Efficient Site-Selective Immobilization of Aldehyde-Tagged Peptides and Proteins by Knoevenagel Ligation

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The aldehyde tag is appropriate to selectively label proteins, prepare antibody-drug conjugates or to immobilize enzymes or antibodies for biotechnological and medical applications. The cysteine within the consensus sequence CxPxR of the aldehyde tag is specifically oxidized by the formylglycine-generating enzyme (FGE) to the non-canonical and electrophilic amino acid C^{α}-formylglycine (FGly). Subsequent reductive amination is a common method for site-directed immobilization, which usually results in poor immobilization efficiency due to the reaction conditions. Here, we introduce a new solid support like agarose modified with an aryl substituted pyrazolone (Knoevenagel reagent) that was obtained in a facile and efficient 2-step synthesis. The modified agarose allowed the site-selective and efficient immobilization of aldehyde-containing small molecules, peptides and proteins – in particular enzymes – at physiological pH (6.2–8.2) without any additive or catalyst needed. In comparison to reductive amination, higher loadings and activities were achieved in various buffers at different concentrations and temperatures.

Introduction

Immobilized proteins play an important role in biochemistry, biotechnology and medicine, e.g. in biosensors, biomedical implants, or heterogenized biocatalysts.[1–3] For these applications immobilization needs to be efficient and stable while maintaining the protein functionality.

Three different methods are used for immobilization: entrapment (**I**), cross-linking (**II**) and binding to a solid support (**III**).[4–6] In **I**, the proteins are entrapped in a membrane device or an organic/inorganic polymer network (e.g. polyacrylamide or silica sol-gel). Potential leaching of the protein has to be avoided. Thus, covalent linkages are often required. For **II**, carrier-free particles in the form of aggregates (cross-linked enzyme aggregates, CLEAs)[7] or crystals (cross-linked enzyme crystals, CLECs)[8] are generated by using bifunctional reagents, such as glutaraldehyde, disuccinimidyl suberate (DSS) or divinyl sulfone (DVS). Especially with regard to biocatalysts, this technique delivers a cost-effective and stable immobilisate with high enzyme density.

For the third method (**III**), binding can occur either via physical (hydrophobic, van der Waals or ionic) interaction/absorption (e.g. affinity tags like cellulose-binding domain[9] or silica-binding tag[10]), or covalent linkages.[5] In the latter case, the side-chain functionalities of natural amino acids like cysteine or lysine are usually used. The primary amino group of lysine side chains or the N-terminus exposed to the surface can react under slightly basic conditions in a nucleophilic substitution with NHS-, CDI-, azlactone- or epoxy-activated resins as well as with aldehydes in a reductive amination. In the latter case, reductive amination with sodium cyanoborohydride can be accomplished in a single reaction step at pH 7.2. However, imine formation is most efficient at pH <5 or 9–10, while the pH optimum for the reduction step is at pH 7.2. Conjugation across cysteine thiols offers a slightly higher selectivity compared to the more abundant lysines. However, cysteines are often present in proteins in their oxidized state, forming disulfides that stabilize secondary and tertiary structures. In order to enable coupling reactions of these cysteine residues, they need to be reduced first, which could be potentially harmful for the protein, or additional unpaired cysteine residues on the protein surface need to be inserted by mutagenesis. The free thiol can react in a nucleophilic substitution/addition with activated disulfides, Michael systems (like maleimides) or iodoacetyl-derivatives. Unfortunately, problems with selectivity and stability as well as loss of activity can occur in coupling via lysine and cysteine.[3]

Over the past few decades, researchers have focused on bioorthogonal methods for site-specific protein immobilization, as a more stable and robust alternative to the aforementioned,

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rather unspecific techniques. One of these alternatives are self-labelling protein tags, like the SNAP-tag (20 kDa),[11] CLIP-tag (20 kDa)[12] and Halo-tag (33 kDa),[13] which react selectively with O^6-benzylguanine, O^2-benzylcytosine and alkyl halide substrates, respectively.

In addition to these tags, enzymatic approaches have been developed, with the enzyme-mediated immobilization using sortase A being a prominent example. This enzyme is a transpeptidase from the gram-positive bacterium *Staphylococcus aureus*, which recognizes the C-terminal consensus sequence LPXTG and transfers a pentaglycine derivative onto the C-terminus. Unfortunately, this reaction is a dynamic equilibrium, resulting in low immobilization rates. However, the efficiency could be significantly improved by directed evolution and substrate design.[14–17]

Other bioorthogonal methods include reactions with unique aldehydes. Glycoproteins and N-terminal serine or threonine residues can be oxidized by treatment with sodium metaperiodate.[18]

Alternatively, an N-terminal glycine can be converted to an α-oxo amide by transamination with pyridoxal-5′-phosphate (PLP).[19] In addition, the formylglycine-generating enzyme (FGE) can be used *in vivo* or *in vitro* to selectively oxidize a cysteine within the consensus sequence CxPxR (aldehyde tag) to C^{α}-formylglycine (FGly).[20–25]

Aldehydes introduced by one of these methods can be addressed by nucleophiles like amines,[26–29] hydroxylamines,[30–32] hydrazines[33–35] or carbon nucleophiles[36–39] under slightly acidic to neutral conditions, forming an imine (pH < 5), oxime (pH < 5), hydrazone (pH 5–6) or C–C double bond (pH 6–7.2) (Scheme 1). Although oximes and hydrazones have higher stabilities compared to imines, reduction with $NaBH_3CN$ is still required for full stability.[3,39] Physiological pH values and better immobilization efficiencies (relative to reductive amination) can be achieved by adding aniline as a nucleophilic catalyst, although it is potentially carcinogenic.[40]

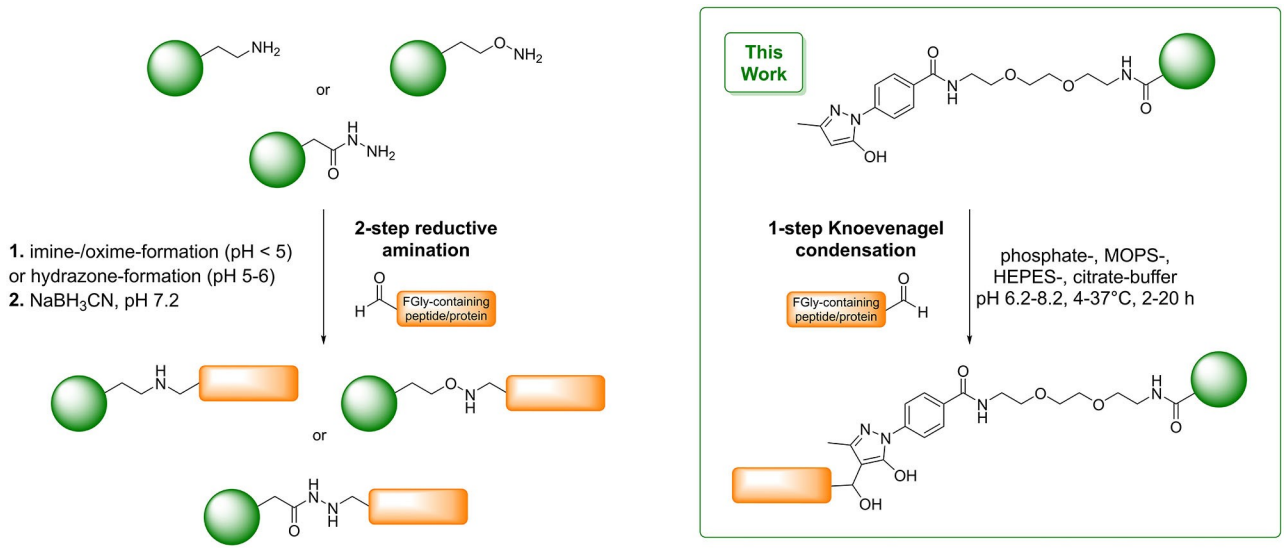
Results and Discussion

In order to selectively address unique aldehydes in ligands, peptides or proteins (enzymes) while overcoming the disadvantages of the well-known reductive amination, a straightforward synthesis of alkyl and aryl-substituted and amine-functionalized Knoevenagel reagents (**4** and **7**) was established in this work. These reagents were subsequently coupled to NHS-activated dry agarose (**8**) and used for site-selective immobilization of aldehyde-containing small molecules, peptides, and proteins under physiological conditions (Scheme 1).

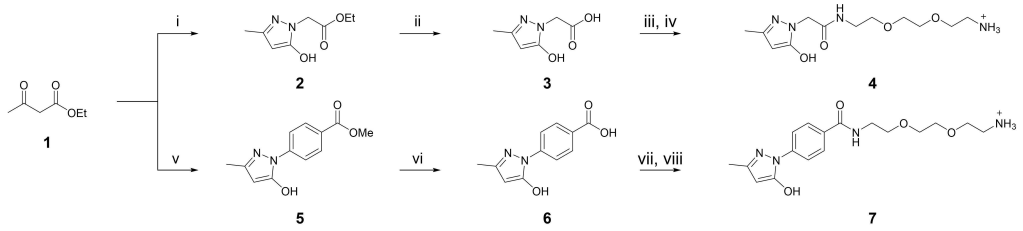
Synthesis of Knoevenagel ligation reagents

The Knoevenagel core segments **3** and **6**, which are also commercially available, were synthesized as described in the literature.[36,38] Problems could be observed especially in the synthesis of **3** after deprotection by LiOH and acidic work-up. Due to the high polarity, the final separation of LiCl was only possible by multiple desalting steps with C18 silica. Subsequently, the amine-functionalized compounds **4** and **7** were obtained by coupling of Boc-PEG_2-NH_2 with EDC·HCl and HOAt, followed by purification and final deprotection using TFA in DCM or HCl in dioxane. Both compounds were used without further purification after evaporation of the deprotection reagent under reduced pressure and lyophilization.

