Total Chemical Synthesis of Proteins without HPLC Purification

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The lab of Professor Oliver Seitz at the Humboldt University in Berlin (Germany) has a long-standing interest in the development of methods which facilitate the synthetic access to proteins for biological studies. Professor Seitz said: "Our first idea was to simplify the synthesis by means of surface-based chemistry. We envisioned the use of fully synthetic protein arrays. Both the synthesis and the biological evaluation of the targeted proteins would proceed in an array format (just as we know it from peptide arrays, but now with folded proteins). We were foreseeing large-scale studies on the influence of post-translational modification on protein–protein interactions." He continued: "While we were working towards achieving this goal (see *Angew. Chem. Int. Ed.* **2016**, *55*, 7252; *J. Am. Chem. Soc.* **2010**, *132*, 11110; *Angew. Chem. Int. Ed.* **2007**, *46*, 4577) we noticed that there is actually an increasing interest in soluble synthetic proteins to guide the development of protein-based drugs. Most recently we learned that chemical protein synthesis may even be an alternative to recombinant synthesis."

According to the authors, the method presented in the *Chem. Sci.* paper has at least six distinct advantages over currently used methods. Firstly, there is the potential for parallelization, whereas traditional methods of protein synthesis rely on HPLC for purification. Professor Seitz explained: "While it is no problem to perform peptide synthesis in parallel, parallelization of HPLC purification is a technological challenge. Let us imagine the parallel synthesis of 100 proteins. The HPLC purification of 100 proteins would call for a sizeable investment into technical infrastructure such as several multiple column HPLC devices, or purification is performed subsequently at the expense of time investment. The method developed by us overcomes the need for HPLC purification. Rather, the instrumental set-up for the entire purification process is based on low-priced filter-equipped plastic syringes and two commercially available 'purification resins'."

Secondly, the presented method saves time. The authors explained that traditional protein synthesis methods require analysis and purification of both intermediary and final products (i.e. peptide fragments and ligation products) which is not only time-consuming but also requires human resources for the preparation of samples, analysis and subsequent lyophilization procedures. "Our approach bypasses the necessity for intermediary analysis. The purity of the intermediary products is not of concern because by-products will automatically be washed away at later stages," said Professor Seitz. Extended native chemical ligation and add-on removal of the ligation auxiliary are usually carried out in solution. However, the Seitz lab approach was different: "We performed both reactions on solid support. This enabled us to remove remaining peptide fragments, excess of reagents or exchange buffers within minutes by simple washing of the resin and consequently helped to further decrease the time required for the whole synthesis procedure," explained Professor Seitz.

The third advantage of this method is the reduction in the amount of waste products. "Considering the large amounts of organic solvents, the excess of protected amino acid building blocks and coupling activators wasted in the course of SPPS, chemical synthesis of peptides/proteins is anything but green. But the use of HPLC-based purification steps also contributes to an increased amount of toxic, in this case aqueous, waste," said Professor Seitz. He remarked: "We calculated that our approach produces approximately 60 times less waste than traditional synthesis with HPLC purification."

"Fourthly, this method has a potential for automation," said Professor Seitz, who explained that the synthesis of a full-length protein usually requires the use of automated SPPS to obtain protein fragments and ligation techniques for the subsequent conjugation of the protein fragments. The synthesis of the protein fragments is performed by using peptide synthesizers in an automated and parallel manner. "The bottleneck of a fully automated and parallel chemical protein synthesis is the necessity for intermediate HPLC purifications, analysis and lyophilization steps," said Professor Seitz, adding: "Our method bypasses these obstacles. The crude peptide fragments obtained after SPPS can be used directly for the HPLC-free purification and peptide ligation process. The stoichiometry or purity of the crude fragments is not important, as the final product will be obtained in high purity anyway."

