High-density immobilization of TCEP on silica beads for efficient disulfide reduction and thiol alkylation in peptides

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ABSTRACT

Tris-(2-carboxyethyl)phosphine (TCEP) linked to agarose beads is widely used for reducing disulfide bridges in proteins and peptides. The immobilization of TCEP on beads allows efficient removal after reduction to prevent its reaction with alkylating reagents and thus interference with conjugation reactions. However, a limitation of agarose TCEP is its relatively low reduction capacity per milliliter of wet beads (about 15 μmol/ml), making it unsuitable for the reduction of disulfides from molecules at millimolar concentrations. In this work, we tested the immobilization of TCEP to a range of different solid supports and found that conjugation to silica gel offers TCEP beads with about 8-fold higher reduction capacity (129 ± 16 μmol/ml wet beads). We show that it allows reducing disulfide-cyclized peptides at millimolar concentrations for subsequent cyclization by bis-electrophile linker reagents. Given the substantially higher reduction capacity, the robust performance in different solvents, the low cost of the silica gel, and the ease of functionalization with TCEP, the silica gel-TCEP is suited for reducing disulfide bridges in essentially any peptide and is particularly useful for reducing peptides at higher concentrations.

INTRODUCTION

TCEP is a powerful reagent widely used for reducing disulfide bonds in proteins, peptides and other disulfide bond-containing molecules[1]. Its strong reductive capability towards disulfide bonds at a wide pH range (1.5-8.5), the high solubility and good stability in aqueous solutions, the compatibility with many functional groups present in the biological systems, and the odourless nature, make TCEP an attractive reducing agent and often the preferred choice.[1a, 2] However, due to the reaction of TCEP with thiol-reactive groups such as maleimides, α-haloacetamides and others, used in reagents for peptide and protein labeling and modification agents,[1c, 3] the reducing agent needs to be quenched or removed for many applications. Methods for quenching TCEP are based on reactions with 4-azidobenzoic acid or azides.[4] Methods involving removal of TCEP are based on dialysis, chromatographic purification or immobilize TCEP. The latter solution is widely applied as TCEP can be efficiently be removed by centrifugation, filtration, or in case of magnetic solid supports even by magnetic capture. For example TCEP immobilized on agarose beads is commercially offered by several providers and is used in many applications.[5] Immobilized TCEP is also used in case reducing agents are not compatible with analytical techniques as in some types of gel electrophoresis or mass spectrometric analysis.

A limitation of TCEP-agarose is the relatively low reducing capacity per volume of wet agarose beads. Commercial providers such as Pierce/ThermoFisher and Sigma-Aldrich/Merck indicate for their products that they reduce at least 8 μmol disulfide bonds per milliliter wet agarose, wherein the actual reducing capacity may be slightly higher depending on the batch. Given that TCEP is typically applied in excess over the biomolecules to ensure quantitative disulfide bond reduction (e.g. 10-fold), and that the beads should not occupy more than half the volume of a reaction in a tube, the application is suited for disulfide

concentrations of around 1 mM or below. In our laboratory, we regularly reduce disulfide bridges in peptides containing two or more thiol groups to subsequently cyclize them by bis- or tris-electrophile reagents.[6] We cyclize the peptides at concentrations as high as possible to facilitate subsequent steps such as purification or high-throughput screening of crude products, but also not too high concentrations so that peptides cyclize rather than react intermolecularly to polymers. An optimal peptide concentration for cyclization reactions is 1—10 mM, but such high concentrations of reduced peptide can currently not be obtained using agarose-TCEP due to the described limited reducing capacity. Reduction of thiol groups in peptides at millimolar concentrations would be of great interest in many other applications too, including for peptide labeling by fluorophores, for crosslinking, or for immobilization to surfaces.

TCEP was immobilized to other supports than agarose with diverse applications in mind, the solid supports that were used being polyethylene glycol (PEG) polymers,[7] silica beads,[8] and magnetic cobalt beads,[9] as briefly described in the following. Miralles *et al.* have conjugated TCEP to a hydrophilic PEG-based beads to efficiently reduce disulfide bonds in aqueous and organic solvents and under microwave irradiation.[7] They conjugated TCEP via a carboxylic acid to amino-functionalized PEG using *O*-benzotriazole-*N,N,N′,N′*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as coupling agent. The TCEP-PEG resin was efficiently applied to reduce disulfide bonds in peptides at concentrations of around 100 μM. The reducing capacity of the PEG-TCEP was not reported but is likely higher than that of agarose-TCEP based on the quantity of immobilized TCEP molecules. Alzahrani and Welham have immobilized TCEP on monolithic silica for reduction of proteins in microfluidic chips.[8] They achieved this by silanization of the silica surface with (3-aminopropyl)triethoxysilane (APTES), followed by coupling of TCEP via a

carboxylic acid to the amine using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) and sulfo-*N*-hydroxysuccinimide (NHS) as coupling agents. The monolithic silica-TCEP was characterized in depth by various analytical methods and successfully applied to reduce the disulfide bonds in insulin that was run through the microchip, but the reduction capacity was not quantified. Most recently, Zwyssig *et al.* immobilized TCEP on magnetic carbon-coated cobalt nanoparticles, which allows fast and efficient removal of the reducing agent with a magnet.[9] The TCEP was conjugated to PEG on cobalt beads by esterification using EDC. The beads were applied to quantitatively reduce disulfide bonds of bovine insulin at a concentration of 400 μM. The reduction capacity of the cobalt beads (number reduced disulfide bonds per volume of solid support) was quantified to be 70 μmol functional TCEP per gram of beads, which is substantially higher than that of agarose-TCEP but we estimated it to be still too low for our envisioned application of fully reducing disulfide bonds of peptides at millimolar concentrations.

Herein, we aim at producing immobilized TCEP with a reduction capacity as high as possible, for reducing disulfide bonds of peptides at high concentration, ideally in the millimolar range, and most preferred even at a double-digit mM concentration. An important goal is also that the production of the TCEP beads is relatively cheap, in order to afford disulfide reduction in peptides at milligram scale and/or to reduce large numbers of different peptides at microgram scale. We further wish that the immobilized TCEP is compatible with aqueous solvent as well as diverse organic ones. Towards this end, we coupled TCEP to a range of commercially accessible, inexpensive solid supports, quantify their reduction capacity, and study in depth the performance and properties of the immobilized TCEP that have the highest reduction capacity. This effort led to the identification of silica beads as the most suited solid support, with the silica-TCEP beads having an around 8-fold higher reduction capacity than commonly

used agarose-TCEP beads and allowing quantitative reduction of peptides at millimolar concentrations.

Results and Discussion

We first established a standardized procedure to quantify the reduction capacity of immobilized TCEP. Such a method was needed in order to accurately and reproducibly determine the performance of commercial and newly developed immobilized TCEP. We chose to quantify the reducing capacity by comparing it to the reducing capacity of free TCEP (assuming that one molecule of free TCEP can reduce one disulfide bridge), and we expressed this in "μmol of functional TCEP per ml wet resin". For quantifying the reducing capacity of free and immobilized TCEP, we incubated both with Ellman's reagent in parallel and quantified the amount of reduced reagents by measuring absorbance at 412 nm, as described before.[2b] With this method, we quantified the reducing capacity of two commercially provided agarose-immobilized TCEP, one being the Pierce™ Immobilized TCEP Disulfide Reducing Gel (ThermoFisher) and the other one being G-Biosciences TCEP Reducing Resin (G-Biosciences). The two agarose-TCEP resins displayed reducing capacities of 15.3 ± 1.3 and 10.4 ± 3.7 μmol of functional TCEP per ml wet resin (Figure 1b).

Compared to free TCEP, which can be dissolved in H_2O at a concentration of more than 150 mM and can thus be used to reduce disulfide bridges in peptides at high millimolar concentration, these reduction capacities were substantially lower, showing the limit of agarose-immobilized TCEP. In order to visualize the limit of the reducing capacity for commercially provided immobilized TCEP, we added increasing volumes of agarose-TCEP to a typical sample volume of 40 μl. The largest volume of wet agarose-TCEP that could still be mixed with the 40 μl sample and allowed subsequent removal of reduced peptide was 50 μl and corresponded to a reducing capacity of 0.77 μmol free TCEP (Figure 1c).