The amine-functionalized Knoevenagel reagents **4** and **7** were prepared on large scale in a 2-step synthesis and were obtained in good to very good yields (61% and 81%, resp.) making this synthesis suitable for large-scale applications (Scheme 2).



Scheme 1. Development of a site-selective, bio-compatible, and efficient immobilization technique using pyrazolone-functionalized agarose.



Scheme 2. Synthesis of alkyl or aryl substituted and amine-functionalized pyrazolones for immobilization on NHS-activated agarose. i) Ethyl hydrazinoacetate hydrochloride, NaOAc, EtOH, reflux, 24 h. ii) LiOH, H_2O, rt, 18 h, 63% after two steps. iii) HOAt, Boc-PEG_2-NHBoc, DIPEA, EDC·HCl, DMF, rt, 24 h, 60.6%. iv) 4 N HCl, DCM, rt, 2.5 h, quant. v) Methyl 4-hydrazino-benzoate, NaOAc, AcOH, 75 °C, 3 h, 57%. vi) 1 N NaOH, rt, 3.5 h, 93%. vii) HOAt, Boc-PEG_2-NHBoc, DIPEA, EDC·HCl, DMF, rt, 16 h, 81.4%. viii) TFA:DCM (2:1), rt, 2 h, quant.

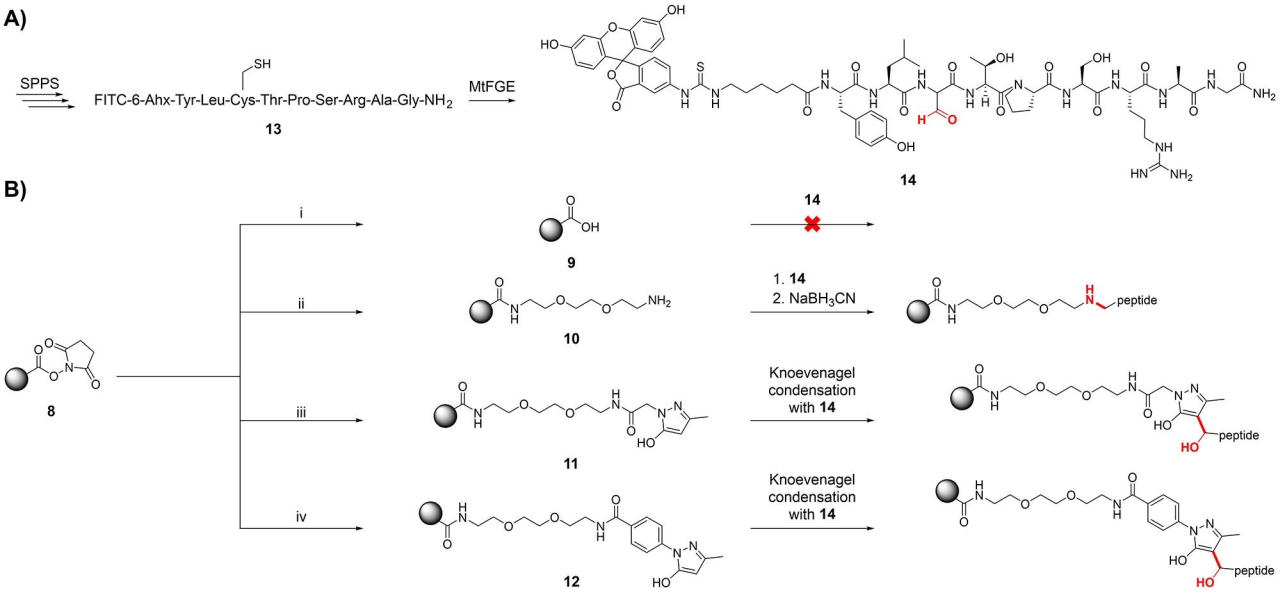
Preparation of agarose

After successful synthesis of the bifunctional Knoevenagel reagents **4** and **7**, the acid- (negative control) and amine-functionalized resins **9** and **10** as well as the pyrazolone-activated resins **11** and **12** were prepared from NHS-activated dry agarose (**8**), which allows identical loadings for later comparisons (Scheme 3B).

The NHS-activated agarose was hydrolyzed with 1 M aqueous NaOH solution providing the acid-functionalized resin **9**. The amine-functionalized resin **10** was prepared using a 1 M solution of H_2N-PEG_2-NH_2 (pH 7.2). High concentrations of the linker were used to suppress double substitution, which would have resulted in lower loading. The bi-functionalized Knoevenagel building blocks **4** and **7** were applied in large excess (ca. 250 eq.) for coupling to the NHS-activated agarose under near-neutral conditions (100 mM phosphate, 150 mM NaCl, pH 7.2) resulting after washing and freeze-drying in orange- to red-colored beads. Excess reagent could be recovered (see Supporting Information for details).

Synthesis of aldehyde-containing fluorophors

We synthesized various aldehyde-containing fluorophors to investigate the immobilization efficiency of the modified agarose beads. The extent of immobilization was determined by the fluorescence decrease in the supernatant. 1-Formyl fluorescein (**15**) was prepared according to the literature by Reimer-Tiemann-formylation. A CxPxR-containing fluorescent peptide (FITC-6-Ahx-Tyr-Leu-Cys-Thr-Pro-Ser-Arg-Ala-Gly-NH_2) (**13**) was synthesized using automated microwave-assisted solid phase peptide synthesis (SPPS). Subsequent enzymatic oxidation of the cysteine to the non-canonical amino acid C^{α}-



Scheme 3. A) Synthesis of FITC-6-Ahx-Tyr-Leu-(FGly)-Thr-Pro-Ser-Arg-Ala-Gly-NH_2 as a test substrate for immobilization. 1. SPPS with TBTU, DIPEA, DMF. Fmoc-cleavage: 20% piperidine in DMF + 0.1% HOBt. 2. MtFGE, bicine-buffer (50 mM bicine, 66.6 mM NaCl, 200 mM arginine, pH 9.0), DTT, $CuSO_4$, 2.5 h, 37 °C, 81.5% over two steps. **B)** Preparation of acid- (negative control), amine- (reference beads) and both pyrazolone-functionalized agarose beads, and subsequent immobilization of **14** via reductive amination or Knoevenagel condensation. i) 1 N NaOH, rt, 30 min. ii) 1 M H_2N-PEG_2-NH_2, pH 7.2, rt, o.n. iii) 12.5 mM **4**, 100 mM phosphate, 150 mM NaCl, pH 7.2, rt, o.n. iv) 12.5 mM **7**, 100 mM phosphate, 150 mM NaCl, pH 7.2, rt, o.n.

formylglycine (FGly) was performed with FGE from *Mycobacterium tuberculosis* (MtFGE) in a bicine buffer containing $CuSO_4$ and DTT upon incubation for 2.5 h at 37 °C. Copper was added for *in situ* reconstitution, which lead to elevated enzyme activity. The FGly-containing peptide **14** was obtained after full conversion and preparative RP-HPLC with an overall yield after 2 steps of 43% (Scheme 3A).

Superior immobilization efficiency under near-physiological conditions with aryl-substituted pyrazolone-functionalized agarose

For immobilization, we first treated the previously prepared beads (**9**, **10**, **11** and **12**) with a 50 μM solution of the FGly-containing peptide **14** (5 mg beads per mL). The suspensions were incubated overnight at 25 °C under near-physiological conditions (100 mM phosphate, 150 mM NaCl, pH 7.2). The imine formed with **10** required an additional reduction step with $NaBH_3CN$ (Scheme 3B and Figure 1A). The remaining fluorescence of the supernatant was then measured in a Tecan reader (λ_{exc} 490 nm, λ_{em} 525 nm). The acid-functionalized beads (**9**) as the negative control did not react with **14**, while 75% (with $NaBH_3CN$) and 77% (without $NaBH_3CN$) of the peptide was immobilized on the amine-functionalized beads (**10**). As already expected from the literature, higher immobilization yields were obtained with the pyrazolone beads **11** (92%) and **12** (98%) (Figure 1A). In order to get a better insight into the kinetics of the amine- and pyrazolone-functionalized beads (**10**-**12**), these immobilization experiments were repeated under the same conditions and samples were taken over 3 hours (every 30 min). A slow linear immobilization of **14** was observed for the amine-beads (**10**). Only 30% of the peptide was immobilized after 3 h. In contrast, a rapid immobilization was detected for both pyrazolone beads, with the aryl-substituted pyrazolone beads reacting slightly faster. The maximum immobilization yield of 96% was observed for **12** after only 2 h, while the alkyl-substituted pyrazolone beads (**11**) showed an immobilization yield of only 84.2% after 3 hours (Figure 1B).

In summary, the degree of immobilization of the FGly-containing peptide could be improved by using alkyl- (**11**) and aryl-substituted (**12**) pyrazolone beads. In particular, resin **12** allowed the highest degree of immobilization (14.2 mg peptide **14** per gram resin) by Knoevenagel reaction (Table S1) under near-physiological conditions without the need of using a potentially harmful reducing agent or additive (Figure 1A and 1B).