A fifth advantage is chemoselectivity vs HPLC purification. "At lengths above approximately 50 amino acids it is often difficult to separate full-length peptides from truncation products," said Professor Seitz. He noted that this problem becomes worse as the length of the target peptide/protein increases. "At large sizes the HPLC elution properties of peptides almost seem to converge. As a result, the single peak observed in HPLC trace may suggest purity but actually many by-products may be hiding underneath that peak," explained Professor Seitz. He continued: "In this case, catch-and-release-based

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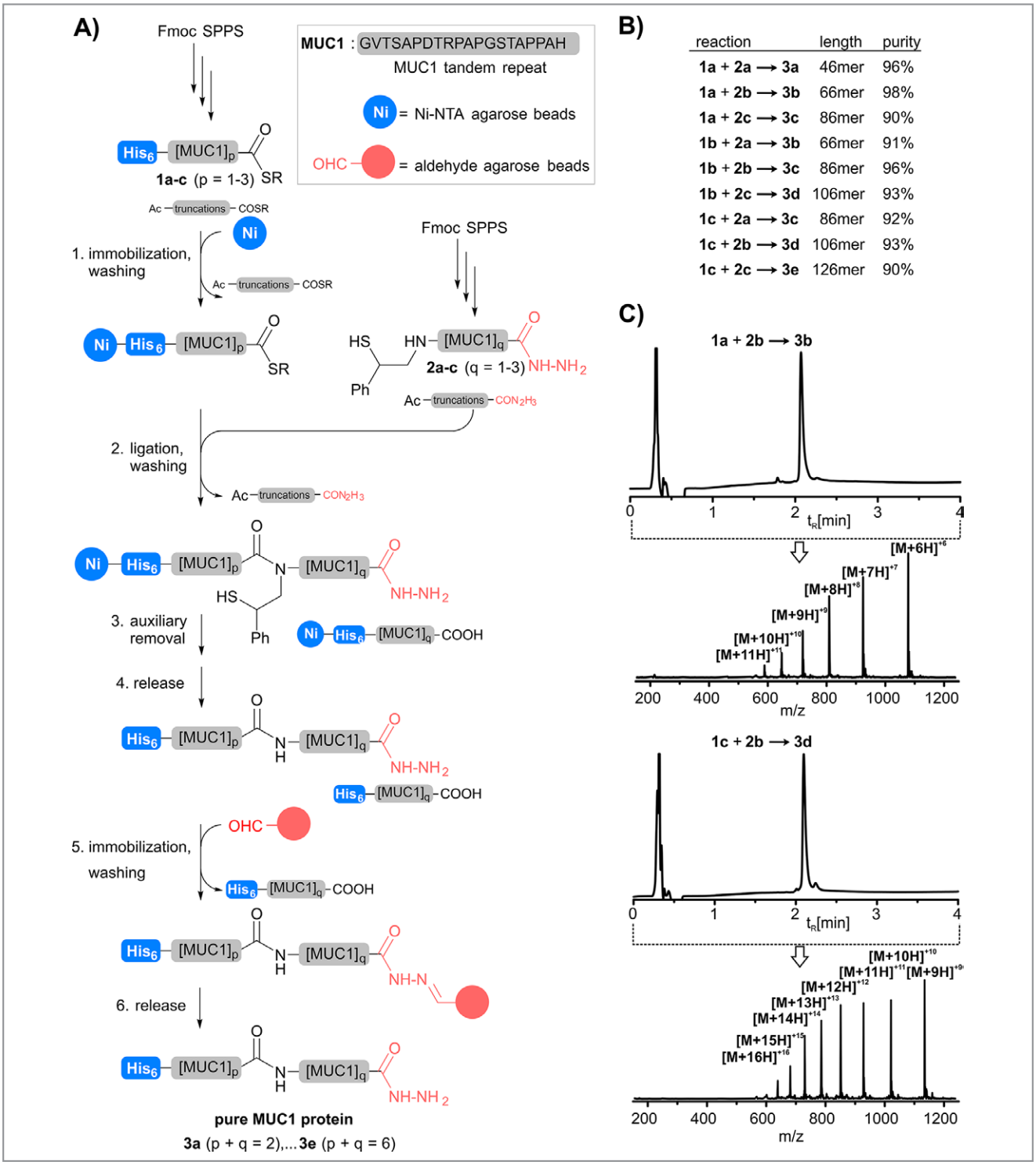


Figure 1 Chemical synthesis of MUC-1 proteins without HPLC purification: A) Non-chromatographic purification is achieved by immobilization reactions via the N-terminal His_6 tag (step 1) and the C-terminal hydrazide (step 5). Peptide thioesters **1a**–**c** and auxiliary-loaded peptides **2a**–**c** are conjugated (step 3) upon extended native chemical ligation which relies on the 2-mercapto-2-phenyl-ethyl auxiliary. The auxiliary is removed (step 4) under mild basic conditions. B) Purities of final MUC-1 proteins **3a**–**e** and C)–D) UPLC-MS analyses of products **3b** and **3d**.

purification methods are superior, because only the full length protein will carry the purification tag, but not the truncations (which may have equal polarity). This is illustrated in Figure 2."