We next performed experiments to assess the capacity and molar excess of commercial agarose-TCEP to quantitatively reduce disulfide-cyclized peptides and to find out the highest concentration of peptide that could be reduced. We synthesized three model peptides of the format Mpa-Xaa-Xaa-Mea, with Xaa being random natural amino acids and Mpa and Mea being mercapto-propionic acid and mercapto-ethylamine, both building blocks containing a thiol group that could oxidize to form disulfide-cyclized peptides (Figure 1d and Supplementary Figure 1). We incubated the peptides (0.4 μmol) in 400 μl water (1 mM final conc.) with different quantities of agarose-TCEP. The reducing capacity of the applied quantities corresponded to those of 2, 4, 8, or 16-fold molar excess free TCEP over the peptide. Analysis of the peptides by LC-MS showed that at eight equivalents were required to completely reduce the peptides in three hours at room temperature. Taking into consideration the maximal volume of agarose-TCEP that can fit into a given volume (if the agarose beads are allowed to occupy around half the volume), we estimated that the maximal concentration of peptide that could be fully reduced by agarose-immobilized TCEP was around 1 mM, which was too low for the peptide library cyclization applications we had in mind.

We next set out to identify a solid phase to which TCEP could be immobilized more densely so that disulfide bridges in higher concentrated peptide samples could be reduced. We aimed at coupling TCEP to solid surfaces via one of its carboxylic acid through an amide bond with an amino group present on the solid phase. We applied seven amino-functionalized resins used for solid-phase peptide synthesis, one agarose solid phase that is used as chromatography support, and silica gel that is used for flash chromatography. We chose these supports because they were previously shown to be suited for molecule immobilization at high density, the presence of suitable chemical groups for TCEP immobilization, and their commercial availability and low price. For

immobilizing TCEP on the silica gel, we first functionalized the material with amino groups by silanization with the silica-reacting reagent APTES similar to previously reported by Alzahrani and Welham who immobilized TCEP on monolithic silica for reduction of proteins in microfluidic chips (Figure 2a).[8] For TCEP immobilization through amidation on all solid supports, we applied the coupling agent EDC and an excess of TCEP over the number of accessible amino groups.

Comparison of the reducing capacity of all TCEP-functionalized solid supports using the above-described Ellman's assay showed that silica gel-TCEP was far superior to all other supports, including the commercially offered agarose-TCEP (Figure 2b). One milliliter wet resin of the silica gel-TCEP had a reducing capacity equivalent to 129 ± 16 μmol of free TCEP (mean value and SD of five silica gel-TCEP batches produced over the course of the project). Compared to the better one of the two commercial agarose-TCEP products, the silica gel-TCEP had thus a more than 8-fold higher reducing capacity. We did not study the reasons for the superior performance of the silica gel over other supports for TCEP immobilization, but it is likely that the highly porous nature of the silica gel, the dense immobilization of functional groups, and the limited swelling were key factors for achieving the high reducing capacity. For illustrating the maximal reducing capacity, we added increasing volumes of the silica gel-TCEP to a typical sample volume of 40 μl. The largest volume of wet silica gel-TCEP that could still be mixed with the 40 μl sample was around 50 μl and corresponded to a reducing capacity of 6.4 μmol free TCEP (Figure 2c). This value was more than 8-fold larger than the one found for agarose-TCEP and suggested that the silica-TCEP beads are suited to reduce peptides at much higher concentration.

In initial applications of the silica gel-TCEP, we observed an unexpected product with a mass that was 32 Da smaller than the reduced peptides, corresponding to the loss of sulfur. Most likely, the peptides were desulfurized by TCEP which is a known side reaction that can be suppressed by suitable scavengers.[10] We found that the side product was substantially reduced or completely suppressed if silica gel-TCEP was acidified prior to use (Supplementary Figure 2). For all subsequent experiments, we thus acidified the silica-TCEP prior to application.

We next quantified the reducing performance of the silica gel-TCEP with the three model peptides described above, with the goal of determining the maximal concentration of peptide that could be reduced with the new TCEP beads. We incubated the peptides (0.4 μmol) in 40.0 μl DMSO (10 mM final conc.) with different quantities of silica gel-TCEP. The reducing capacity of the applied quantities corresponded to those of 1, 2, 4, or 8-fold molar excess free TCEP over the peptide. Note that the concentration of peptide applied was 10-times higher compared to the above reference experiment performed with agarose-TCEP as we expected that the higher reduction capacity of silica gel-TCEP would be suited to reduce such a high peptide concentration. Four equivalents were sufficient to fully reduce peptides in three hours at room temperature (>96% reduced peptide; Figure 2d). This was much better compared to the agarose gel-TCEP beads that reduced peptide having a 10-fold lower concentration (1 mM) and for which an 8-fold molar excess was required to fully reduce the model peptides. Based on the improved reducing capacity (8-fold) and the smaller excess needed for quantitative reduction of model peptides (4-fold versus 8-fold), we concluded that the silica gel-TCEP can be used to reduce peptide at a 16-fold higher concentration than with the agarose-TCEP. While we showed that peptide at a concentration of 10 mM can be fully reduced, we estimate that even higher concentrations (e.g. 20 mM) can

be treated with the silica gel-TCEP for full disulfide bond reduction. The estimation that slightly higher concentrated peptide can be reduced with silica gel-TCEP too (e.g. 2-fold higher) is based on the observation that the amount of silica gel-TCEP needed for reducing 10 mM peptide occupied a relatively small volume in the tube (Figure 2c, tube with 1.6 μmol functional silica gel-TCEP) and that the amount of TCEP beads could still be increased.

We next tested if the thiol groups of the reduced peptides could be alkylated after removing the silica gel. As a reaction, we chose the cyclization by the bis-electrophilic reagent **1** as shown in Figure 1a, that involved two consecutive reactions, of which the second one is intramolecular. The reaction of the peptides in volumes of 20 μl at a concentration of 1 mM with four equivalents of **1** in ammonium bicarbonate buffer/DMSO (1:1, v/v, pH 8) for 3 h led to quantitative cyclization (Figure 3 and Supplementary Figure 3). No disulfide-cyclized peptide was observed, indicating that the peptides were quantitatively reduced and did not partially oxidize back after removal of the TCEP beads or during the alkylation reaction.

Depending on the chemical reaction and the reagents needed for chemically modifying the reduced thiol groups, specific solvents may be required, and we thus tested the performance of silica gel-TCEP in a range of different solvents. We dissolved lyophilized model peptides in 40 μl water, methanol (MeOH), acetonitrile (MeCN), DMSO or DMF at a concentration of 10 mM (0.4 μmol) or as high as the solubility allowed, incubated the peptides with 12 μl wet silica gel-TCEP (corresponding to a reducing capacity of 1.6 μmol free TCEP, 4 equiv.) for 3 h at RT and analyzed the peptidic species by LC-MS before and after reduction. The peptide was reduced with an efficiency between 96 and 100% (Figure 4a), showing that the silica-immobilized TCEP can flexibly be

applied in many different solvents. The study with the different solvents was performed with only peptide A (and peptide B in case of MeCN as peptide A was not soluble in this solvent), wherein the peptides were chosen randomly. Given the clear result of essentially full reduction in all the solvents, we expect that peptides with different sequences are likely efficiently reduced in the different solvents too.

After removal of immobilized TCEP from peptides, the thiols may partially oxidize back, which would lead to incomplete modification of the peptides. The risk of back-oxidation is particularly high for peptides containing two or more thiol groups as they can react intramolecularly. For the synthesis of cyclic peptides as shown in the example above, partial back-oxidation would lead to product mixtures of linker-cyclized peptide and disulfide-cyclized peptide. In order to assess the propensity of dithiol peptides to oxidize and to find conditions that limit oxidation, we tested the stability of a randomly chosen peptide (peptide G) in different solvents and at different pHs (Figure 4b). As solvents, we used water containing either 10% DMSO, 10% DMF or 20% acetonitrile, which are mixtures that are suited for dissolving most peptides and that are compatible with thiol-alkylation reactions. For testing different pH conditions, we added HCl to the solvent-buffer mixtures to reach a pH of 6, 4 or 2. We followed back-oxidation by LC-MS analysis of samples taken over a time span of 24 hours. While the model peptide fully oxidized in 10% DMSO at all pH values, it remained reduced at pH 2 in 10% DMF and 20% MeCN (Figure 4b). We subsequently tested the back-oxidation of two additional, randomly picked peptides (peptides H and I) to test if the effects of different solvents and pH are peptide sequence-dependent. The two additional peptides neither back-oxidized at low pH (2) and in 10% DMF or 20% MeCN and thus behaved similarly (Supplementary Figure 4). We concluded that peptides may best

reduced at low pH and ideally in buffer containing either DMF or MeCN, in order to prevent rapid back-oxidation.