Further increase in immobilization efficiency enables the immobilization of proteins

In order to further increase the immobilization efficiency of **12**, the stoichiometry of beads versus peptide was reduced (2 mg

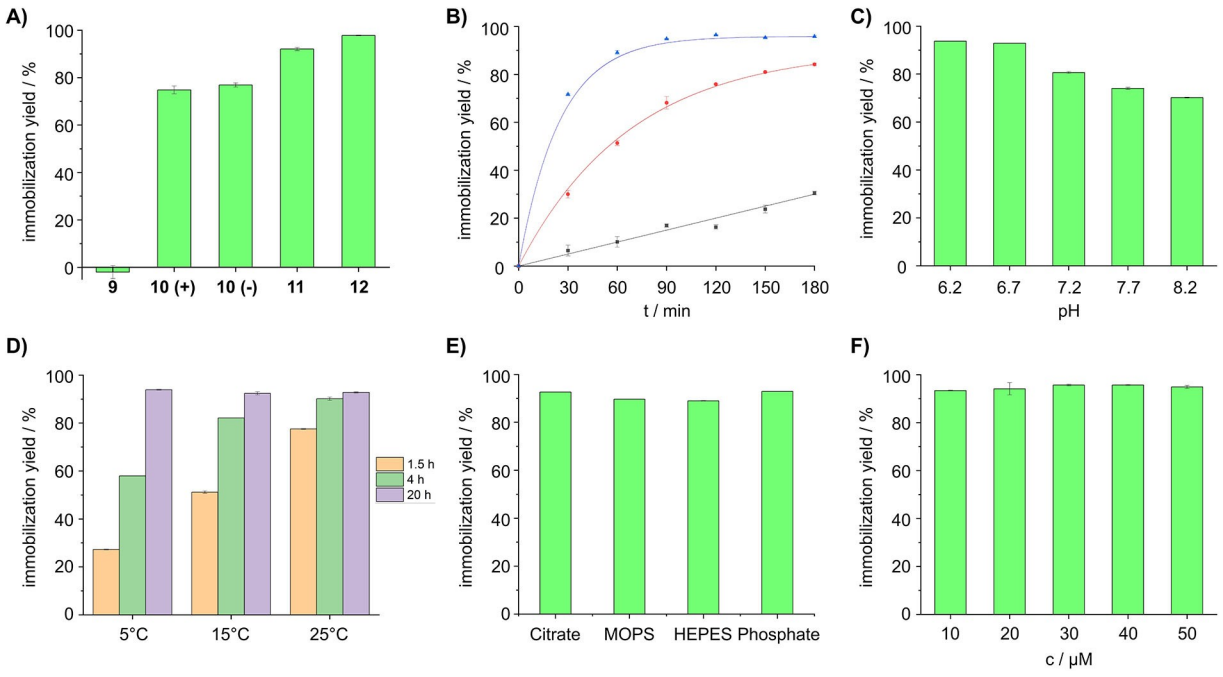


Figure 1. Immobilization screening with the FGly-containing peptide **14**. Residual fluorescence in the supernatant was measured to determine the degree of immobilization. Samples were measured in duplicates. **A)** Immobilization levels after 20 h at 25 °C with **9**–**12** in phosphate buffer (100 mM phosphate, 150 mM NaCl, pH 7.2). The fluorescence of **10** was measured after treatment with (+) and without (−) $NaBH_3CN$. **B)** Kinetic measurements for **10**–**12** (black: **10**, red: **11**, blue: **12**) in phosphate buffer described before. Fluorescence was measured every 30 minutes over a total period of 3 hours. **C)** Degree of immobilization after 20 h at 25 °C with **12** in phosphate buffer at different pH (6.2, 6.7, 7.2, 7.7 and 8.2). **D)** Measurement of immobilization levels for **12** in phosphate buffer (pH 6.7) after 1.5 h, 4 h and 20 h at 5 °C, 15 °C and 25 °C. **E)** Immobilization levels after 20 h at 25 °C with **12** in different buffers (Citrate, MOPS, HEPES, phosphate). **F)** Degree of immobilization with **12** after 20 hours at 25 °C in phosphate buffer (pH 6.7) with different concentrations of peptide **14** (10-50 μM).

beads per mL of a 50 μM fluorophor solution) and a pH screening was performed (Figure 1C). Compared to the previous experiments, a lower degree of immobilization was obtained at higher pH (7.7 and 8.2). However, immobilization efficiency comparable to the amine functionalized beads (for pH 7.2) could still be observed at pH 8.2. Interestingly, immobilization improved by reducing the pH value from 7.2 to 6.7 (81% vs 93%). At even lower pH (6.2), no significant increase in immobilization could be obtained (94%).

This result is consistent with observations in the literature and is probably related to the protonation of the aldehyde. Further experiments were performed at pH 6.7 and the dependence of the degree of immobilization on temperature, buffer and concentration was tested (Figure 1D–F). As expected, slower kinetics was observed at lower temperatures (5 °C and 15 °C). However, all experiments reached about the same final immobilization yield (93–94%) after 20 h (Figure 1D). Furthermore, the compatibility of different buffers (citrate, MOPS, HEPES, phosphate) with Knoevenagel chemistry was tested. Among all tested buffers, no remarkedly differences in the immobilization yield was observed. Slightly better results were found for phosphate (93%) and citrate (93%) compared to MOPS (90%) and HEPES (90%) (Figure 1E). Finally, immobilization was tested with lower concentrations (10-50 μM) of the peptide. In all cases, almost the same immobilization yield (between 93% and 96%) could be obtained after 20 h (Figure 1F).

Furthermore, immobilization experiments with 1-formyl fluorescein (**15**) led to similar results, with the best immobilization levels being achieved with the aryl-substituted pyrazolone beads **12** (Figure S17).

Overall, the optimized reaction conditions resulted in immobilization of about 34.8 mg of peptide per gram beads (Table S1), which corresponds to an improvement by a factor of about 2.5 (compared to the initial yield of 14.2 mg). Notably, the beads could be stored for several months at −20 °C without losing activity, underlining, on top of the superior immobilization properties, the applicability of this technique. Moreover, it is conceivable that this resin is also stable at 4 °C or rt due to the general stability of pyrazolones. In case of an industrial application, this would have to be investigated in more detail.

FGE as a biocatalyst for the generation of aldehydes in proteins

Two approaches are possible to introduce an aldehyde moiety using the FGly tag methodology: either the co-expression with FGE, resulting in an *in vivo* conversion of the cysteine residue to C^{α}-formylglycine, or an *in vitro* conversion by incubating the purified tagged protein with purified FGE.

For the latter case, human FGE and bacterial MtFGE were expressed and purified. The MtFGE used was labelled with the aldehyde tag sequence CTPSR so that an FGE immobilisate could be tested for conversion in addition to the soluble enzyme. This would avoid contamination by the enzyme (Figure 2A). Since the recombinant CTPSR-tagged MtFGE was able to convert its own tag *in vivo*, the purified protein already contained the C^{α}-formylglycine residue (Figure S20). First immobilization experiments were unsuccessful as most of the protein precipitated during incubation at 37 °C, which is due to the instability of non-reconstituted FGE at slightly acidic pH (pH 6.7)[38] close to the isoelectric point of the protein (pI_{calc}: 6.33). Recently, FGE activity and stability has been demonstrated to be strictly dependent on a copper cofactor and that overexpression leads to insufficient copper loading.[41] Therefore, copper reconstitution was performed, which led to a greatly increased loading (0.01 to 0.88 mol Cu/FGE) as measured by ICP-MS. This resulted in a significant increase in stability, as the protein was much less prone to precipitation.

Subsequent immobilization experiments on **12** with optimized immobilization conditions yielded an active immobilisate (Figure S21). It has to be noted that an active immobilisate was only obtained in the absence of EDTA, possibly because EDTA would chelate the crucial copper cofactor.

Purification and FGly-conversion of transaminase

The suitability of our novel immobilization technique for protein immobilization was examined using the (*S*)-selective transaminase of *Vibrio fluvialis* (VfTA). For this purpose, the aldehyde tag sequence CTPSR was introduced C-terminally between the transaminase sequence and His_6-tag, generating VfTA-CTPSR-His_6. Optimized expression and purification of VfTA-CTPSR-His_6 yielded in 15–20 mg protein per gram of wet cell pellet (Figure S18).

In case of the transaminase, *in vivo* conversion experiments with MtFGE were not successful (data not shown). Therefore, the conversion was performed after expression and purification with three different FGE variants: soluble human FGE, soluble MtFGE and immobilized MtFGE (Scheme 2B). All resulted in quantitative FGly content, as shown by subsequent labeling with the aldehyde-reactive fluorophor **16**[38] (Figure 2B–2D). In this respect it is worth mentioning that the use of the immobilized FGE did not lead to contamination of the transaminase (Scheme 2D).

Site-selective immobilization of transaminase

After successful site-specific introduction of an unique aldehyde moiety, immobilization experiments with our newly established pyrazolone beads (**12**) were performed. The first step comprised screening of the optimal immobilization temperature. For this, 100 μg of transaminase (40 μM solution) per mg beads were incubated for 24 hours at different temperatures. Pyruvate (2.5 mM) and *rac*-1-phenylethylamine (PEA) (2.5 mM) were used as substrates in the activity measurements of the immobilized transaminase (Figure 3A). The highest activity could be measured after immobilization at 37 °C, while a considerably lower relative activity of 27% was achieved after immobilization at 22 °C, and only 3.7% after immobilization at 4 °C, demonstrating

