Partially Shielded Enzymes Capable of Processing Large Protein Substrates

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We report the first method of enzyme protection enabling the production of partially shielded enzymes capable of processing substrates as large as proteins. We show that partially shielded sortase retains its transpeptidase activity and can perform bioconjugation reactions on antibodies. Moreover, a partially shielded trypsin is shown to outperform its soluble counterpart in terms of proteolytic kinetics. Remarkably, partial enzyme shielding results in a drastic increase in temporal stability of the enzyme.

Owing to their outstanding performances in terms of specificity and turnover rates, enzymes have found applications in a large range of industrial sectors. Indeed, the application spectrum of enzymes outstretches from low-added value applications (e.g., laundry detergent, food processing enzymes, chemical production, cosmetics) to extremely high value applications (e.g., enzyme for proteomics, biological therapies). One of the main stumbling blocks to the wider implementation of enzymes is the relative fragility of most of proteins under process conditions. This has triggered the interest of scientists to develop strategies for enzyme stabilisation. Approaching this challenge from a biochemical perspective, scientists have developed several protein engineering methods mainly through rational design or directed evolution. From a chemical perspective, enzyme immobilisation or encapsulation techniques have been demonstrated to reach high enzyme stability along with the possibility to retain and reuse the enzyme when operated under continuous flow conditions. It is, however, often found that support-immobilised enzymes produced by enzyme adsorption, entrapment or encapsulation suffer from enzyme leaching resulting in the loss of catalytic activity of the biocatalytic material and contamination of the resulting product. Enzyme immobilisation via covalent binding minimises this leaching and thus often appears to be the method of choice for immobilising enzymes. Since the early 1960’s, efforts have been made in designing robust carrier-bound immobilised enzymes with tailor-made chemical and physical properties including different shapes, sizes, porosities, aquaphilicities (i.e., the capacity of water-adsorption of the support material) and binding functionalities. Additional embedment of the enzyme in a protective matrix was also shown to enhance enzyme stability owing to a set of interactions established between the enzyme surface and the protective material. For example, Braun et al. reported the first successful enzyme trapping in a sol-gel matrix produced through the polycondensation of tetramethoxysilane. Although enzyme activity was decreased after its embedment, an improved enzyme thermal stability was observed. Reetz et al. described a method for producing efficient and stable lipase biocatalysts by embedding of the enzyme in hydrophobic sol-gel matrix. Yan et al. developed a method for trapping and protecting enzyme into DNA nanocages. Wang et al. recently achieved a biomimetic enzyme cascade reaction system through hydrogel-encapsulated enzymes. Liang et al. developed an enzyme protection strategy using metal-organic framework systems. In this context, we have reported a chemical strategy to covalently immobilise and fully shield enzymes in an organosilica layer at the surface of silica particles (SPs). This strategy allowed the enzyme to resist a large set of denaturing stress conditions while keeping its biocatalytic activity. However, fully embedded enzymes allow only small substrates to reach the enzyme active site by diffusion through the protective layer. It is remarkable that, in spite of the large amount of literature available for chemical encapsulation of enzymes, no method has been described involving enzymes having large substrates (e.g., proteins, nucleic acids, polymers) due to mass transfer issues.
We report herein a method allowing the immobilisation and partial shielding of enzymes on SPs in a thin organosilica layer. This partial shield was expected to enable enzyme stabilisation through a set of non-covalent interactions established between the protein surface and the protective organosilica shield, yet allowing access of large protein substrates to the enzyme active site for the biocatalytic reaction. We established a proof of concept by applying this method to two relevant and significantly different systems, namely sortase and trypsin enzymes, in order to demonstrate the versatility and efficacy of our method. On one hand, we took advantage of the transpeptidase activity of sortase for the continuous production of antibody-drug-conjugates (ADCs). On the other hand, we explored the proteolytic activity of partially shielded trypsin enzymes.