We tested further measures for preventing rapid back-oxidation of the dithiol peptides, this time over several days, one being the storage of the peptide solutions at -20°C and one the closing of the microwell plates containing the peptides by DMSO-soaked microclime lids. For this experiment, we incubated a model peptide in DMSO for six days and analyzed samples at different time points by LC-MS (Figure 4c). We additionally tested in this experiment the influence of peptide concentration on oxidation (1 mM, 5 mM, 10 mM, 20 mM), as well as simultaneously monitored the uptake of water into the DMSO samples and the increase of the sample volumes (Supplementary Figure 5). At room temperature and without a lid on the microtiter plate, thiol oxidation took place already on the first day to a large extend and the peptide was fully oxidized after six days. At room temperature and covered with a lid, a small quantity of oxidized peptide was observed after 1-2 days and around 25% of the peptide remained reduced after six days. Peptides covered with lid and stored at -20°C oxidized only marginally over the first two days and more than 75% was still reduced after six days. Taking these results together, dithiol peptides do rapidly oxidize after removal of the TCEP beads if no precaution is taken but the oxidation can be controlled to a large extent if peptides are kept in an appropriate solvent or away from humidity and at low temperature. If the application allows, it is best to remove the TCEP beads just before the thiol groups are to be chemically modified.

Finally, we assessed the stability of the silica gel-TCEP beads at different conditions to find out how they are best kept for short time periods and stored over longer times. We kept the beads at different temperatures (room

temperature, 4°C, -20°C) and under different atmospheres (air, nitrogen, vacuum) for more than 100 days and tested the reducing capacity with Ellman's reagent (Figure 4d). The temperature had the strongest effect, wherein storage at -20°C was best and retained around 80% of the reducing capacity after 112 days. Without any storage precautions (room temperature and air in tubes), the beads still kept more than 50% of their reducing capacity, showing that they are rather stable and that no particular storage measures need to be taken when using the beads over several days.

CONCLUSIONS

We have developed TCEP beads that have an around 8-fold higher reduction capacity than the widely applied and commercially used agarose-TCEP. We achieved this by immobilizing TCEP on silica gel, which is a highly porous, non-swelling and cheap solid-phase material that is compatible with a wide range of solvents. We have shown that the silica gel-TCEP beads are suited to efficiently reduce disulfide bridges in peptides. Due to the higher reduction capacity, the new beads allow quantitatively reducing disulfide bridges in peptide samples having concentrations of 10 mM and likely at even slightly higher concentrations. We furthermore showed that the beads are suited to reduce disulfide-cyclized peptides that could, due to the efficient removal of the immobilized TCEP, be immediately cyclized by bis-electrophile linker reagents. While we have tested the silica gel-TCEP on short peptides only, it is likely that thiol groups in peptides of different size and shape are efficiently reduced too. The silica gel-TCEP beads were found to be stable and can be stored for several months at -20°C or for days at RT without losing much of their reducing capacity. The silica gel-TCEP bead may be particularly attractive for applications where disulfide bridges of peptide at millimolar concentrations need to be reduced. The beads may be also applied to recover partially oxidized peptide stock solutions in organic solvents. The broad compatibility with numerous functional groups, solvents and longer-term storage possibilities are further beneficial aspects of these high capacity TCEP beads.

MATERIALS AND METHODS

General considerations

Unless otherwise indicated, all reagents were purchased from commercial sources and used without further purification. Solvents were not anhydrous, nor were they dried prior usage. The following abbreviations are used in the manuscript: MeCN (acetonitrile), APTES (3-aminopropyl)triethoxysilane), DCM (dichlormethane), DMF (dimethylformamide), DMSO (dimethylsulfoxide), EDC (*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimid-hydrochlorid), HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate), Mea (mercapto-ethylamine), MES (2-morpholin-4-ylethanesulfonic acid), Mpa (mercapto-propionic acid), NMM (*N*-methylmorpholine), NMP (*N*-methylpyrrolidone), TCEP (tris(2-carboxyethyl)phosphine), TFA (trifluoro acetic acid), THF (tetrahydrofuran), TIS (triisopropyl silane), SiO_2 (silica gel)

Quality of chemicals

Ammoniumbicarbonate (Sigma-Aldrich, 99-101%), MeCN (Fisher Chemical, >99.8%), (3-aminopropyl)triethoxysilane (Sigma-Aldrich, 99%), 2,6-bisbromomethyl pyridine (sigma-Aldrich, 98%), dichlormethane (Sigma-Aldrich, >99.9%), DMF (Biosolve Chimie Sarl, >99.5%), DMSO (Sigma-Aldrich, >99.5%), EDC (Carl Roth GmbH + Co KG, >99%), fluorenylmethoxycarbonyl (Fmoc) amino acids and derivatives (GL Biochem Shanghai Ltd, >99%), HATU (GL Biochem Shanghai Ltd, >99%), MES (Apollo Scientific, >99.5%), NMP (VWR, 99.5%), NMM (Sigma-Aldrich, >98%), phenol (Acros Organics, >99%), piperidine (Acros Organics, 99%), potassium cyanide (Sigma-Aldrich, 99%), sodium chloride (Carl Roth GmbH + Co KG, >99.5%), pyridine (Sigma-Aldrich, 99%), TCEP (Chem Scene, >98%), TFA (Sigma-Aldrich, 99%), THF (Fisher

Chemical, >99.5%), TIS (Sigma-Aldrich, 98%), silica gel (SiliaFlash® P60, Silicycle).

Peptide synthesis

Peptides were synthesized at a 50 μmol scale on an Intavis Multipep RSi synthesizer using 5 ml polypropylene (PP) reactors containing polyethylene (PE) frits (V051PE76, Multisyntech GmbH) and cysteamine 4-methoxytrityl resin (49.6 mg, 50 μmol, 1.01 mmol/g, S8066787 106, Novabiochem). Resin in each column was washed with DMF (4 × 1000 μl) for resin swelling. Coupling was performed with 417 μl of Fmoc amino acid (209 μmol, 4.2 equiv., final conc. 0.22 mM), 392 μl of HATU (196 μmol, 4.0 equiv., final conc. 0.21 mM), 125 μl of NMM (500 μmol, 10 equiv., final conc. 1.27 M,) and 5 μl of NMP. All components were premixed for one minute prior addition to the resin. The reaction mixture was left for one hour without shaking. The final volume of the coupling reaction was 939 μl. Coupling was performed twice. Then, the resin was washed with 4 × 1000 μl of DMF. Fmoc deprotection was performed using 800 μl piperidine/DMF (1:4, v/v) for 5 minutes and was repeated once. Next, the resin was washed with 4 × 1000 μl of DMF. At the end of the peptide synthesis, the resin was washed with 2 × 1000 μl of DCM. The resin was deprotected and cleaved using 4 ml TFA/H_2O/TIS (95:2.5:2.5, v/v/v) for 3 h at room temperature. The TFA of the filtrate was evaporated under a continuous stream of nitrogen in a 15 mL canonical PP tube (greiner bio-one, 188271).

Peptide purification

Peptide from a 50 μmol-scale synthesis was dissolved in 10 ml of a MeCN and H_2O mixture (10:90, v/v), filtered through a teflon (PTFE) syringe filter (25 mm diameter, 0.22 μm pore size; BGB) and run over a preparative column (XTerra Prep MS C18 OBT 10 μm, 19 × 250 mm) using preparative HPLC system

(Waters 2535). A flow rate of 16 ml/min and a linear gradient from 0 to 40% solvent B over 39 min (A: 99.9% H_2O and 0.1% TFA; B: 99.9% ACN and 0.1% TFA) were applied. Absorbance was monitored at 220 nm. Fractions containing the desired peptide were pooled together, frozen (liquid nitrogen) and lyophilized until dry white fluffy powder was obtained.

Disulfide cyclization of peptides

Monomeric disulfide-cyclized peptide was formed by incubating a 1 mM solution of purified reduced peptide in 60 mM NH_4HCO_3 (pH 8) containing 50% MeCN (v/v) for three days under rotation (10 rpm) at room temperature. The samples were frozen (liquid nitrogen) and lyophilized again. Oxidized peptides were dissolved in 10 ml solvent mixture of MeCN and H_2O (10:90, v/v) and purified by HPLC. The fractions were frozen (liquid nitrogen) and lyophilized to obtain a fluffy white powder. The purity and identification of disulfide cyclized peptide was confirmed by UHPLC-MS.

LC-MS analysis of peptides

Peptides were analyzed by LC-MS analysis with a UHPLC and single quadrupole MS system (Shimadzu LCMS-2020) using a C18 reversed-phase column (Phenomenex Kinetex 2.1 mm × 50 mm C18 column, 100 Å pore, 2.6 μM particle) and a linear gradient of solvent B (acetonitrile, 0.05% formic acid) over solvent A (H_2O, 0.05% formic acid) at a flow rate of 1 ml min^{-1}. For all samples, a gradient of 0 to 100% MeCN within 10 min was applied and UV at 220 nm was used when not otherwise mentioned. Mass analysis was performed in positive ion mode. 100 μL polypropylene (PP) HPLC microvial (Shimadzu, 980-14379) with PP and teflon caps (Shimadzu, 980-18425) were used for all samples. For the assessment of reduced peptide content, the

samples were injected within 3 min after sample preparation to avoid back-oxidation.