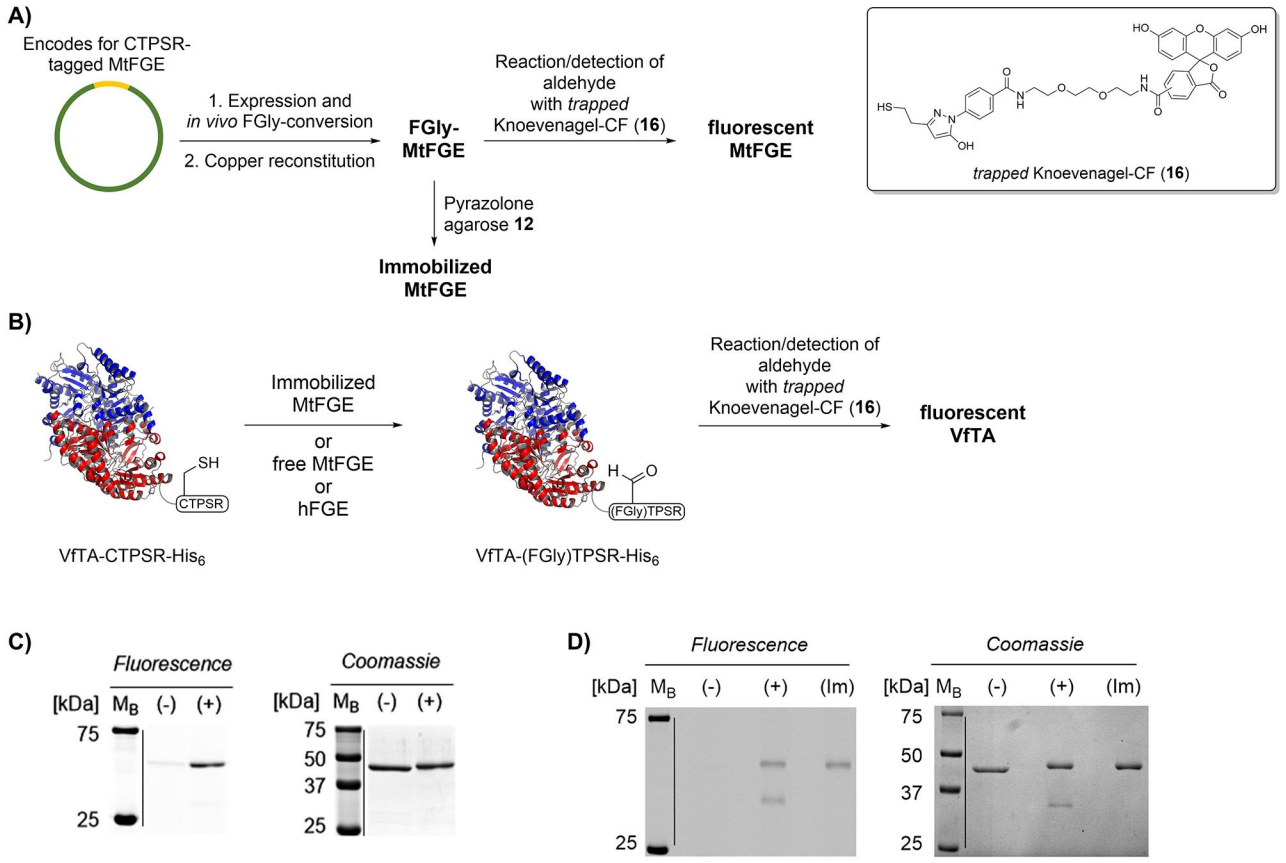


Figure 2. A) Expression, *in vivo* FGly-formation and copper reconstitution of MtFGE. Afterwards, the FGly-content was determined by coupling to a fluorescent dye (*trapped* Knoevenagel-CF; **16**)[28] or to optimized pyrazolone beads (**12**). **B)** Schematic figure of transaminase being converted by different FGEs and subsequently fluorescent labeling. **C)** Fluorescence labelling of purified VfTA-CTPSR-His_6 before (−) and after conversion with human FGE (+) with *trapped* Knoevenagel-CF (**16**). Transaminase (5 μg) in phosphate buffer (100 mM phosphate, 150 mM NaCl, pH 6.7) was incubated with 1 mM **16** overnight at 37 °C, followed by 12.5 % SDS-PAGE analysis with a 250 kDa protein ladder with 25 kDa and 75 kDa fluorescent bands. In-gel fluorescence was detected at 515 nm, followed by Coomassie staining. A slightly shifted distinct fluorescent band was detected for the treated protein, indicating a quantitative conversion of the aldehyde tag. MW markers and samples are from the same SDS gel, but only relevant samples are shown. Full SDS gels and fluorescence images can be found in figure S19. **D)** Fluorescence labelling of purified VfTA-CTPSR-His_6 before (−) and after conversion with MtFGE (+) and immobilized MtFGE (Im) using the same protocol as in B). The untreated protein, not containing the FGly residue, was not detected by the aldehyde-specific fluorescent labelling, while transaminase converted with soluble or immobilized MtFGE showed a distinct fluorescent band at 515 nm of similar intensity, demonstrating a quantitative conversion with both methods. As MtFGE was itself aldehyde-tagged, it was detected as well as a lower running band in sample (+). The absence of an MtFGE signal in the sample converted with immobilized FGE (Im) shows that no protein leakage has occurred.

the high kinetic dependence of the immobilization reaction (Figure 3B).

Accordingly, all further immobilizations were carried out at 37 °C. Experiments with different amounts of protein per mg beads (Figure 3C) showed that the activity correlates with the amount of protein immobilized (determined by the Bradford assay of the supernatant after immobilization). Addition of more protein lead to more protein being immobilized (with saturation of the beads reached at about 150 μg immobilized protein per mg of beads, data not shown). However, the activity was proportional to the immobilized protein up to 100 μg protein per mg bead and did not increase if more protein was coupled – most likely because of conformational reasons, inaccessible areas for substrate (buried areas), or aggregation. Furthermore, high protein loads might lead to mass transport limitation.[42] Aggregation and denaturation during the immobilization at higher protein concentrations, in combination with the shear forces caused by the agitating beads, could also be a possible reason for protein losses and was indeed observed at concentrations ≥ 200 μg protein (per mg bead).

Kinetic measurements were done with soluble and immobilized transaminase (Figure 3F) at 22 °C and 37 °C. As expected, higher temperatures resulted in faster production of acetophenone for both free and immobilized transaminase. However, in contrast to the soluble enzyme, the immobilized transaminase reached a lower conversion if incubated at 22 °C. At 37 °C, free and immobilized transaminase showed comparable reaction kinetics.

Reusability and storage stability

A key feature for the heterogenization of biocatalysts is their reusability and operational stability. We examined the perform-