Solubility is yet another advantage of this method. Chemical protein synthesis is frequently faced with the problem of dealing with sparingly soluble peptide fragments. "While the crude material obtained after SPPS may still have sufficient solubility, the solubility problem becomes pressing during HPLC purification," said Professor Seitz. He continued: "The use of large amounts of denaturing agents such as guanidinium hydrochloride (added also in the native chemical ligation step) is not an option for HPLC as this will cause column overloading or even column damage. It is an advantage that our method tolerates the use of guanidinium hydrochloride."

State-of-the-art purification of proteins or peptides is done by HPLC-based methods and provides a purity of the final product greater than 95% (typical high quality level offered from many peptide manufacturing companies). Professor Seitz remarked: "We were able to obtain similar purities (90–98%) by using our HPLC-free purification approach, yet, as mentioned before, our method appears to be faster and cheaper. The quality of the final products should be sufficient for biological studies to guide screening efforts. Compared to proteins from recombinant sources, we have better batch-to-batch repeatability."

However, the method does have a few limitations. "Our case study involved the synthesis of mucin proteins. We selected a comparatively easy-to-form His-Gly bond," said Professor Seitz. He continued: "Still, the on-resin native chemical ligation required 24 hours. Even then, the ligation was not complete. How will the solid-supported native chemical ligation proceed when more challenging ligation sites are targeted? This will be problematic. Yet there is an easy solution to this problem. Rather than immobilizing the N-terminal fragment prior to ligation, the ligation fragments may be mixed in solution phase prior to immobilization. Native chemical ligation proceeds much faster in solution. After the solution-phase ligation, the affinity capture resin will be added to selectively extract products that contain the full-length N-terminal fragment."

Professor Seitz concluded: "We foresee two types of application. In one scenario, the method will be used for the parallel synthesis of proteins for subsequent screening in biological and biomedical research. Here, the full potential of chemistry can be unraveled in the synthesis of protein modifications not accessible by biological methods. In the second scenario, the method will be used to facilitate production of proteins when recombinant methods may either not provide access to the targeted modification or produce undesired by-products with batch-to-batch variations."

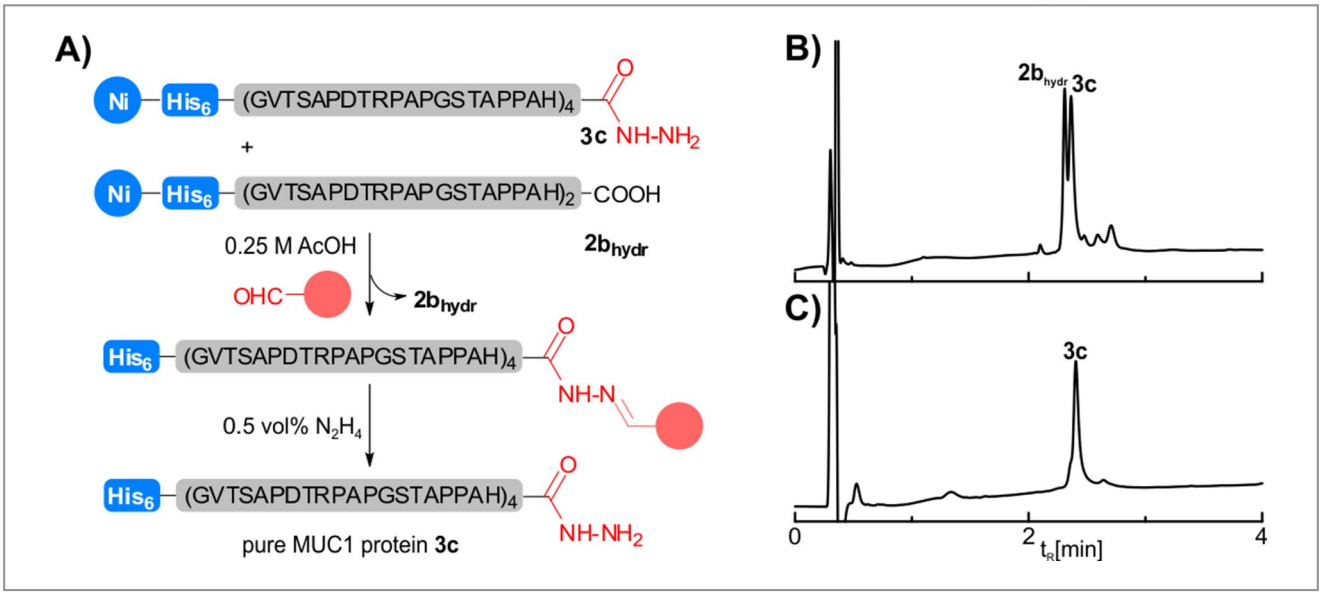
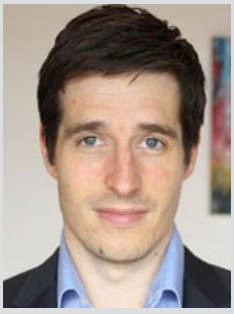