For analyzing HPLC-purified peptides (reduced, disulfide-cyclized and linker-cyclized peptides), a 20 µl sample of the desired fraction was transferred and analyzed using an injection volume of 2 µl.

For analyzing peptides treated with agarose-TCEP beads (around 1 mM peptide), 10 µl of supernatant were transferred into a microvial and 1 µl was injected for analysis.

For analyzing peptides treated with silica gel-TCEP beads (around 10 mM peptide) or peptide cyclized by bis-electrophile reagents, samples of 2 µl were diluted with 18 µl buffer to reach a final buffer concentration of 60 mM NH_4HCO_3 and a DMSO content of 10%. Samples of 3 µl were injected for analysis.

Quantification of functional TCEP of resins

Around 5 mg of immobilized TCEP (dry resin) was added to a well of 96-well PS microtiter plate (Greiner Bio-One, 655061). To the resin, 80 µl of 150 mM NH_4HCO_3 (pH 8) containing 10% DMSO was added, followed by 20 µl of 20 mM Ellman's reagent in ammonium bicarbonate buffer (pH 8). An immediate color change from transparent colorless to yellow was observed upon addition if functional TCEP was present. This solution was diluted with ammonium bicarbonate buffer (typically a factor 1 to 12) to reach an absorption value of around 0.5 on a Nanodrop 8000 instrument (d = 1 mm, 412 nm, 2 µl). The concentration of functional TCEP was determined by correlating the absorbance with a calibration curve using the same solvents and procedures and a freshly prepared solution of free TCEP (A_{412} = 0.1-1.0). For quantifying the functional TCEP of commercial agarose-TCEP, beads (21.5 mg) from a ~1.0 ml of suspension was washed three times with water, filtered on a microscale column filter (Intavis, 35.103) and about 3 mg added to a well of a

microtiter plate. The gel was transferred by plastic instead of a metal spatula to avoid risks of TCEP inactivation as suggested in the product description.

Space occupation of immobilized TCEP

Immobilized TCEP was added to microscale column filters (Intavis, 35.103). In case of agarose-TCEP (ThermoFisher; 15.3 ± 1.3 μmol/ml), 0, 25, 50, 75, 100, 150, 200, and 400 μl settled beads, corresponding to 0, 0.38, 0.77, 1.1, 1.5, 2.3, 3.1, 6.1 μmol of functional TCEP, were transferred. In case of dry silica gel-TCEP, 0, 2.7, 5.4, 8.1, 10.8, 16.2, 21.6 or 43.2 mg wet resin, corresponding to 0, 0.4, 0.8, 1.2, 1.6, 2.4, 3.2, 6.4 μmol of functional TCEP (batch loading = 149 ± 19 μmol/g), were transferred and wetted with DMSO (200 μl). The solvent was removed by positive pressure (rubber suction cup) and the different wet bead amounts were placed into the eight tubes containing 40 μl of DMSO (silica gel-TCEP) or water (agarose-TCEP). The resin was settled by gravity and a picture from the “hanging” samples (Olympus OMD EM-5, Olympus M. Zuiko Premium 60/2.8 ED Macro lens) was taken on a black background.

Preparation of silica gel-TCEP

Silica gel (15.2 g, 230-400 mesh) was transferred into a dry round bottom flask (250 ml) and suspended in toluene (150 ml) followed by the addition of APTES (30 ml, 129 mmol). The suspension was refluxed for 2 h, cooled down and filtered using reduced pressure. The filter cake was washed with toluene (3 × 30 ml) and DCM (3 × 30 ml) before drying it at air and under reduce pressure overnight (RT, 0.15 mbar). Afterward, the functionalized silica beads were grafted by heating for 2 h at 120 °C before cooling down for drying under vacuum (RT, 0.10 mbar). The grafted beads showed purple coloration in the Kaiser test and the total mass increased by 3.25 g, which corresponds to roughly 2 mmol/g primary amine if fully grafted.

In a 50 ml PP canonical falcon tube (greiner bio-one), TCEP*HCl (3.30 g, 11.4 mmol, 2.9 eq.), MES (0.651 g, 3.34 mmol), and NaCl (0.585 g, 10.0 mmol) were dissolved in millipore water (23 ml) and the pH was adjusted from 1.6 to 6.8 using 10 M NaOH solution. The solution was adjusted to the final volume (33 ml) with millipore water. Afterward, EDC*HCl (0.730 g, 3.81 mmol, 0.95 equiv.) was added to the TCEP buffer solution, dissolved the solid quickly by shaking the tube vigorously, and added the amino-functionalized silica beads (2.00 g, 4.00 mmol, 1.0 equiv.) into the solution. The suspension was incubated for 3 h at room temperature under rotation (20 rpm) before filtering in a 20 ml reaction column (CEM, 99.278). The resin was washed with water (3 × 10 ml) and THF (3 × 10 ml). The resin was dried inside the syringe using a nitrogen stream before placing it under reduced pressure overnight (RT, 0.05 mbar) to afford dry immobilized TCEP silica beads.

Several different attempts were undertaken to further increase the reducing capacity. The use of smaller mesh size (625-2500, increased surface area), larger excess of TCEP*HCl over EDC*HCl (3:1) in respect to the maximal theoretical primary amine loading, the addition of hydroxysulfosuccinimide (1.6 equiv.) and variation of the pH (6.5, 7.5, 8.0) did not lead to a higher reducing capacity. The reducing capacity of the various silica gel-TCEP batches ranged from 107 ± 9.5 to 151 ± 8 μmol/ml wet resin (Supplementary Table 1).

Immobilization of TCEP on diverse supports

NovaPEG-NH_2 (188 mg, 100 μmol, 0.53 mmol/g, S7256726 833 Novabiochem), ChemMatrix-AM-NH_2 (100 mg, 100 μmol, 1.0 mmol/g, BCBW3297, Sigma Aldrich), PEGA-NH_2 (238 mg (F = 8.2), 100 μmol, 0.42 mmol/g, S7786915, Novabiochem), TentaGel S-NH_2 (385 mg, 100 μmol,

BCBX7246, Sigma Aldrich), NovaGel-AM-NH_2 (145 mg, 100 μmol, 0.69 mmol/g, S529238425, Novabiochem), PS-AM-NH_2 (71.9 mg, 100 μmol, 1.39 mmol/g, 9952639, apptec), SiO_2-APTES-NH_2 (84 mg, 1.2 mmol/g, 230-400 mesh, 100 μmol) or high density AM agarose gel (2.0 ml, 50 μM/ml suspension, I123R-1005, ABT) were added into pre-weighed disposable 5 ml PP syringes with PE filters (Multisyntech GmbH) followed by the addition of a buffer solution (2.6 ml) containing 0.34 M TCEP*HCl (260 mg, 900 μmol) in 0.1 M MES and 0.3 M NaCl at an adjusted pH of 6.8. In case of the agarose ABT gel, the storage buffer was filtered off and the gel was washed with water (3 × 3 ml). EDC*HCl (58 mg, 300 μmol) was added to each reaction container and dissolved through shaking. The coupling reaction was performed by incubating the tubes for 3 h at RT under rotation (20 rpm) before filtration. Each syringe was washed with water (3 × 3 ml) and THF (except agarose) prior to drying overnight under a high vacuum (0.08 mbar, RT). The net dry weight of each resin inside the syringe was determined to calculate the reductive capability per unit of mass.

Determination of resin swelling factor

The swelling factor was determined with water for Thermo Scientific Pierce agarose gel, G-Biosciences agarose gel, ABT agarose gel and Tentagel S as well as SiO_2-APTES-NH_2 (230-400 mesh). Between 100 and 500 mg of resin was added into a 5 ml PP syringe reactor with PE frit (Multisyntech GmbH) and fully swollen by adding 3 ml of water and one-hour of incubation at RT. The water was filtered off and the level of wet swollen resin was determined. The occupying volume was calculated by the height of the solid inside the column and the syringe's inner diameter. The same resin was then lyophilized and the volume determined in the same way. The swelling factor was calculated by dividing the swollen wet resin volume by the dry solid volume.

Quantifying disulfide reduction in peptides

Agarose-TCEP gel suspension (ThermoFisher; reducing capacity of 15.3 ± 1.3 μmol/ml wet beads as determined using Ellman's reagent) was pipetted into a microscale column filter (Intavis, 35.103) and washed with millipore water (3 x 100-800 μl) by applying positive pressure. The added volumes of wet agarose-TCEP gel was 0, 50, 100, 200 und 400 μl, corresponding to 0, 0.8, 1.5, 3.1 and 6.1 μmol and around 0, 2, 4, 8 and 16 equiv. respectively compared to the to the peptide sample (40 μl, 0.400 μmol, 1 equiv). The wet resin was added to 1 mM of oxidized peptide in 400 μl of solvent composed of 90% aqueous buffer (66 mM NH_4HCO_3 in ddH_2O, pH 8.0) and 10% DMSO (0.4 μmol peptide). The 1.5 ml PP tubes were gently rotated (20 rpm, Stuart rotator). At one and three hours of incubation, 10 μl supernatant (UHPLC-MS samples) were taken and immediately analyzed by LC-MS.

