the serum to recognize certain epitopes of the antigens. In contrast, sera will detect only linear epitopes in denatured proteins resolved by SDS-PAGE

Screening HA *Influenza* constructs expressed in eukaryotic cell shows specific interaction

Protein folding and post-translational modification of HA and NA may significantly affect their biological activity and immunogenicity. Therefore, we expressed four seasonal HA proteins in HEK 293 T cells: HA A/Vietnam/1194/2004 (H5N1), HA A/Panama/2007/1999 (H3N2), HA A/New York/396/2005 (H3N2), HA A/California/7/2009 (H1N1). Each of these HA variants were equipped with an N-terminal ER signal peptide and a C-terminal LPETG motif and their expression and post-translational modification were assessed in eukaryotic cells (Figure 6A). Glycanase, Sialidase A and O-Glycanase sensitivity and distinct migration patterns on an acrylamide gel demonstrate that the exogenously expressed proteins have acquired their typical post-translational modifications (Figure 4 SI), suggesting that they also acquire the correct fold. Next, cell extracts solubilized under mild lysis conditions were incubated overnight on Gly3 derivatized glass slides in the presence of SrtA. A cellular extract not expressing any of the HA variants served as negative control.

After incubation with SrtA, each well was probed with a specific neutralizing antibodies raised against influenza anti-A/New York/HA or the anti-A/Vietnam/1194/04 HA(21) (Figure 6B). The FI of each protein was normalized for background FI. The specificity of the two different sera rigorously matched the HA protein that was spotted on the array. The FI of each protein was normalized for background FI. The specificity of the two different sera rigorously matched the HA variant that was spotted on the array. In addition, when the array was interrogated using an Influenza serum able to recognize different Flu strains, we were able to show that all the HA proteins were immobilized on the chip (Figure 5 SI). As the specific anti-HA antibodies used here have neutralizing activity against intact viral particles²¹, they are likely to be conformation-specific, suggesting that in our assay the immobilized proteins at least partially retained proper folding.

DISCUSSION

In summary, we have developed a general strategy to build protein arrays using the specificity of the sortase transpeptidase reaction to attach proteins of different molecular size, structure and activity to a solid support. The immobilization method described here only requires the insertion of a small LPXTG motif at the C-terminus of the overexpressed protein, with minimal or no impact on the expression and folding of the substrate. The reaction takes place only in the presence of the enzyme SrtA, and results in the exclusive conjugation of the selected substrate to the pre-activated glass side, even in the presence of a complex cellular extract. As discussed above, proteins can be immobilized onto different kinds of solid supports by chemical reactions with aldehyde², epoxide²⁷, photo-crosslinking agents²⁸ or amine groups, but with no control over the reaction. Alternatively, GST, His₆, or biotinylated tags are often used in order to obtain a uniformly oriented protein array. Although successfully used, these approaches require purification of the engineered substrates before conjugation to the solid support. Sortase has an excellent enzymatic activity under a wide range of experimental conditions, enabling substrate extraction, labeling and covalent immobilization to occur simultaneously without intermittent purification steps. In addition, the specificity of the reaction allows anchoring of the recombinant protein to the solid support in a site-specific manner. In a previous study, the transpeptidase activity of SrtA was tested to link a purified model protein to a biacore chip²⁴. Here we show that different proteins can be successfully linked to a solid support and we are now able to control critical parameters, i.e concentration of Gly₃ and SrtA, which allow optimization of the reaction on the glass slide.

In a recent study, a proteome microarray expressing around 5000 candidate tumor antigens has been developed for the identification of novel autoantibody biomarkers in early detection of breast cancer. The following screening of sera has lead to the identification of 28 biomarkers from patients with heterogeneous breast cancers²⁹. Following this approach an entire bacterial, viral, or eukaryotic genome can be randomly (or rationally) cut in order to generate fragments that can be expressed in the most suitable expression system and immobilized via sortase onto solid supports for subsequent screening²³. To this end we successfully expressed antigens from *Influenza A* virus and selectively immobilized the recombinant proteins onto a glass slide. We confirmed that all expressed antigens are efficiently immobilized on the slide and that they are recognized by the respective serum. In this work, we used the small genome of *Influenza A* as a proof of concept to build a Sortag-protein array and we envision that such a protein chip can easily become an efficient means for the early detection of seasonal flu. Additionally, it can be scaled up to proteins of larger genomes in order to develop a protein array for high throughput screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Galli Grazia, Roberta Cozzi, Annemarte van der Veen, Carlo Zambonelli and Antonello Covacci for helpful discussion and critical review of the article. We are grateful to Barbara Capecci for providing us with HA constructs and specific antibodies. We thank Thomas DiCesare for assistance with the original art work.