In this study, SPs were used as carriers to immobilise the selected enzymes (Fig. 1a). They were produced following a method reported previously. Partial amino-modification of the SPs surface was carried out in water using (3-aminopropyl)triethoxysilane (A) to yield SPs-NH₂. Here, we used a genetically engineered sortase A from S. aureus (SrtA) and a non-methylated recombinant porcine trypsin (Try). SrtA and Try were covalently anchored to SPs-NH₂ using glutaraldehyde as cross-linker to yield SPs-SrtA and SPs-Try, respectively. Protein quantification assays performed on the reaction supernatants showed immobilisation of 11.4 µg SrtA and 2.3 µg Try per mg of SPs-NH₂, corresponding to immobilisation yields of 81% and 84%, respectively (see ESI for details). Attempts to characterize the systems produced with AFM failed, certainly owing to protein drying and unfolding phenomena at the surface of the nanoparticles. In the next step, the growth of partial organosilica layers, aiming at protecting the enzyme yet maintaining accessibility to large protein substrates, was performed. SPs-SrtA-AT and SPs-Try-AT were obtained by reacting tetra-ethyl-orthosilicate (T) and A with SPs-SrtA and SPs-Try, respectively. SrtA is a monomeric and globular enzyme that can be approximated, based on the protein structural analysis (PDB code: 1IJA), to a sphere of 4.1 nm in diameter. We aimed at producing a protective layer thinner that the protein diameter not fully covering the enzyme and maintaining the active site accessible to large substrates. Sample aliquots were collected at increasing reaction durations and imaged by scanning electron microscopy (SEM). From the SEM micrographs (Fig. 1b), the surface of all SPs produced appeared fairly smooth with the exception of that measured after 4 hours of reaction where an increased surface roughness was observed. The layer growth kinetics, monitored by statistical analysis of scanning electron micrographs (Fig. 1c), revealed a non-linear growth kinetics with layers of 0.3, 0.7, 2.7 and 4.3 nm after reaction durations of 1, 2, 3 and 4 hours, respectively (Fig. 1c, Table S4). The polydispersity index remained mainly unchanged with values below 0.0014.

![Fig. 1](image-url) (a) Procedure of enzyme immobilisation; (b) SEM micrographs of bare SPs and SPs-SrtA-AT after 1, 2, 3 and 4 hours of organosilica layer growth reaction; (c) kinetics of layer growth over time measured on SEM micrographs; every point is the average of at least 100 SPs. Scale bars represent 200 nm.

![Fig. 2](image-url) (a) Enzyme kinetics of SPs-SrtA (○) and SPs-SrtA-AT after 1 (▲), 2 (●), 3 (♦) or 4 (■) hours reaction time with A and T; (b) Specific activity of SPs-SrtA (layer thickness = 0 nm) and SPs-SrtA-AT after 1 h, 2 h, 3 h and 4 h incubation with A and T.
In order to verify that the biocatalytic activity of the immobilised and partially shielded enzyme was maintained in the presence of the protective layer, we used an established reaction based on an artificial substrate. It consists in a carboxyfluorescein-based FRET pair (5'-FAM-OXL) linked to a LPXTG polypeptide chain. Cleavage of the LPXTG substrate causes a decrease of the FRET effect leading to an increase of fluorescence intensity ($\lambda_{ex}/\lambda_{em} = 490/520$ nm; Fig. 2a, Table S1).

It is also of note that after the layer growth reaction, the particles were allowed to “cure” at $20^\circ$C for 12 h; this curing reaction has been demonstrated to be beneficial for recovery of enzyme activity.\(^{14}\)

SrtA immobilisation led to a decrease of enzyme activity (Fig. S1, Fig. 2). The results presented in Table S1 showed the lowest activity for SPs-SrtA with an initial velocity value of $0.4$ nM.min\(^{-1}\). Interestingly, an increase of the SrtA activity was observed with the increase of the layer thickness, to reach a maximum of $4.2$ nM.min\(^{-1}\) for a layer thickness of 2.7 nm. This result confirmed that the presence of a thin organosilica shield does not hinder sortase A activity. For a layer of 4.3 nm, thicker than the enzyme diameter, a drastic drop in activity was measured at 2.9 nM.min\(^{-1}\). This result is consistent with our initial hypothesis that the protection layer thickness must not exceed the protein diameter. Moreover, this result suggests that the thickness of the thinnest layer required to protect the enzyme is 2.7 nm. Indeed, a thinner layer is not sufficient for stabilising the enzyme whereas a thicker layer hampers the enzyme active site accessibility. Since the artificial FRET substrate is, however, significantly smaller than the real target protein, it may diffuse in the protection layer explaining the residual activity measured with a layer of 4.3 nm.