Silica gel-TCEP beads (reducing capacity of 149 ± 19 μmol/g wet beads) were weighed out into a microscale column filter (Intavis, 35.103) and acidified using HCl proportional to the amount of beads. For example, 2.7 mg dry silica gel-TCEP beads were suspended with a solution of 100 μl of 40 mM HCl in 1,4-dioxane. The quantity of beads used were 0, 2.7, 5.5, 11 and 22 mg of dry beads, having 0, 400, 800, 1600 and 3200 nmol functional TCEP. The solvent was removed by positive pressure and washed with DMSO (3 × 100 μl). The DMSO wet silica resin was transferred to 200 μl PP tubes containing 10 mM of oxidized peptide in 40 μl of DMSO (0.4 μmol peptide). The 1.5 ml PP tubes were gently rotated (20 rpm, Stuart rotator). At one and three hours of incubation, 2 μl supernatant (UHPLC-MS samples) were taken, diluted and immediately analyzed by LC-MS. It is worth mentioning that the TCEP silica beads have an optimal reduction time (about 3 h). They should not be used for more than about six hours since a start of a continous decrease in peak intensity (LC-MS) was observed from six hours on.

Cyclization of reduced peptide by alkylating agents

Solvents and reagents were added to a 0.3 mL PP microvial (Shimadzu, 980-18425) in the following order. A volume of 14 μl reaction solution containing 71% of NH_4HCO_3 (85.7 mM) and 29% DMSO (pH 8), 4 μl of 20 mM 2,6-bis(bromomethyl)pyridine (BBMP) in DMSO (4 equiv.), and 2 μl of 10 mM reduced peptide in DMSO (20 nmol, 1 equiv.). The final concentrations in the reactions were 1 mM peptide, 4 mM BBMP, 60 mM NH_4HCO_3 and 50% DMSO. The reaction mixture was well mixed with a pipette and incubated for 3 hours at room temperature. The reaction mixture was analyzed by UHPLC-MS.

Testing disulfide reduction in different solvents

To each test solution inside a 200 μl PCR tube (TreffLab, 96.09852.9.01), acidified immobilized TCEP silica beads (10.1 mg/sample, ~1.6 μmol, 4 equiv., loading = 158 μmol/g) were added and incubated for 3 hours at room temperature under rotation (20 rpm). Samples of 2 μl were diluted with 18 μl aqueous 150 mM NH_4HCO_3 buffer containing 10% DMSO and analyzed by UHPLC-MS.

Storage stability determination of silica gel-TCEP

A single uniform batch of immobilized TCEP on silica (1.0 g, 173 ± 9.5 μmol/g dry resin) was split (15 mg) into 6 × 7 (42) HPLC glass vials (Schmidlin Labor, LPP 11 09 0500) with septum-based screw caps (Brown, 155630) for tight closing. Seven different storage conditions were tested, and for each storage condition, six vials with silica were stored together. The conditions parameters included either room temperature, 4 or -20 °C with either nitrogen or air atmosphere. One condition consisted of keeping the beads at vacuum (< 0.1

mbar) at room temperature. The nitrogen atmosphere was introduced by placing all appropriate samples into a desiccator and the septum caps untightened. By pulling a vacuum and refilling the atmosphere with a nitrogen balloon (3×), the air atmosphere in the vials was exchanged by nitrogen. Afterward, the lids were closed quickly and tightly right after opening the desiccator and before storage. For each time point, one sample vial was taken for analysis, which also corresponded to the first time opening of the sample vial. Before the functional immobilized TCEP was determined, the silica beads were homogenized by gently shaking the vial. The functional TCEP quantification of the beads was realized using the Ellman’s procedure.

SUPPORTING INFORMATION

One supplementary table (summary of different supports with immobilized TCEP) and five supplementary figures (LC-MS analysis of peptides, effects of acidification, LC-MS data of cyclization reactions, back-oxidation data, storage data). The authors have cited no additional references within the Supporting Information.

ACKNOWLEDGMENTS

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KEYWORDS

cyclic peptide, disulfide bridge, immobilized TCEP, TCEP, TCEP-agarose

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FIGURES

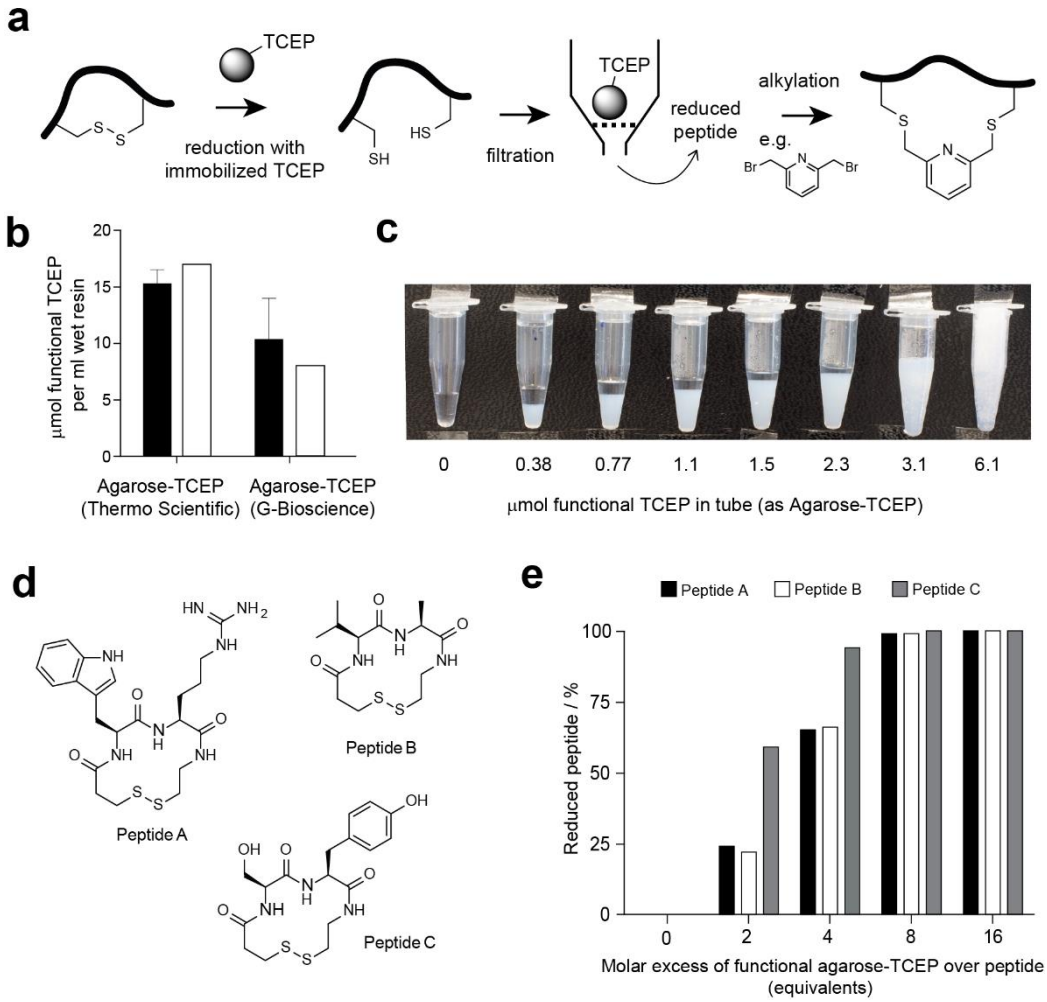


Figure 1. Disulfide bond reduction by immobilized TCEP. (a) Schematic depiction of strategy for reducing disulfide bonds, removal of immobilized TCEP by filtration, and alkylation of reduced thiol groups, shown for a cyclization reaction. (b) Capacity of commercially provided agarose-TCEP to reduce Ellman's reagent. The capacity is indicated as μmol of functional TCEP per ml wet resin, determined using Ellman's reagent and using free TCEP as reference. The black bars show mean values of three independent measurements. SDs are indicated. The white bars indicate the reduction capacity indicated by the commercial provider. (c) Visual presentation of volume occupied by TCEP immobilized on agarose beads in a volume of 40 μl

water (beads from Thermo Fisher, 15.3 ± 1.3 μmol functional TCEP per ml wet resin). The quantity of functional TCEP (as agarose-TCEP beads) is indicated. (d) Model peptides cyclized by disulfide bridges, used to test the reduction capacity of immobilized TCEP. (e) Model peptides (0.4 μmol) in 400 μl water (1 mM final conc.) were incubated with the indicated molar excess of functional agarose-TCEP (from ThermoFisher, 15.3 ± 1.3 μmol functional TCEP per ml wet resin determined by Ellman's reagent) for 3 h at RT, and analyzed by HPLC.