ABBREVIATIONS

BSA	Bovine Serum Albumin
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMF	<i>N, N'</i> -Dimethylformamide
DSC	<i>N,N</i> -Disuccinimidyl carbonate
Fmoc	9-Fluorenylmethyloxycarbonyl
HOBT	1-Hydroxybenzotriazole
NMP	<i>N</i> -Methyl-2-pyrrolidone
MFI	Mean fluorescence intensity
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate

References

1. Zhu H, Snyder M. Protein chip technology. *Curr Opin Chem Biol.* 2003; 7:55–63. [PubMed: 12547427]
2. Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidlingmaier S, Houfek T, Mitchell T, Miller P, Dean RA, Gerstein M, Snyder M. Global analysis of protein activities using proteome chips. *Science.* 2001; 293:2101–5. [PubMed: 11474067]
3. Kindermann M, George N, Johnsson N, Johnsson K. Covalent and selective immobilization of fusion proteins. *J Am Chem Soc.* 2003; 125:7810–1. [PubMed: 12822993]
4. Camarero JA, Kwon Y, Coleman MA. Chemoselective attachment of biologically active proteins to surfaces by expressed protein ligation and its application for “protein chip” fabrication. *J Am Chem Soc.* 2004; 126:14730–1. [PubMed: 15535692]
5. Ton-That H, Mazmanian SK, Faull KF, Schneewind O. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH(2)-Gly(3) substrates. *J Biol Chem.* 2000; 275:9876–81. [PubMed: 10734144]
6. Ton-That H, Liu G, Mazmanian SK, Faull KF, Schneewind O. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc Natl Acad Sci U S A.* 1999; 96:12424–9. [PubMed: 10535938]
7. Kruger RG, Otvos B, Frankel BA, Bentley M, Dostal P, McCafferty DG. Analysis of the substrate specificity of the *Staphylococcus aureus* sortase transpeptidase SrtA. *Biochemistry.* 2004; 43:1541–51. [PubMed: 14769030]
8. Wu Z, Guo X, Wang Q, Swarts BM, Guo Z. Sortase A-catalyzed transpeptidation of glycosylphosphatidylinositol derivatives for chemoenzymatic synthesis of GPI-anchored proteins. *J Am Chem Soc.* 2010; 132:1567–71. [PubMed: 20078124]
9. Popp MW, Antos JM, Ploegh HL. Site-specific protein labeling via sortase-mediated transpeptidation. *Curr Protoc Protein Sci.* 2009; Chapter 15(Unit 15):3. [PubMed: 19365788]
10. Popp MW, Antos JM, Grotenbreg GM, Spooner E, Ploegh HL. Sortagging: a versatile method for protein labeling. *Nat Chem Biol.* 2007; 3:707–8. [PubMed: 17891153]