We next tested the activities of the SPs produced on antibody substrates (Fig. 3a, Fig. S2). To do so, bioconjugation reactions of recombinant monoclonal antibodies (mAbs), C-terminally tagged with a sortase recognition motif, to penta-glycine-modified fluorescein isothiocyanate (Gly5-FITC) were performed using SPs-SrtA-AT with a layer of 2.7 nm and free SrtA; reaction products were analysed by SDS-PAGE. After image processing, bioconjugation efficiencies on heavy chain (HC) and light chain (LC) of both free and immobilised enzymes were compared (Fig. 3b and 3c).

The study of the bioconjugation efficiencies on the HC showed that for a reaction duration as short as 2 hours, the transpeptidation reaction is completed with both, SPs-SrtA-AT and free SrtA. In contrast, the kinetics of bioconjugation on the LC using SPs-SrtA-AT appears to be slightly slower than that obtained using free SrtA. This result can most likely be attributed to a lower accessibility of the LC conjugation site to the immobilised enzyme’s active site. However, it clearly demonstrates that despite the presence of the protective layer, a substrate as large as an antibody can be processed by immobilised and partially shielded enzymes.

In order to assess the stability of the partially shielded enzyme, SPs-SrtA-AT and free SrtA were freeze-dried, stored under different storage conditions ($-20^\circ$C, $4^\circ$C and $20^\circ$C) and tested after increasing storage duration (Table S2 & S3). The soluble SrtA did not show any remaining activity after only 3.5 months when stored at both $4^\circ$C and room temperature (Table S3) whereas SPs-SrtA-AT exhibited remarkable stability with no relevant loss of activity after 7 months at any of the tested storage conditions (Table S2). These results clearly confirmed...
the protective effect of the partial shield on the immobilised enzyme. Further, we explored the possibility of partial shielding with trypsin. SPs-Try-AT were prepared as described above. Trypsin is a dimeric enzyme with an estimated diameter of 5.5 nm based on protein structural analysis (PDB code: 1AVW). \(^{22}\) SPs-Try-AT were produced with a protective layer thinner than the protein diameter (3.4 nm; Fig. S4). In order to assess the digestion kinetics of SPs-Try-AT, we incubated the biocatalyst with a HEK cell lysate. The digestion efficiency was compared to that of free trypsin. Reaction products were analysed by SDS-PAGE/Comassie staining (Fig. S5). Remarkably, the immobilised trypsin digested the HEK cell proteome within only 2 hours, i.e. 3 times faster than its soluble counterpart. To identify the different fragments generated by tryptic digestion, LC-MS/MS analysis of the different tryptic HEK cell lysate digests were performed (Fig. 4). Fragment spectra interpretation was conducted in silico with \(^{8}\)MaxQuant and a transproteome search was done against a recent Swissprot human protein sequence database. The results showed that SPs-Try-AT allowed better identifications of protein group (Fig. 4a) and peptide forms (Fig. 4c) with peptide spectrum matches increasing from 40120 with soluble trypsin to 50881 for the immobilised one (Fig. 4b). Moreover, the use of SPs-Try-AT allowed us to significantly reduce the number of missed cleavages from 7% to 4% (Fig. 4d). This set of results confirmed that immobilisation and partial protection of trypsin allows outperforming soluble trypsin in terms of protein/peptide identifications and proteolysis kinetics.

In summary, we demonstrated that our method enables the production of highly stable partially shielded enzyme capable of either conjugating oligo-glycine-containing molecule to mAbs or highly specific protein hydrolysis. We expect our strategy to be applicable to a large range of enzymes. Our findings open the opportunity to produce nanobiocatalysts capable of processing large substrates such as proteins. These newly designed nanobiocatalysts can be implemented in different industrial applications such as the on-line enzyme-mediated manufacturing of ADCs or proteomics. The financial support from the Swiss commission for technology and innovation (CTI/KTI, grant agreement 16437.1 PFEN-NM) is gratefully acknowledged. We also acknowledge project funding by the European Commission, within the Horizon 2020 (PROLIFIC project, BBI-JU - grant agreement 790157).

Conflicts of interest
There are no conflicts to declare.

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