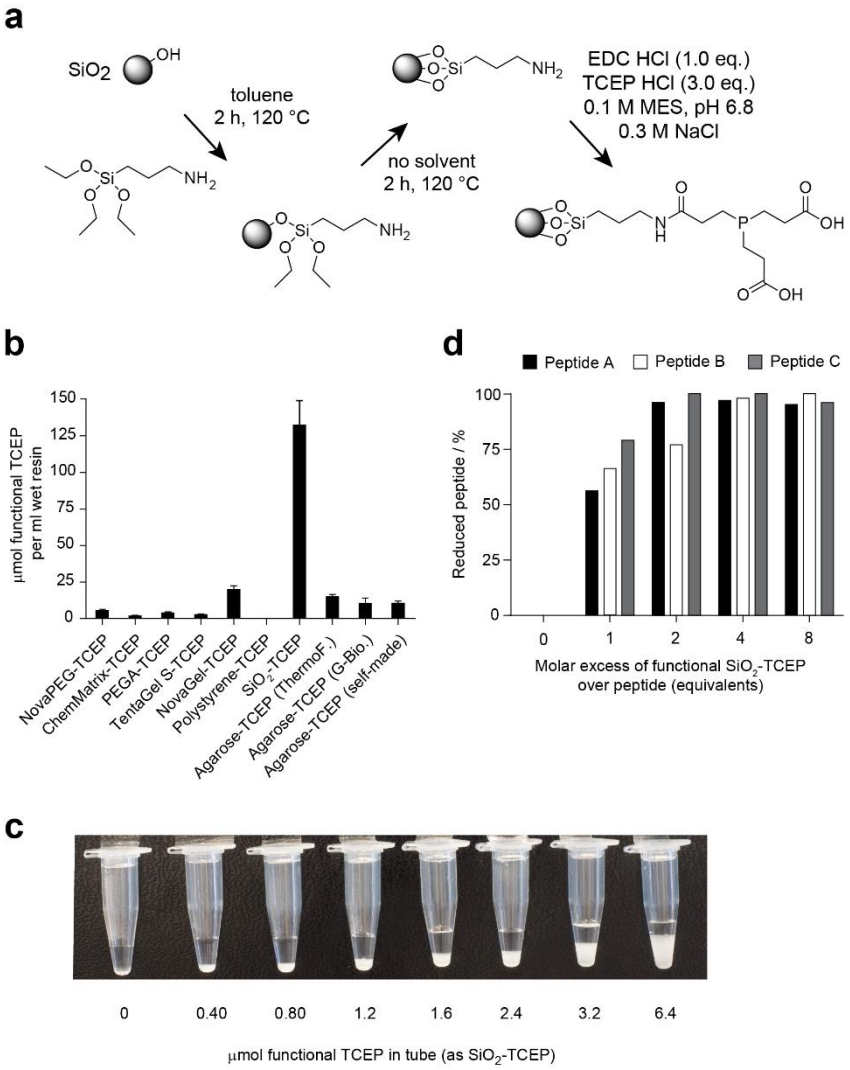


Figure 2. Immobilization of TCEP on silica beads and characterization. (a) Strategy for functionalizing silica with amino groups and subsequent functionalization with TCEP similar to the strategy reported by Alzahrani and Welham.[15] (b) Reduction capacity of ten different TCEP-functionalized solid supports. Six of the solid supports are peptide synthesis resins, one is silica gel, and three are based on agarose beads (two commercial, one custom-made). The capacity is indicated as µmol of functional TCEP per ml wet resin, determined using Ellman's reagent and using free TCEP as reference. Mean values and SDs of three measurements (technical replicates) are shown. For

silica gel-TCEP, the mean value and SD is shown for five different batches (Supplementary Table 1). (c) Visual presentation of volume occupied by TCEP immobilized on silica gel in a volume of 40 μl DMSO (131 ± 16 μmol functional TCEP per ml wet beads). (d) Model peptides (0.4 μmol) in 40 μl DMSO (10 mM final conc.) were incubated with the indicated molar excess of functional SiO_2-TCEP (batch with reducing capacity of 131 ± 16 μmol functional TCEP per ml wet resin determined by Ellman's reagent) for 3 h at RT, and analyzed by HPLC.

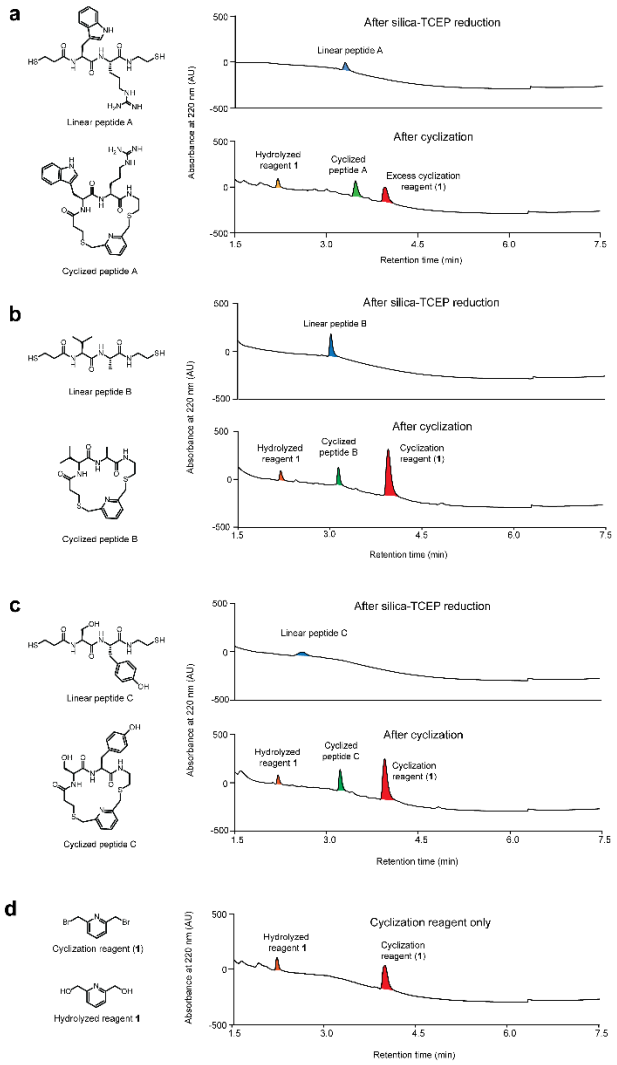


Figure 3. Alkylation of thiols after reduction with silica-TCEP beads. Three model peptides were reduced and cyclized with the bis-electrophilic reagent 2,5-bis(bromomethyl)pyridine (**1**). Peptides were analysed by LC-MS before and after alkylation. (a) Peptide A. (b) Peptide B. (c) Peptide C. (d) LC-MS analysis of cyclization reagent without peptide. Peaks of linear peptides (blue), cyclized peptides (green), cyclization reagent (red) and hydrolized cyclization reagent (yellow) are highlighted in color.