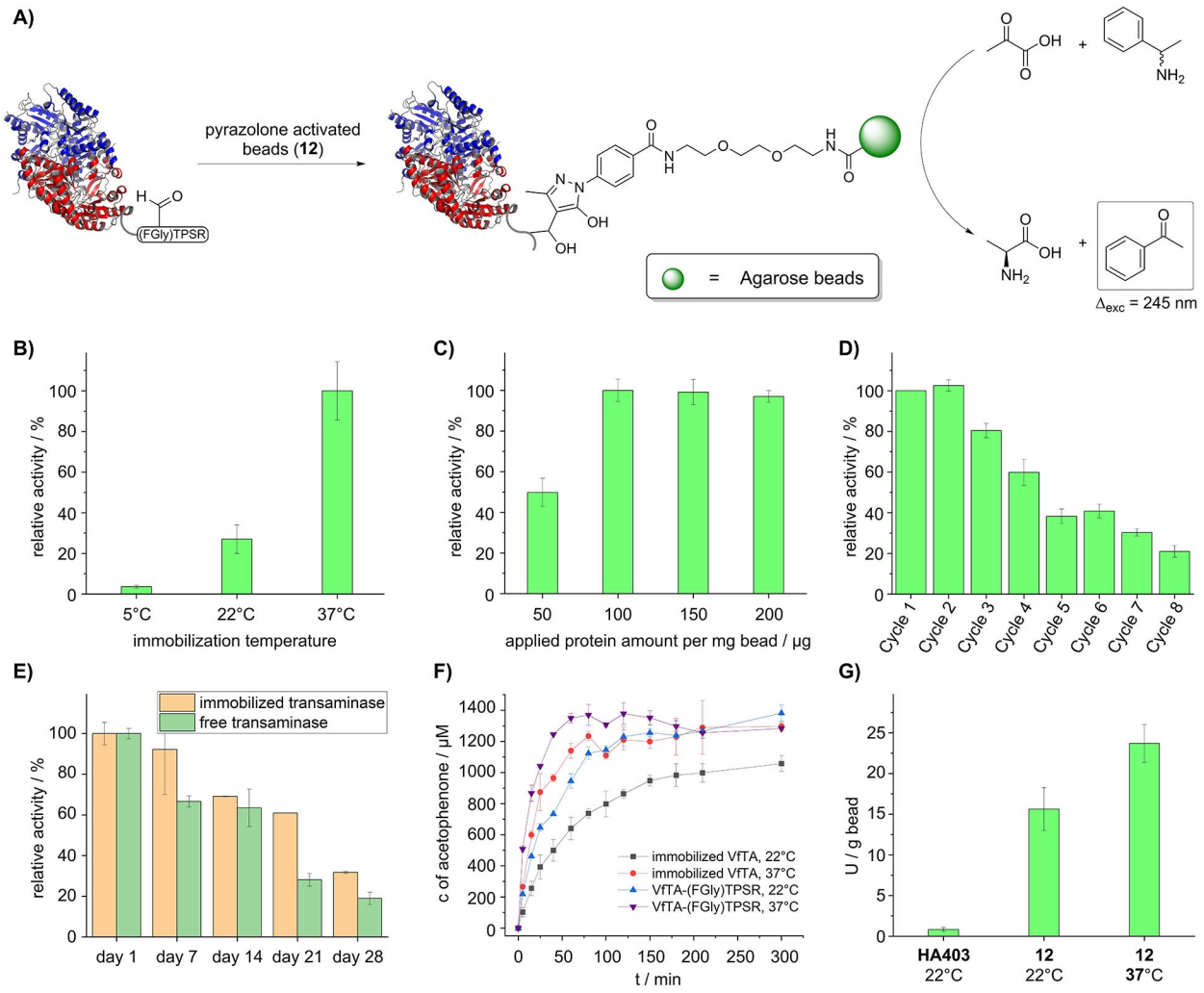


Figure 3. A) Immobilization and activity assay of VfTA-CTPSR-His_6 with converted aldehyde tag (C^{α}-formylglycine(FGly)TPSR). The aldehyde moiety is used for coupling to pyrazolone-functionalized beads (**12**). All activity assays were performed using 2.5 mM pyruvate and 2.5 mM *rac*-PEA as substrates (0.5 % DMSO, pH 8.0) whereby the product acetophenone could be detected at 245 nm which was used for determination of the immobilized enzyme activity. **B)** Screen for optimal immobilization temperature. Transaminase was immobilized on **12** at different temperatures (4, 22, 37 °C) for 24 hours. Further immobilizations were done at 37 °C as most efficient immobilization temperature. **C)** Screen for optimal loading efficiency. Different protein amounts of transaminase per mg beads (as indicated) were immobilized at 37 °C for 24 hours. The activity was proportional to the amount of immobilized protein until 100 µg per mg bead. Further increase of protein amounts did not yield higher activities. **D)** Reusability. Immobilized transaminase was used in repeated cycles (up to cycle 8) of catalysis at 37 °C for 50 minutes with washing steps in between. After 8 cycles the immobilized transaminase still showed 21 % remaining activity. **E)** Storage stability. Immobilized and free transaminase were stored at 4 °C and the activity was determined every 7 days for 4 weeks. The immobilized transaminase (orange) showed slightly better storage behavior compared to its soluble counterpart (green). **F)** Kinetic courses. The reaction of immobilized and soluble transaminase (VfTA-CTPSR-His_6) was followed by measuring the production of acetophenone at 245 nm over time at 22 °C and 37 °C. Whereas the kinetic behavior was similar at 37 °C the immobilized transaminase showed slower conversion at 22 °C. **G)** Comparison of immobilized activity. The transaminase was immobilized on **12** with optimized (pH 6.7 at 37 °C) and on commercially available amine beads (ReliZyme HA403/S) with recommended (pH 5.0 at 4 °C, reduction with 50 mM $NaBH_3CN$) conditions and activity was measured at 22 °C for both immobilisates and 37 °C additionally for VfTA bound at carrier **12**. Immobilization at **12** resulted in about 20x (22 °C) or 29x (37 °C) higher activities compared to immobilization on amine beads under recommended conditions. All immobilization procedures were performed in duplicates using 100 mM phosphate buffer with 1 mM EDTA, 50 mM NaCl and 40 µM transaminase, and the immobilized activity was subsequently determined.

ance of the immobilized transaminase in repeated rounds of catalysis. As shown in Figure 3D, the immobilized transaminase could be used in several catalytic cycles, with about 21 % remaining activity after cycle 8. Protein leakage of transaminase can be excluded as a reason for the loss of activity since no catalytic activity was observed in the supernatant after removal of the beads (data not shown). Another common effect of immobilizing an enzyme is an increased storage stability. Immobilized and soluble transaminase were stored at 4 °C and the activity was determined every 7 days over 4 weeks (Figure 3E). After 28 days the immobilisates retained about 32 % of their initial activity, compared to only about 19 % in case of the free transaminase.

As shown, the activity under storage or operation is decreasing over time continuously. In case of the transaminase used in this study, the enzyme is active as a homodimer and is

dependent on the cofactor PLP.[43–44] In the course of the reaction cycle, the covalent imine bond between the cofactor and the active site lysine is replaced by an imine bond between PLP and the substrate (referred to as external aldimine). The enzyme then transfers the substrate's amino group to yield the modified cofactor pyridoxamine-5′-phosphate (PMP) and the ketone product, which is able to leave the active site. Importantly, also PMP and PLP as the external aldimine can dissociate from the enzyme, resulting in a cofactor-free and hence inactive transaminase until new PLP reaches the active site. Alternatively, the enzyme might unfold, or the dimer might dissociate, if not both monomers are immobilized.[45–47] The latter results in inactive immobilized monomers with no possibility of dimer association and, hence, in decreased operational stability. Addition of higher PLP concentrations in the washing buffer (0.5 mM) and extended incubation times during the washing steps (15 min) did not show any positive effect (data not shown). However, higher PLP concentrations (up to 5 mM) as well as the use of different buffers showed stabilizing effects regarding the storage and thermal stability of transaminases.[45–47] Higher PLP concentrations might also accelerate the slow PLP binding and dimer association kinetics of transaminases.[48–49] Additionally, ketone substrates showed positive effects on the stability of transaminases while amine substrates negatively affected the stability.[45,47] In general, strategies to stabilize the protein of choice using various additives (e.g., glycerol, DMSO) could also be investigated for their ability to increase storage and operational stability.[50–53]

Comparison: Knoevenagel ligation vs reductive amination.

Reductive amination is the most common immobilization technique used for aldehyde-tagged proteins so far. While this approach gave satisfactory immobilization results, the necessity for an acidic pH and a reducing agent are detrimental for sensitive proteins. The immobilization protocol presented in this study works at near-neutral pH without the need of reducing agents. Therefore, it is suitable for immobilization of enzymes that do not tolerate the reaction conditions required for a reductive amination. As shown in Figure 4A, the transaminase is unstable even under slightly acidic conditions, with a small degree of precipitation observed at pH 6.7, while at pH 5 only 14% of protein was soluble. However, the protein remained fully soluble under basic conditions (pH 8).

A stable linkage is to be preferred to minimize enzyme leaching after immobilization on the solid support. Reductive amination protocols recommend the reduction of the imine formed between the aldehyde and the amine group to a stable secondary amine. $NaBH_3CN$ is often used as a reducing agent because of its imine-selectivity. However, as PLP, the cofactor of transaminases, is also bound to the active site by imine formation, it might be reduced as well if exposed to $NaBH_3CN$.[43] Incubation with only 25 mM $NaBH_3CN$ (half of what is usually used for the reduction step) already resulted in a loss of activity of more than 68% (13-32% and 13–23% residual activity for the Cys- and the FGly-transaminase, respectively) while 50 mM $NaBH_3CN$ almost completely inactivated the transaminase (3-13% and 1–6% residual activity for the Cys- and the FGly-protein, respectively; Figure 4B). Removal of the reducing agent after treatment by dialysis did not restore activity (2.6% residual activity; Figure 4C), proving that the loss in activity a) was not due to $NaBH_3CN$ interfering with the activity assay and b) that the deactivation was indeed irreversible.

For the sake of completeness, a direct comparison of the newly established Knoevenagel immobilization protocol with immobilization via reductive amination was done. For this, the transaminase was immobilized on pyrazolone resin **12** and in parallel on commercially available amine beads (ReliZyme HA403/S).

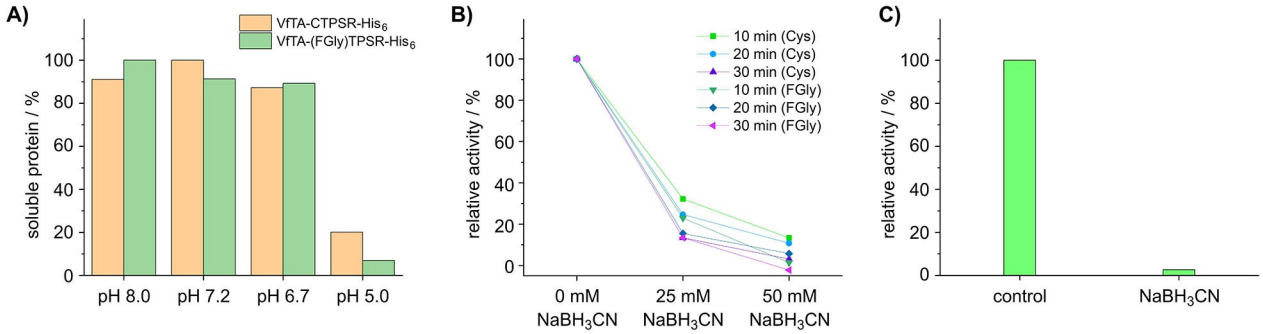


Figure 4. Inactivation of transaminase by $NaBH_3CN$. A) Tagged transaminase (non-converted (Cys) and oxidized transaminase (FGly)) was incubated at different pH values (as indicated) for 2 h at 22 °C. After centrifugation, the protein amount in the supernatant was determined via Bradford assay. Physiological and slightly basic pH-values did not affect the solubility negatively whereas the acidic pH (5.0) led to high precipitation of transaminase. Non-converted and converted species did not show differences in behaviors. **B)** Transaminase (Cys- and FGly-protein; see above) was incubated with different concentrations of $NaBH_3CN$ (as indicated) for different times (as indicated) at 4 °C and the activity of transaminase was determined afterwards. The initial activity (without reducing agent) was set as 100%. Almost total loss of activity was observed if transaminase was incubated with 50 mM of $NaBH_3CN$ – while the incubation time played a minor role. **C)** Transaminase (non-converted cysteine residue) was incubated with 50 mM $NaBH_3CN$ and dialyzed afterwards for removal of $NaBH_3CN$.The residual activity was determined under analogous conditions with untreated transaminase being used as the control. Again, the incubation with $NaBH_3CN$ caused almost total loss of activity (2.6% residual activity) compared to the control. Activity measurements were done as mentioned in the methods with 2.5 mM of each substrate (pyruvate and *rac*-PEA).

The amount of protein immobilized (based on the Bradford assay with the supernatants after immobilization) was comparable for both methods (data not shown). However, the activity of the immobilisates differed markedly. The enzyme immobilized on amine beads retained only about 3.5–5 % of the activity of the Knoevenagel immobilisate (Figure 3G). This result underscores the advantages of the newly established immobilization protocol especially for enzymes that are sensitive to the conditions needed for reductive amination.

Conclusion

Reductive amination requires low pH-values for the initial imine/oxime/hydrazone formation and potential harmful $NaBH_3CN$ that reduces the hydrolysis-sensitive C–N double bond. In our experiments, the immobilization of a fluorescent formylglycine-containing peptide (**14**) via reductive amination showed slow linear kinetics under physiological conditions.

Therefore, we established a facile and efficient 2-step synthesis of bifunctional alkyl or aryl and amine substituted pyrazolones, which were immobilized on NHS-activated dry agarose. The pyrazolone agarose beads (**12**) showed the highest efficiencies in a screening with the FGly-containing peptide **14**, with superior immobilization rates obtained at near-neutral pH (6.7) across a wide range of buffers and concentrations without using any reducing agents or additives (Table 1).