11. Antos JM, Popp MW, Ernst R, Chew GL, Spooner E, Ploegh HL. A straight path to circular proteins. *J Biol Chem.* 2009; 284:16028–36. [PubMed: 19359246]
12. Antos JM, Miller GM, Grotenbreg GM, Ploegh HL. Lipid modification of proteins through sortase-catalyzed transpeptidation. *J Am Chem Soc.* 2008; 130:16338–43. [PubMed: 18989959]
13. Antos JM, Chew GL, Guimaraes CP, Yoder NC, Grotenbreg GM, Popp MW, Ploegh HL. Site-specific N- and C-terminal labeling of a single polypeptide using sortases of different specificity. *J Am Chem Soc.* 2009; 131:10800–1. [PubMed: 19610623]
14. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Gargiullo PM, Brammer TL, Cox NJ, Tumpey TM, Katz JM. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med.* 2009; 361:1945–52. [PubMed: 19745214]
15. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem.* 2000; 69:531–69. [PubMed: 10966468]
16. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. *Science.* 2009; 324:246–51. [PubMed: 19251591]
17. Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, Najar TA, Lu X, Joyce JG, Shiver JW, Casimiro DR, ter Meulen J, Liang X, Varadarajan R. Design of an HA2-based *Escherichia coli* expressed influenza immunogen that protects mice from pathogenic challenge. *Proc Natl Acad Sci U S A.* 2010; 107:13701–6. [PubMed: 20615991]
18. Lu H, Khurana S, Verma N, Manischewitz J, King L, Beigel JH, Golding H. A rapid Flp-In system for expression of secreted H5N1 influenza hemagglutinin vaccine immunogen in mammalian cells. *PLoS One.* 2011; 6:e17297. [PubMed: 21386997]
19. Lin SC, Huang MH, Tsou PC, Huang LM, Chong P, Wu SC. Recombinant trimeric HA protein immunogenicity of H5N1 avian influenza viruses and their combined use with inactivated or adenovirus vaccines. *PLoS One.* 2011; 6:e20052. [PubMed: 21655326]
20. Audouy SA, van der Schaaf G, Hinrichs WL, Frijlink HW, Wilschut J, Huckriede A. Development of a dried influenza whole inactivated virus vaccine for pulmonary immunization. *Vaccine.* 2011; 29:4345–52. [PubMed: 21514345]
21. Alberini I, Del Tordello E, Fasolo A, Temperton NJ, Galli G, Gentile C, Montomoli E, Hilbert AK, Banzhoff A, Del Giudice G, Donnelly JJ, Rappuoli R, Capecchi B. Pseudoparticle neutralization is a reliable assay to measure immunity and cross-reactivity to H5N1 influenza viruses. *Vaccine.* 2009; 27:5998–6003. [PubMed: 19665606]
22. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif.* 2005; 41:207–34. [PubMed: 15915565]
23. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science.* 2000; 289:1760–3. [PubMed: 10976071]
24. Clow F, Fraser JD, Proft T. Immobilization of proteins to biacore sensor chips using *Staphylococcus aureus* sortase A. *Biotechnol Lett.* 2008; 30:1603–7. [PubMed: 18414796]
25. Chan L, Cross HF, She JK, Cavalli G, Martins HF, Neylon C. Covalent attachment of proteins to solid supports and surfaces via Sortase-mediated ligation. *PLoS One.* 2007; 2:e1164. [PubMed: 18000537]
26. Wu S, Proft T. The use of sortase-mediated ligation for the immobilisation of bacterial adhesins onto fluorescence-labelled microspheres: a novel approach to analyse bacterial adhesion to host cells. *Biotechnol Lett.* 2010; 32:1713–8. [PubMed: 20623314]
27. Zhu H, Klemic JF, Chang S, Bertone P, Casamayor A, Klemic KG, Smith D, Gerstein M, Reed MA, Snyder M. Analysis of yeast protein kinases using protein chips. *Nat Genet.* 2000; 26:283–9. [PubMed: 11062466]
28. Kanoh N, Honda K, Simizu S, Muroi M, Osada H. Photo-cross-linked small-molecule affinity matrix for facilitating forward and reverse chemical genetics. *Angew Chem Int Ed Engl.* 2005; 44:3559–62. [PubMed: 15880755]
29. Anderson KS, Sibani S, Wallstrom G, Qiu J, Mendoza EA, Raphael J, Hainsworth E, Montor WR, Wong J, Park JG, Lokko N, Logvinenko T, Ramachandran N, Godwin AK, Marks J, Engstrom P, Labaer J. Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer. *J Proteome Res.* 2011; 10:85–96. [PubMed: 20977275]

Figure 1. Expression of *Influenza A* virus protein array for Sortagging

SDS-Page analysis of bacterial lysates containing *Influenza* virus proteins stained with Coomassie Blue. The library represents each of 10 cDNA's encoding the viral genome (Lanes 1–11: HA chain A 38 kDa; HA chain B 24 kDa; NA/C₂ 15 kDa; NA/C₃ 9 kDa; M1 28 kDa; M2 26 kDa; NS1 30 kDa; NS2 25 kDa; PA 78 kDa; PB1 85 kDa; PB2 83 kDa; NP 55 kDa). Lane 13 represents purified SrtA protein.