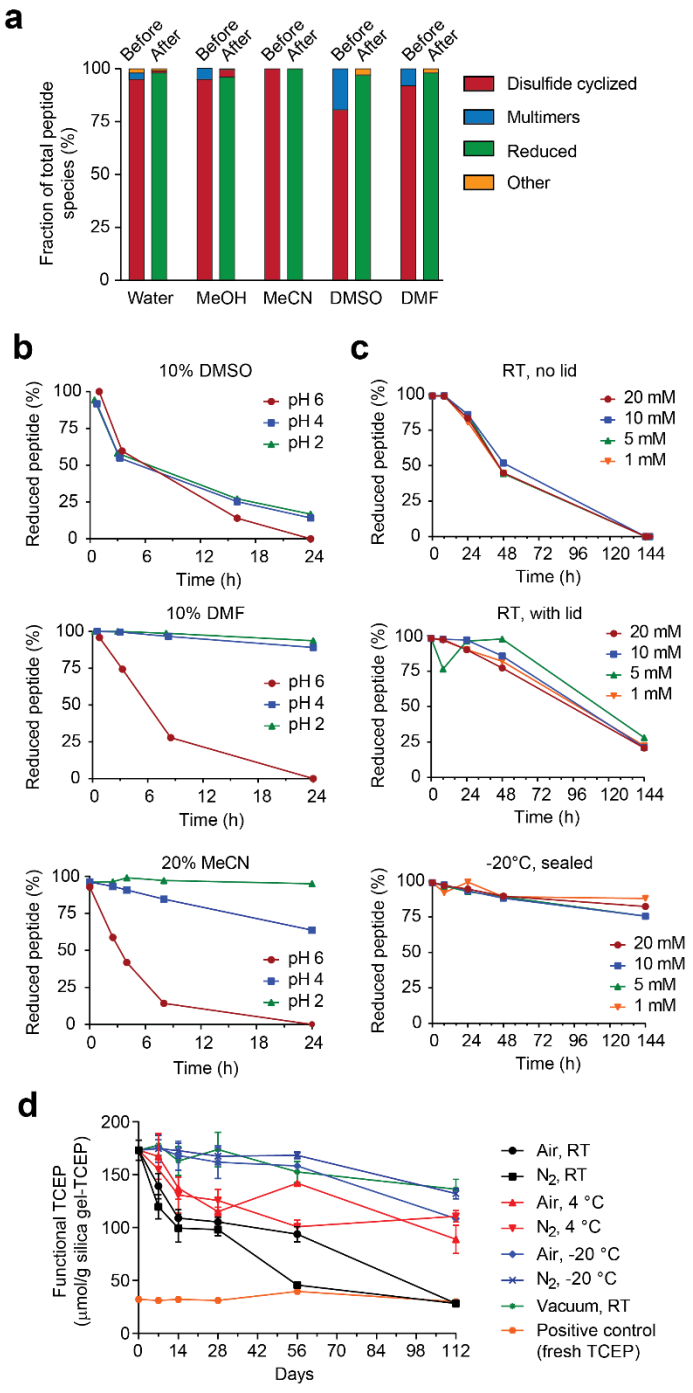
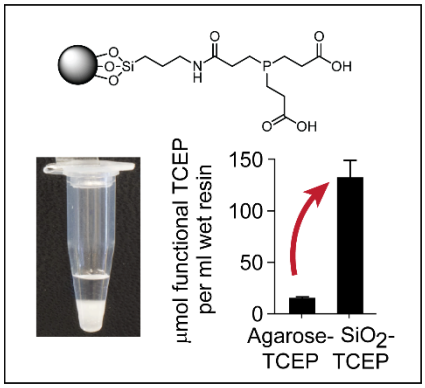


Figure 4. Solvent compatibility, back-oxidation of peptides, and stability of silica gel-TCEP. (a) Reduction of dithiol peptide by silica gel-TCEP in different solvents. Products were identified and quantified (absorbance at 220 nm) by LC-MS before and after reduction. Experiments were performed with peptide A

for all solvents except MeCN, for which peptide B was used for solubility reasons. (b) Back-oxidation assessed with model peptide G (1 mM; Supplementary Figure 4), incubated in mixtures of 60 mM NH_4HCO_3 buffer and the indicated organic solvents, at the indicated pH, and monitored over 24 hours. (c) Back-oxidation of model peptide C at different concentrations in DMSO, stored at the indicated temperatures in 384 PP microwell plates covered with the indicated lids, and monitored by LC-MS over 7 days. (d) Storage stability of silica gel-TCEP beads. For each time point and condition, vials containing 15 mg of beads (173 ± 9 μmol/g) were stored in triplicate and the reducing capacity analyzed at the indicated time points using Ellman's reagent.

Table of Contents

TCEP immobilized on agarose is widely used for efficient reduction of cysteines in peptides and proteins, but the reducing capacity is rather low. Herein, we have compared different solid supports for TCEP immobilization and found that silica-TCEP has an 8-fold higher reduction capacity, allowing thiol reduction of peptides at millimolar concentrations.



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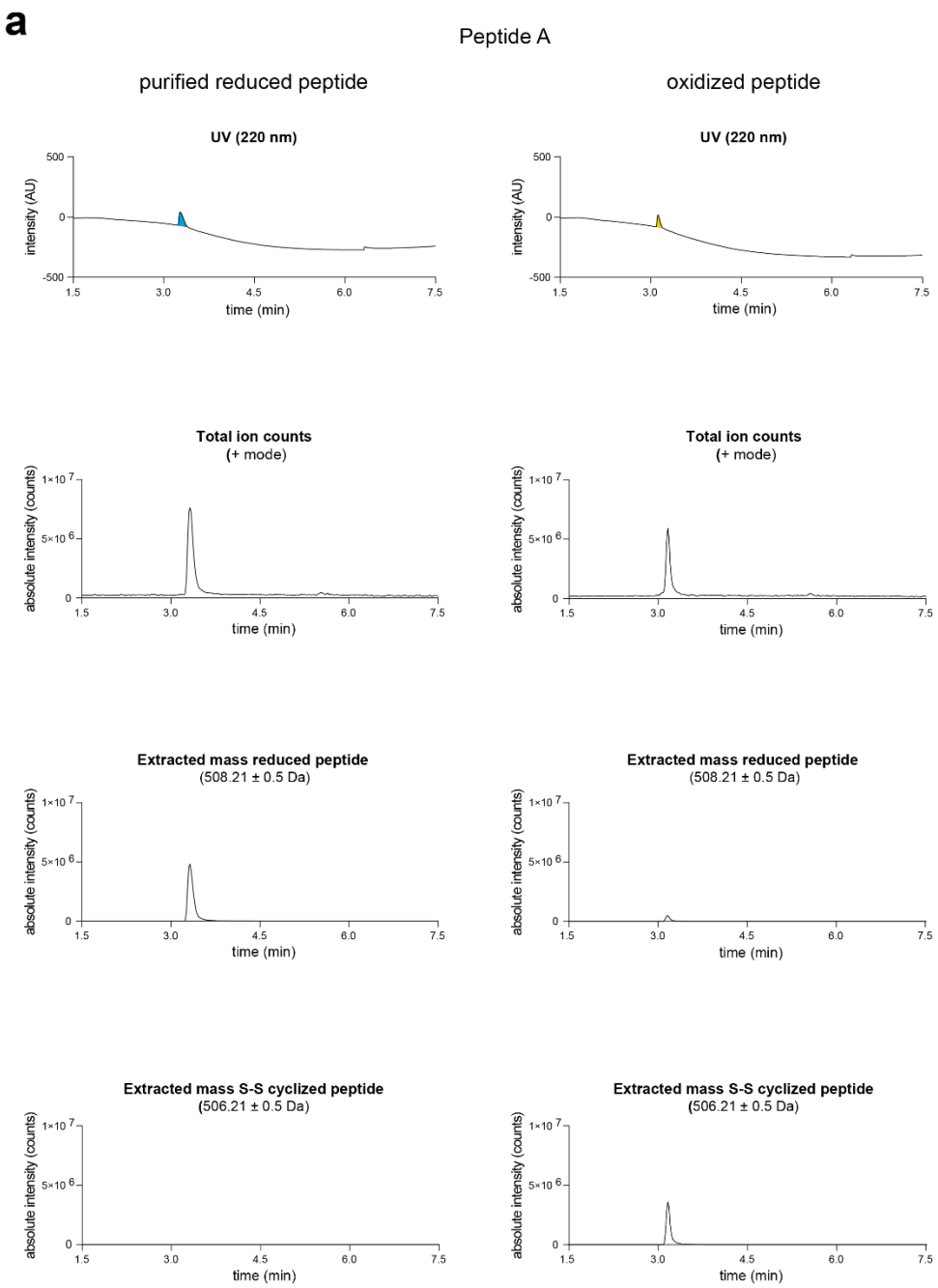
SUPPLEMENTARY TABLE