The FGly-containing transaminase (VfTA), generated by hFGE, soluble, or immobilized MtFGE (immobilized on **12**) *in vitro*, was used as model enzyme to test the applicability of our immobilization method. Subsequent screening with **12** resulted in quantitative immobilization at physiological pH without any additive or catalyst needed. The immobilized transaminase showed similar kinetics and slightly enhanced storage stabilities compared to the soluble enzyme and could be reused multiple cycles without leakage proving the stable coupling. Compared to reductive amination the novel immobilization technique showed markedly higher activities and enabled gentle immobilization by avoiding protein denaturation at acidic pH and reduction of protein intrinsic imines (i.e., imine between the cofactor PLP and transaminase).

Table 1. Reductive amination vs Knoevenagel condensation.

Reductive Amination	Knoevenagel condensation
Selective towards FGly and other aldehydes	Selective towards FGly and other aldehydes
2 steps	1 step
pH < 4.5 (imine/oxime formation) pH 5–6 (hydrazone formation) pH 7.2 (reduction)	pH 6.2–8.2
Reductant ($NaBH_3CN$) and/or additive (aniline) is needed (potentially harmful/carcinogenic)	Low cost 2-step synthesis of Knoevenagel reagent No catalyst/additive is needed
C–N-bond	Stable C–C-bond
Low-high loading obtained	Moderate-high loading obtained

Overall, the presented site-selective immobilization method opens up new possibilities for the synthesis of complex immobilisates. In particular, the synthesis of biosensors (like SPR chips) with improved binding properties seems possible. Additionally, industrial application is conceivable due to the long-term stability of the resin, high efficiency and selectivity, cost-effective synthesis, and good recovery rate of **7**.

Experimental Section

General information

Oxygen and moisture sensitive reactions were carried out using Schlenk technique and an argon atmosphere. Chemicals and solvents were purchased from commercial distributors and used without further purification. NHS-activated dry agarose was bought from Thermo Fisher Scientific™. Ac-YLCTPSRAALLTGR-NH_2 was purchased from JPT Peptide Technologies GmbH. Anhydrous solvents were distilled over desiccants. Distilled solvents (petroleum ether, EtOAc and DCM) were used for extractions and column chromatography (silica gel with a diameter of 0.040-0,063 mm (Merck)). For substrate detection on Merck aluminium plates with silica gel 60 and fluorescent indicator F254, UV (254 nm) and/or staining solutions (bromocresol, potassium permanganate or ninhydrin) were used. HPLC analysis was performed on an Agilent 1200 series LC–MS system with a C18 Luna (100×2 mm, particle size 3 μM, Phenomenex) using a linear acetonitrile gradient from 5–95 % in 12 min (+0.1 % formic acid. Absorption was monitored at 220 nm. For some compounds, RP-HPLC was used for purification (LaChrom HPLC system (Merck Hitachi) with Interface D-7000, UV detector L-7520 and pump L-7150) and performed with a C18 Jupiter column (250×21.2 mm, particle size 10 μm, flowrate 10 mL/min, Phenomenex). ^{1}H- and ^{13}C-NMR spectra were recorded with a BRUKER DRX 500 or BRUKER Avance 500 (298 K). Chemical shifts are given relative to residual solvent signals. The multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet) with coupling constants given in Hertz [Hz].

All biology reagents were mainly purchased from Merck (Darmstadt, Germany), VWR (Hannover, Germany), Carl Roth (Karlsruhe, Germany) or Thermo Fisher Scientific (Waltham, USA) with analytical grade. DNAse I was used from AppliChem, lysozyme from Fluka Analytical and KAPA HiFi HotStart ReadyMix PCR Kit from Roche. *E. coli* BL21 (DE3) was purchased from New England Biolabs.

Chemical synthesis:

The synthesis of the Knoevenagel core segments **3** and **6** as well as a Boc-protected PEG-linker, 1-formyl fluorescein (**15**) and *trapped* Knoevenagel-CF (**16**) were synthesized similar to literature known procedures and can be found in the supporting information.

MePzMePEG$_2$NHBoc

MePzMeOH (**3**) (240 mg, 1.54 mmol), HOAt (313.8 mg, 2.3 mmol, 1.5 eq.), Boc-PEG_2-NHBoc (572.5 mg, 2.3 mmol, 1.5 eq.) and DIPEA (401.1 μL, 2.3 mmol, 1.5 eq.) were dissolved in DMF (3.5 mL) and EDC·HCl (442 mg, 2.3 mmol, 1.5 eq.) was added over 30 min as a suspension in DMF (2.5 mL). The reaction mixture was stirred for 24 h at rt. Finally, the Boc-protected amine was purified directly via RP-HPLC and obtained as a yellow oil (360 mg, 60.6 %). ^{1}H-NMR (500 MHz, $CDCl_3$, mixture of tautomers): 1.43 (s, 9H, Boc-H), 2.10 (s, 3H, -CH_3), 2.25 (s, 3H, –CH_3), 3.28 (t, ^3J = 5.49 Hz, 2H, PEG–H), 3.52

(m, PEG–H and –CH_2=), 4.34 (s, 2H, –CH_2–CO–), 4.78 (s, 2H, –CH_2–CO–), 5.57 (s, 1H, –NH–). LC–MS (ESI): r_t = 5.6 min, *m/z* = 387.24 $[M+H]^+$. HRMS (ESI): Exact mass calc. for $[C_{17}H_{30}N_4O_6H]^+$: 387.22381, found: 387.22217.

MePzMePEG$_2$NH$_3^+$ Cl$^-$ (4)

MePzMePEG$_2$-NHBoc (360 mg) was dissolved in DCM (10 mL) and 4 M HCl in dioxane (10 mL) was added dropwise. After stirring the reaction mixture for 2.5 h at room temperature the solvent was removed, and the residue was freeze dried. The amine (quant.) was obtained as a yellow/orange oil and was used without further purification. ^{1}H-NMR (500 MHz, D_2O, mixture of tautomers): 2.12 (s, 3H, -CH_3), 2.29 (s, 3H, -CH_3), 3.21 (t, ^3J = 5.02 Hz, 2H, PEG–H), 3.46 (t, ^3J = 5.41 Hz, 2H, PEG–H), 3.65 (t, ^3J = 5.36 Hz, 2H, PEG–H), 3.71 (s, 4H, PEG–H), 3.76 (m, 2H, PEG–H), 4.40 (s, 1H, –CH=, reduced integral due to H–D-exchange), 5.65 (s, 1H, -NH-, reduced integral due to H–D-exchange). LC–MS (ESI): r_t = 1.6 min, *m/z* = 287.18 $[M+H]^+$. HRMS (ESI): Exact mass calc. for $[C_{12}H_{22}N_4O_4H]^+$: 287.17138, found: 287.17150.

MePzArPEG$_2$NHBoc

MePzArOH (**6**)[28] (585.5 mg, 2.68 mM, 1 eq.), HOAt (365.4 mg, 2.68 mM, 1 eq.), H_2N-PEG$_2$-NHBoc (1000 mg, 4.03 mM, 1.5 eq.) and DIPEA (701.5 µL, 4.03 mM, 1.5 eq.) were dissolved in DMF (5 mL) and EDC·HCl (772 mg, 4.03 mM, 1.5 eq.) was added in portions. The reaction mixture was stirred for 16 at rt and then the solvent was removed under reduced pressure. The crude was suspended in EtOAc and acidified with NH_4Cl. The organic phase was washed with brine, dried over $MgSO_4$ and the solvent was removed under reduced pressure. The Boc-protected pyrazolone was purified via column chromatography (10% MeOH in DCM) and obtained as a pale-yellow solid (980 mg, 81.4%). R_f: 0.15. ^{1}H-NMR (500 MHz, $CDCl_3$): 2.21 (s, 3H, –CH_3), 3.30 (m, 2H, –PEG–H), 3.45 (s, 2H, –CH_2–CO–), 3.54 (t, ^3J = 5.29 Hz, PEG–H), 3.66 (m, 8H, PEG–H), 5.00 (s, 1H, –NH–), 6.74 (s, 1H, -NH-), 7.83 (d, ^3J = 8.42 Hz, 2H, Ar–H), 7.98 (d, ^3J = 8.46 Hz, 2H, Ar–H). LC–MS (ESI): r_t = 6.6 min, *m/z* = 449.26 $[M+H]^+$. HRMS (ESI): Exact mass calc. for $[C_{22}H_{32}N_4O_6H]^+$: 449.23946, found: 449.23742.

MePzArPEG$_2$NH$_3^+$ TFA$^-$ (7)

MePzArPEG$_2$NHBoc (850 mg) was dissolved in TFA:DCM (2:1, 30 mL) and stirred for 2 h at rt. Afterwards, the solvent was removed under reduced pressure and the residual oil was dissolved in 0.1% TFA in H_2O (350 mL). After freeze drying, the product (quant.) was obtained as an orange-red oil, which was used without further purification. ^{1}H-NMR (500 MHz, D_2O, mixture of tautomers): 2.19 (s, 3H, -CH_3), 2.28 (s, 3H, -CH_3), 3.16 (t, ^3J = 5.29 Hz, 2H, PEG–H), 3.61 (t, ^3J = 5.55 Hz, 2H, PEG–H), 3.73 (m, 8H, PEG–H), 7.62 (d, ^3J = 8.30 Hz, 2H, Ar–H), 7.73 (d, ^3J = 8.40 Hz, 2H, Ar–H), 7.82 (d, ^3J = 8.44 Hz, 2H, Ar–H), 7.86 (d, ^3J = 8.34 Hz, 2H, Ar–H). LC–MS (ESI): r_t = 3.8 min, *m/z* = 349.20 $[M+H]^+$. HRMS (ESI): Exact mass calc. for $[C_{17}H_{24}N_4O_4H]^+$: 349.18703, found: 349.18676.

FITC-6-Ahx-Tyr-Leu-Cys-Thr-Pro-Ser-Arg-Ala-Gly-NH$_2$ (13)

Peptide synthesis: The peptide was synthesized on a 0.11 mmol scale using 4-fold excess of reagents (TBTU, DIPEA and amino acid) and automated and microwave assisted SPPS (CEM Liberty) starting from Fmoc-Rink amid resin (250 mg, 0.456 mmol/g). Fmoc-cleavage was performed using 0.1 M HOBt in 20% piperidine in DMF.