Figure 2. Laser scan analysis of derivatized slides

A) The BSA-layer of a super amine glass slide was conjugated to a fluorescent peptide (TMR-Gly₃), allowing direct visualization of the coupling reaction with a microarray slide fluorescence scanner. B) The concentration of Gly₃ peptide was varied between 100 μM and 200 mM during derivatization to find the optimal nucleophile concentration required to facilitate the transpeptidation reaction. Subsequently, 25 μM of fluorescent purified protein mCherry-LPETG-His₆ was immobilized by Sortagging using 150 μM of SrtA in sortase reaction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM CaCl₂) on the Gly₃-modified glass slides and the immobilized mCherry was detected with a microarray slide fluorescence scanner.

Figure 4. Effect of different concentrations of cellular lysate on protein immobilization to Gly₃ glass slides

A) The lower part depicts direct visualization of immobilized NA/C₂ on a Gly₃ derivatized slide. Increasing concentrations of *Influenza* virus protein in crude extract of NA/C₂ ranging from 1.5 μM to 9 μM were incubated in the absence or presence of SrtA and detected by Anti-Flag 647 antibody. Upper part: graphical representation of the fluorescence intensity showing enzymatic activity. Data were analyzed using Image J software. B) Linearity and sensitivity of the protein array by experiments with lysates in triplicate. Lower part: a SDS-Page analysis of the different cellular protein concentrations used on in the Sortagging reaction.

Figure 5. Probing an immobilized library of *Influenza virus* proteins

A) 3 μ M of *Influenza virus* proteins in crude extract were incubated in the presence of 50 μ M Sortase A and detected by H1N1 serum. Graphical representation of fluorescence intensity of the constructed protein array shows some interaction with H1N1 serum. The total extract from *E. coli* cells expressing NA/C₂ Flag-tag was printed on the array in the presence or absence of Sortase A, verifying that immobilization of the proteins and subsequent detection with specific antibodies was sortase-dependent. B) Visualization of the detected immobilized proteins after fluorescence slide scanning. C–D) Graphical representation of fluorescence intensity of constructed protein array shows some interaction with specific antibodies against NS and PB proteins. E) SDS-Page analysis of bacterial lysates containing *Influenza virus* proteins from the library blotted against H1N1 antibodies (Lanes 1–12: HA chainA 38kDa; NA/C₂ 15 kDa; HA/A₁ 7 kDa; HA/A₃ 23 kDa; M1 28 kDa; M2 26 kDa; NS1 30 kDa; NS2 25 kDa; PA 78 kDa; PB1 85 kDa; PB2 83 kDa; NP 55 kDa).

Figure 6. Probing an immobilized HA proteins array produced in HEK293 T cells

A) HA tag immunoblot showing the expression of HA full length in HEK 293 T cells. The higher MW of bands is correlated to post translational glycosylation processes. B) 0.5 μ M of *Influenza* virus proteins in mammalian cell crude extract were incubated in the presence of 50 μ M Sortase A and detected by Influenza anti-H3N2/New York or anti-A/Vietnam/1194/04 HA sera produced in mouse and sheep and subsequently recognized by anti-mouse Alexa 555 and anti-sheep Alexa 647, respectively. Fluorescence intensity of constructed protein array shows the interaction of HA sera with its specific antigen.

Scheme 1. Sortagging immobilization of an expressed cDNA protein library

A) Super amine glass slides were derivatized with Gly₃ attached to a layer of bovine serum albumin (BSA). B) Bacterial lysates or eukaryotic expressing proteins containing the LPETG motif are incubated in separate wells in the presence of Sortase A. As a result, proteins are covalently linked to the slide. C) Sortagged conjugates are detected using specific anti sera. D) The slide is scanned to visualize protein interactions.