Figure 2 Reactivity-based purification as an alternative for difficult chromatographic purification: A) Chemoselective catch-and-release from aldehyde-agarose enables the separation of MUC1- protein **3c** from hydrolyzed peptide thioester $\mathbf{2}_{bhydr.}$ B)–C) UPLC analysis of the mixture of **3c** and $\mathbf{2}_{bhydr}$ and the purified MUC-1 protein **3c**.

About the authors

Prof. O. Seitz

Oliver Seitz was born in Frankfurt/Main (Germany) and studied chemistry at the Johannes-Gutenberg University Mainz (Germany). There he obtained his Ph.D. in organic chemistry with Horst Kunz. After postdoctoral research with Chi-Huey Wong at the Scripps Research Institute in La Jolla (USA) he moved to the Technical University Karlsruhe (Germany). He became group leader in the Department of Chemical Biology at the Max-Planck Institute for Molecular Physiology and obtained the *venia legendi* in organic chemistry from the Technical University Dortmund (Germany). In 2003, he was appointed Full Professor at the Humboldt University of Berlin (Germany). He is broadly interested in fashioning nucleic acid and protein molecules and their conjugates to enabling tools for the life sciences.



S. Loibl

Simon Loibl received his diploma (2011) in chemistry at the Humboldt University of Berlin (Germany) for his work with Oliver Seitz on LNA-enhanced FIT hybridization probes for DNA/RNA detection. The diploma thesis included an internship at the University of Southern Denmark where he worked in the group of Jesper Wengel. Simon is currently completing his Ph.D. work in the Seitz group. His thesis will describe the development of highly efficient N^{α}-auxiliaries for extended native chemical ligation.

Dr. R. Zitterbart

Robert Zitterbart studied chemistry at the Humboldt University of Berlin (Germany). For his diploma thesis, he worked in 2010 with K. P. C. Vollhardt at UC Berkeley (USA). In 2011, he started his Ph.D. work under the mentorship of Oliver Seitz on the chemical synthesis of protein domain arrays. After completion of his Ph.D. in 2016, he started a spin-off with two friends, which is based on a patent-pending technology for the HPLC-free purification of peptides. The start-up 'EnviroPep' was awarded with the first prize of Berlin's business plan competition (BPW) in 2015 and received funding in 2016 from the EXIST program financed by the Bundesministerium für Wirtschaft und Energie and the European Social Fund.

Dr. Z. Harpaz

Ziv Harpaz studied at Ben-Gurion University of the Negev (Israel) and completed B.Sc. and M.Sc. degrees in chemistry. In 2010, he joined the Seitz group at the Humboldt University in Berlin (Germany), where he was awarded his PhD in chemistry with specialization in bioorganic chemistry. He now works as a chemistry lecturer at Berlin Medical College (Germany).