Coupling of FITC: After the coupling of Fmoc-6-Ahx-OH, the Fmoc protection group was manually removed (3x 15 min with 0.1 M HOBt in 20% piperidine in DMF) and the resin was suspended in a solution of FITC (134 mg, 3 eq.) in DMF (3 mL) and DIPEA (120 µL, 6 eq.) was added. The reaction mixture was stirred for 3 h at rt and the resin was finally washed with DMF (5x), DCM (5x) and diethyl ether (5x).

Cleavage: The peptide was cleaved with a mixture of TFA:H_2O:TIS (95/2.5/2.5) and a spatula tip of DTT. The mixture was stirred for 3 h at rt and finally precipitated in a mixture of heptane and MTBE (1:1). 1/6 of the crude was purified via RP-HPLC and the desired peptide was obtained as a yellow solid (10 mg, 52.2%). LC–MS (ESI): r_t = 6.0 min, *m/z* = 734.81 $[M+2H]^{2+}$. HRMS (ESI): Exact mass calc. for $[C_{68}H_{89}N_{15}O_{18}S_2H_2]^{2+}$: 734.80485, found: 734.80423.

FITC-6-Ahx-Tyr-Leu-(FGly)-Thr-Pro-Ser-Arg-Ala-Gly-NH$_2$ (14)

The cysteine-containing peptide (**13**) (5 mg) was dissolved in 1 mL of a bicine buffer (150 mM bicine, 200 mM NaCl, 600 mM Arg, pH 9.0) and a solution of 100 mM DTT (150 µL) and 150 µM $CuSO_4$ (1 mL) were added. Finally, the reaction was started with the addition of non-reconstituted MtFGE (5 mg/mL, 1 mL) and was stirred for 2.5 h at 37 °C. After filtration and freeze drying, the orange solid was dissolved in H_2O (1.5 mL) and purified via RP-HPLC. The FGly-containing peptide (4 mg, 81.5%) was obtained as a yellow/orange solid. LC–MS (ESI): r_t = 6.0 min, *m/z* = 1450.61 $[M+H]^+$. HRMS (ESI): Exact mass calc. for $[C_{68}H_{87}N_{15}O_{19}SH_2]^{2+}$: 725.80845, found: 725.80949.

Preparation of acid functionalized agarose beads

NHS-activated dry agarose (50 mg) was suspended in a 1 M aq. solution of NaOH (1 mL) and shaken for 30 min. After filtration, the agarose was washed with H_2O (3x), 1 M HCl (3x), H_2O (3x), phosphate buffer (100 mM phosphate, 150 mM NaCl, pH 7.2, 3x) and H_2O (3x). Finally, the beads were lyophilized in an aqueous solution of 0.1% TFA.

Preparation of amine functionalized agarose beads

A 1 M aq. solution of H_2N-PEG$_2$-NH_2 (pH 7.2, 3 mL) was added to NHS-activated dry agarose (30 mg) and was shaken overnight. After removal of the supernatant, the resin was resuspended in 1 M aq. NaOH (3 mL) and incubated for 15 min. Finally, the resin was washed and worked up as describe above.

Preparation of methylene- and phenyl-bridged pyrazolone agarose beads

NHS-activated dry agarose (25 mg) was suspended in a 12.5 mM solution of the amine-functionalized pyrazolone (**4** or **7**) in a phosphate buffer (100 mM phosphate, 150 mM NaCl, pH 7.2) and was shaken overnight. After filtration, the resin was prepared as already described for the amine functionalized agarose beads. Here, lyophilization or a final washing step with isopropanol and drying in high vacuum can be used before storing the beads at −20 °C.

Biochemical experiments:

Expression of non-reconstituted and CTPSR-tagged MtFGE

Expression: The expression of MtFGE in *E. coli* was performed as already published.[34,38] For this, 1.5 L of main culture (LB-broth) was

cultivated until an optical density (OD_{600}) of 0.6 was reached. The cells were then cooled to 18 °C, induced with 250 μM IPTG and shaken overnight. The centrifuged pellet was then resuspended in lysis buffer (50 mM TRIS, 500 mM NaCl, 20 mM imidazole, protease inhibitor (1 mM), DNAse I (spatula tip), pH 7.5), mechanically lysed using french press (3x1000 psi) and the cell debris was removed via centrifugation (4 °C, 10,000 rpm, 30 min) and filtration (pore size 0.22 μm).

Purification: The protein was purified via Ni^{2+}-NTA affinity chromatography (HisTrap HP, 1 mL, GE Healthcare) using an ÄKTA system (ÄKTA explorer). The eluted protein was analyzed by SDS-PAGE, pooled and rebuffered using Milipore Centricon (10 kDa Cutoff).

SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 5% stacking gel and 10%, 12.5 or 15% running gel. Bisacrylamide was used as crosslinker. Protein samples were denatured with 4×Lämmli buffer plus 20% of 2-mercaptoethanol or DTT. After completion, the gel was washed with water (5 min), treated with Gel-fixing solution and then washed with water and stained with staining solution (Coomassie Brilliant Blue).

Expression of hFGE

The expression and purification of hFGE from insect cells was performed exactly as described by Peng et al.[21]

Cloning and expression of transaminase

Cloning: For FGly-conversion the aldehyde-tag was inserted downstream of the open reading frame of the (*S*)-selective transaminase of *Vibrio fluvialis* (VfTA; see supporting information section 5 and 7, encoded in the expression vector pET24b). For this purpose, the plasmid pET24b-X-CTPSR-His_6 was created with pET24b as backbone, 'X' as potential gene of interest and CTPSR/His_6 as aldehyde tag/His-tag coding sequence. For overlap extension PCR the coding sequence of VfTA was amplified using add-on primers with complementary nucleotide sequences to match flanking sequences within the insertion region in pET24b-X-CTPSR-His_6.[54–55] Using the obtained amplicon as a megaprimer without further purification in the following OE-PCR and pET24b-X-CTPSR-His_6 as backbone pET24b-VfTA-CTPSR- His_6 was obtained. Further information (sequences, pcr programs and concentrations) are listed in the supporting information (section 5 and 7).

Expression: For heterologous protein production, *E. coli* BL21 (DE3) was transformed with pET24b-VfTA-CTPSR-His_6. Transformed cells were used either to directly inoculate overnight precultures or to generate glycerol stocks. Expression cultures in LB-medium were inoculated by overnight cultures to an optical density at 600 nm (OD600) of 0.1 and incubated at 37 °C to an OD600 of 0.6–0.7. Expression was induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and performed at 30 °C and 170 rpm. After 22 hours, cells were harvested by centrifugation for 20 min at 6500×g and 4 °C. Cell pellets were stored at −20 °C.

Purification: The cell pellet was resuspended in lysis buffer (10 ml/g cell wet weight; 50 mM Tris-HCl pH 8.0, 15 mM imidazole, 150 mM NaCl, DNAse I (spatula tip), 1 mM AEBSF, 0.1 mg/ml lysozyme, 0.1 mM PLP) and incubated at room temperature for 30 min. After disruption by sonication at 4 °C for 3x10 min (Branson Sonifier cell disruptor B15) the suspension was centrifuged (30 min, 18.500×g, 4 °C), the supernatant was filtered (0.22 μm filter) and then applied onto a 1 ml HisTrap™ HP column (Ni^{2+}-NTA affinity chromatography, Cytiva) using the ÄKTA system (ÄKTA explorer). Protein was eluted by using elution buffer (50 mM Tris-HCl pH 8.0, 300 mM imidazole, 150 mM NaCl) and step gradients (10, 20, 60, 100%) while VfTA-CTPSR-His_6 was eluted at 180 mM imidazole in 15 ml. Purification was monitored at 280 nm (aromatic amino acids), 388 nm (free PLP) and 425 nm (imine between PLP and enzyme-lysine) while the difference of 425 and 388 nm displayed the purification of VfTA-CTPSR-His_6 specifically. Elution fractions were analyzed by SDS-PAGE and VfTA-CTPSR-His_6 containing fractions were directly dialyzed in several steps using Tris-buffer (25 mM TRIS, 50 mM NaCl, pH 9.0). If needed, protein solutions were concentrated via speedvac at 40 °C (vacuum concentrator RVC 2–18). The optimized expression and purification yielded in about 90 mg VfTA-CTPSR-His_6 per liter expression medium (5 g wet weight).

Copper reconstitution of non-reconstituted MtFGE

303 μL of non-reconstituted MtFGE (1 mg, 30.5 μM) was diluted with 682 μL of TRIS buffer (10 mM TRIS, 150 mM NaCl, pH 7.5) and 15 μL of aq. $CuSO_4$ (10 mM) was added. The mixture was incubated for 1 h at 25 °C. Then, 3 μL of EDTA (100 mM) was added to solubilize the protein precipitated during reconstitution by copper chelation and to remove the excess of copper. Finally, the protein was rebuffered via PD10 column and concentrated by using Milipore Centricon (10 kDa Cutoff). In the end, a total yield of 68% was obtained of the reconstituted MtFGE.

Sample preparation for ICP-MS of reconstituted and non-reconstituted MtFGE

212.5 μg of non-reconstituted MtFGE (6 mg/mL, 35.4 μL) and 212.5 μg of reconstituted MtFGE (3.1 mg/mL, 68.5 μL) were diluted to 200 μL with HPLC water. In addition, the same volume of buffer of the non-reconstituted (35.4 μL) and reconstituted (68.5 μL) enzyme were diluted to 200 μL with HPLC water. 200 μL of each sample (4 in total) were mixed with 200 μL of 65% HNO_3 (Merck), vortexed and were finally made up to 5 mL with HPLC water. After centrifugation at 12.000 rpm for 60 min at 4 °C, the supernatant was sent to Eurofins for ICP-MS measurement.

Fluorescent-labeling of FGly-containing proteins with trapped Knoevenagel-CF (16)

To assess a successful FGly conversion of aldehyde-tagged transaminase and FGE, the respective protein was incubated overnight with 1–2 mM *trapped* Knoevenagel-CF (**16**) in immobilization buffer (protein was either dialyzed into the buffer or overlayed with a 10x stock) at 22–37 °C with shaking at 750 rpm. The conversion was analyzed via SDS-PAGE and by in-gel fluorescence detection (LAS-3000, Fujifilm, filter FL—Y515) followed by Coomassie staining.

FGly conversion of VfTA-CTPSR-His_6

The conversion of cysteine to formylglycine (FGly) within the aldehyde tag by human FGE was performed in dialysis buffer (see above) supplemented with 3 mM DTT and human FGE using a molar ratio of 25:1 (transaminase:hFGE). Conversion with MtFGE was performed in bicine buffer (50 mM bicine, 67 mM NaCl, 200 mM Arg, pH 9.0) supplemented with 5 mM DTT. Either the soluble MtFGE was added in a 3:1 ratio (transaminase:MtFGE) or the reaction solution was added to 4 mg MtFGE immobilisate

equilibrated with bicine buffer. Incubation was performed at 37 °C for 4 hours at 650 rpm.

Immobilization experiments:

Immobilization of FITC-6-Ahx-Tyr-Leu-(FGly)-Thr-Pro-Ser-Arg-Ala-Gly-NH_2 (14) and 1-formyl fluorescein (15)

A solution of FITC-6-Ahx-Tyr-Leu-(FGly)-Thr-Pro-Ser-Arg-Ala-Gly-NH_2 (**14**) or 1-formyl fluorescein (**15**) (10–50 μM) was prepared by diluting a 10 mM solution of the fluorophor (in DMF) in buffer (100 mM phosphate/ MOPS/HEPES/citrate, 150 mM NaCl, pH 6.2–8.2). The acid- (**9**), amine- (**10**), methylene-bridged pyrazolone- (**11**) and phenyl-bridged pyrazolone (**12**) agarose beads were suspended in the above describe peptide solution (2–5 mg/mL). For reduction with $NaBH_3CN$: A 1 M stock solution of $NaBH_3CN$ in water was added to yield a final concentration of 50 mM and stirred for 30 min. Samples were taken after a specific time and the residual fluorescence of the supernatant was measured in a Tecan reader (λ_{exc} 490 nm, λ_{em} 525 nm). Samples were measured in duplicates. The supernatant was then returned to the reaction mixture. During the measurement, the suspension was cooled on ice.

Immobilization of VfTA-CTPSR-His_6

Immobilization of VfTA-CTPSR-His_6 on pyrazolone beads was done in PCR-tubes to allow continuous shaking of beads within the protein solution. Therefore, PCR-tubes were submitted with beads (1, 2, 4 or 8 mg per 100 μl) and filled with protein solution (100 mM phosphate buffer, 50 mM NaCl, 1 mM EDTA, pH 6.7, 40 μM protein; 50, 100, 200 or 400 μg protein/mg bead). The suspension was incubated for 20–24 h at different temperatures (temperature studies: 4, 22, 37 °C, final batches at 37 °C) and 1400 rpm. Finally, the beads were washed (2x immobilization buffer supplemented with 0.5 M NaCl; 2x immobilization buffer), incubated with 25 mM pyruvate in immobilization buffer at 37 °C for 1 hour (blocking of free residues) and washed twice in enzyme buffer (50 mM Tris-HCl pH 8.0, 0.075 mM PLP).

The immobilization of VfTA-CTPSR-His_6 on amine support (Relizyme HA403/S) via FGly was also done in PCR-tubes. The beads were submitted (2 mg per 100 μl) and protein solution (50 mM acetate buffer, pH 5.0, 40 μM protein, 200 μg protein/mg bead) was added. The suspension was incubated under continuous shaking for 24 h at 4 °C and 1400 rpm. The beads were washed as mentioned above, treated with $NaBH_3CN$ (50 μl/mg bead, 50 mM, 20 min at 4 °C) and washed again three times with enzyme buffer.

All beads were stored in enzyme buffer at 4 °C and analyzed by activity assay.

Immobilization of MtFGE

For immobilization of MtFGE, 4 mg of pyrazolone beads **12** were weighed in a 2 mL reaction tube. To this, 150 μg MtFGE were added in 200 μL immobilization buffer without EDTA (100 mM phosphate buffer, 50 mM NaCl, pH 6.7). The suspension was incubated for 20–24 h at 37 °C and 650 rpm. Finally, the beads were washed (2x immobilization buffer supplemented with 0.5 M NaCl; 2x immobilization buffer), incubated with 25 mM pyruvate in immobilization buffer at 37 °C for 1 hour (blocking of free residues) and washed twice in TRIS buffer (10 mM TRIS, 150 mM NaCl, pH 7.5).

Immobilization yield

The immobilization yield was determined using the protein amount initially applied (set to 100 %) minus the percentage of protein measured in all washing buffer as determined by Bradford assay with using bovine serum albumin as standard.[56]

Activity assay of VfTA-CTPSR-His_6

The specific activity of free enzymes was measured using acetophenone as the substrate.[57] 120 μl of enzyme solution in enzyme buffer (50 mM Tris-HCl, 0.075 mM PLP, pH 8.0) was submitted to a 96-well UV-microtiter plate (UV-Star®, Greiner Bio-One) and pre-warmed to the appropriate temperature. Addition of 30 μl reaction solution (50 mM Tris-HCl, 0.075 mM PLP, 12.5 mM pyruvate, 12.5 mM *rac*-1-phenylethylamine (PEA), 2.5 % DMSO, pH 8.0) started the reaction while the increase in absorbance at 245 nm was measured in 1 minute intervals using a TECAN reader (Spark™ 10 M). Samples were measured in triplicates and the specific activity was calculated based on the initial reaction velocity.

For analyzing the activity of immobilized enzyme, the storage buffer was removed via centrifugation. Afterwards, reaction solution was applied (50 mM Tris-HCl, 0.075 mM PLP, 0.5 % DMSO, 2.5 mM pyruvate, 2.5 mM *rac*-PEA; pH 8.0, 400 μl per mg bead) and was shaken for 5 minutes at 22 °C. After centrifugation (20,000×g) 100 μl of the supernatant was analyzed at 245 nm within the TECAN reader while the reaction buffer served as blank. Samples were measured in duplicates.

Analyzing the storage stability of immobilized enzyme, different batches were prepared in parallel and stored at 4 °C. The batches were analyzed in 7 day intervals starting from day 1 with batch 1. The reusability was studied by repeated cycles of reactions while the beads were washed twice with storage buffer between each cycle. For optimization of reusability, reactions were performed at different temperatures (22 °C, 37 °C) and/or with different reaction times (20, 50 minutes). Furthermore, in case of one batch, the washing step was extended to 15 minutes with 1 mM PLP and the reaction was done with 0.5 mM PLP.

For kinetic analysis, the reaction was performed at 20 and 37 °C. 100 μl supernatant was analyzed at 245 nm in 5-minute distances. To maintain reaction conditions, the supernatant was immediately given back to the reaction after measurement.

For determining the amount of produced acetophenone, 12 $mM^{-1}cm^{-1}$ was used as extinction coefficient.

Activity Assay for His_6-CTPSR-MtFGE

The specific activity of immobilized His_6-CTPSR-MtFGE was measured by an adapted version of the *in vitro* assay described before for human FGE.[24] Accordingly, the immobilisate was incubated with 80 nmol (0.2 mM) substrate peptide (acetyl-YLCTPSRAALLTGR-amide) in 400 μL enzyme buffer (150 mM bicine, 200 mM NaCl, 45 μM $CaCl_2$, pH 9.3), supplemented with 5 mM DTT, at 37 °C and 1100 rpm. After short centrifugation, 20 μL of the supernatant was mixed with 5 μL 10 % TFA (2 % final concentration). Conversion of the cysteine residue within the substrate peptide to C^{α}-formylglycine was determined by co-crystallization of the sample 1 : 1 with 4-chloro-α-cyanocinnamic acid matrix (20 mg mL^{-1}, Sigma), followed by an on-target washing step with 0.1 % TFA, and analyzed by MALDI-ToF-MS (UltrafleXtreme, Bruker Daltonics) in reflector positive mode (m/z 500–5000). Based on the signal intensities for the unconverted peptide (1562. 84 Da, $[M+H^+]$ and the peptide containing the formylglycine residue (1544.83 Da, $[M+H^+]$), the

percentage conversion was calculated using the following equation:

$$FGly\ conversion\ [\%] = \frac{Intensity\ (FGly\ peptide) \times 100}{Intensity\ (Cys\ peptide) + Intensity\ (FGly\ Peptide)}$$

FGly conversion of VfTA-CTPSR- His_6 with immobilized MtFGE

After equilibration of the MtFGE immobilisate with enzyme buffer, 100 μg VfTA-CTPSR-His_6 in 150 μL enzyme buffer, supplemented with 5 mM DTT and 0.2 M arginine, was added to the beads and the suspension incubated for four hours at 37 °C and 650 rpm. As a control, VfTA-CTPSR-His_6 was also converted with free MtFGE (30 μg) under the same conditions. Successful conversion of the cysteine to the C^{α}-formylglycine residue was verified by aldehyde-specific fluorescence labelling (see above).

Author Contributions

N.J. synthesized the aldehyde-reactive fluorophor, designed, and synthesized the acid-, amine-, and pyrazolone-functionalized beads, and prepared aldehyde-containing peptides and small molecules whose immobilization via Knoevenagel condensation was established and optimized, and wrote mainly the paper under the supervision of M.H., U.T.B., G.F.v.M., and N.S. T.H. worked on the cloning, expression, purification, and immobilization of VfTA. T.B. performed immobilization experiments and FGly conversions with MtFGE. S.A. expressed and purified hFGE and MtFGE and performed copper reconstitution.

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Conflict of Interest

The authors declare no conflict of interest.

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