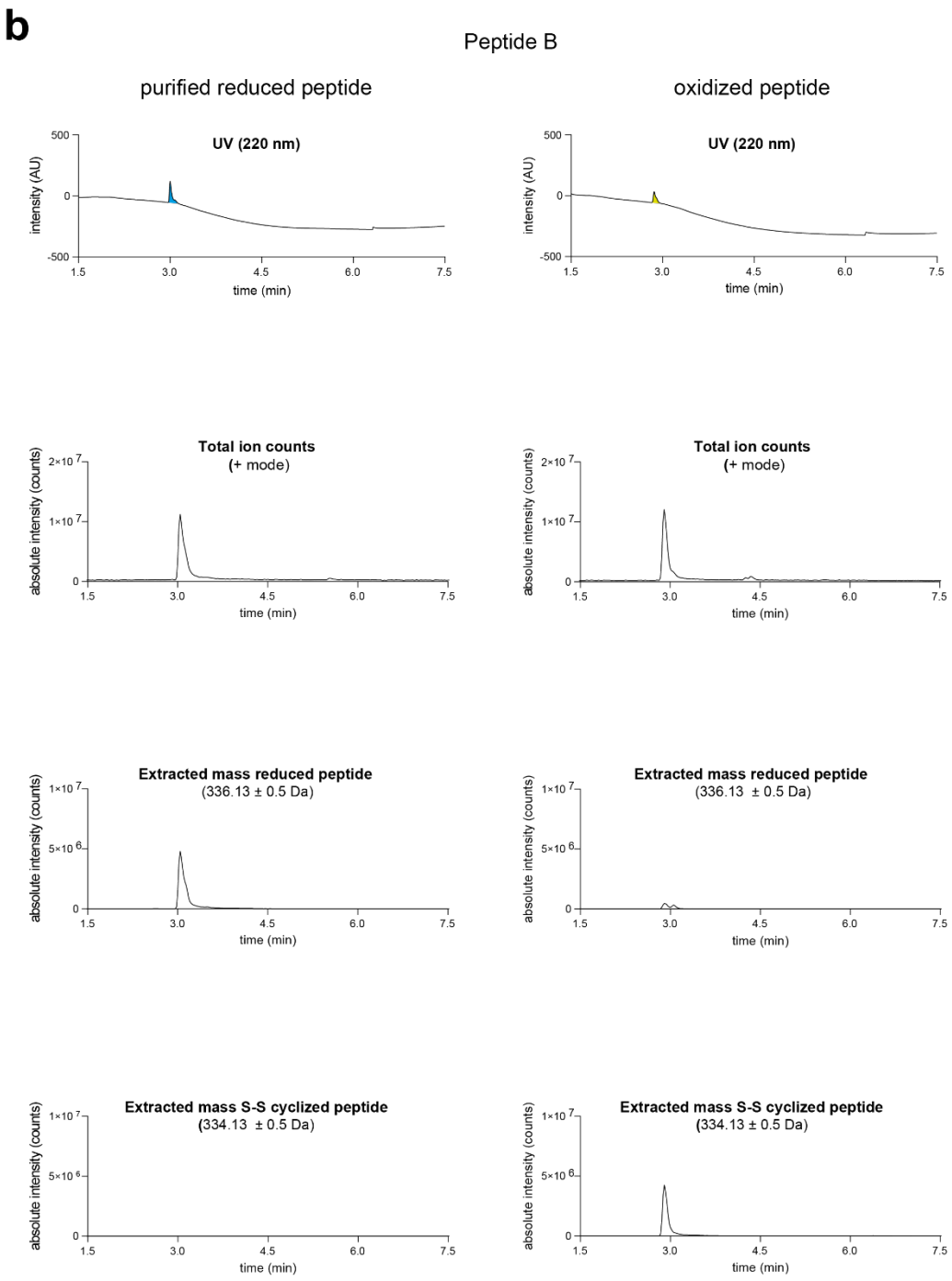
Supplementary Table 1. Reduction capacity of nine different TCEP-functionalized solid supports. Six of the solid supports are peptide synthesis resins, one is silica gel, and two are based on agarose beads. The capacity is indicated as μmol of functional TCEP per mg dry resin and μmol of functional TCEP per ml wet resin, determined using Ellman's reagent and using free TCEP as reference. The swelling factors of each different solid support for water is indicated. DMSO swelling factor was determined only for silica gel-TCEP beads. For agarose-TCEP, the DMSO swelling factor could not be determined (N.D.) due to the limited solubilization.

Solid support	Reducing capacity (μmol/g dry resin)	Reducing capacity (μmol/ml wet resin)	Swelling (H_2O)	Swelling (DMSO)
NovaPEG	63 ± 7.2	5.7 ± 0.7	11	N.D.
ChemMatrix	27 ± 1.9	2.5 ± 0.2	11	N.D.
PEGA	62 ± 13	3.9 ± 0.8	16	N.D.
TentaGel S	12 ± 0.4	3.4 ± 0.1	3.6	N.D.
NovaGel	40 ± 2	20 ± 2.5	2	N.D.
PS	0 ± 1	0.4 ± 1.0	1	N.D.
Silica gel (batch 1)	122 ± 11	107 ± 9.5	1.1	1.1
Agarose (Pierce)	230 ± 19	15 ± 1.3	15	n.c.
Agarose (G-Biosciences)	156 ± 56	10 ± 3.7	15	n.c.
Silica gel (batch 2)	134 ± 16	118 ± 14	1.1	1.1
Silica gel (batch 3)	149 ± 19	131 ± 16	1.1	1.1
Silica gel (batch 4)	158 ± 5	139 ± 4	1.1	1.1
Silica gel (batch 5)	173 ± 9	151 ± 8	1.1	1.1

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Quality control of purified peptides A to G (a-g) by LC-MS. The left panels show the analysis of reduced and purified peptide, and the right panels show the analysis of oxidized peptide. The top panels show UV traces recorded at 220 nm and the panels below the total ion counts recorded over the indicate LC elution times. Panels below show extracted masses of reduced and oxidize peptide.



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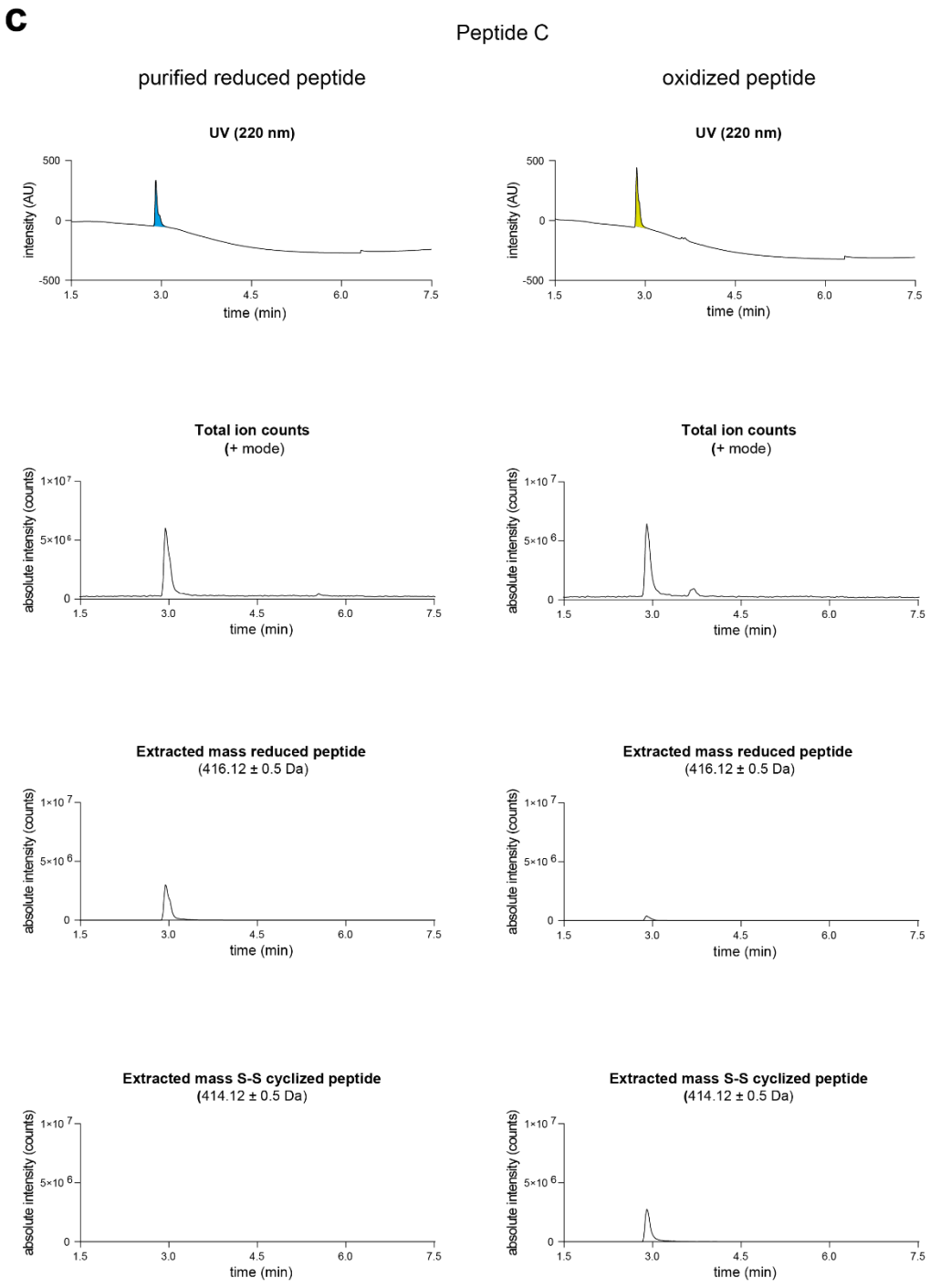

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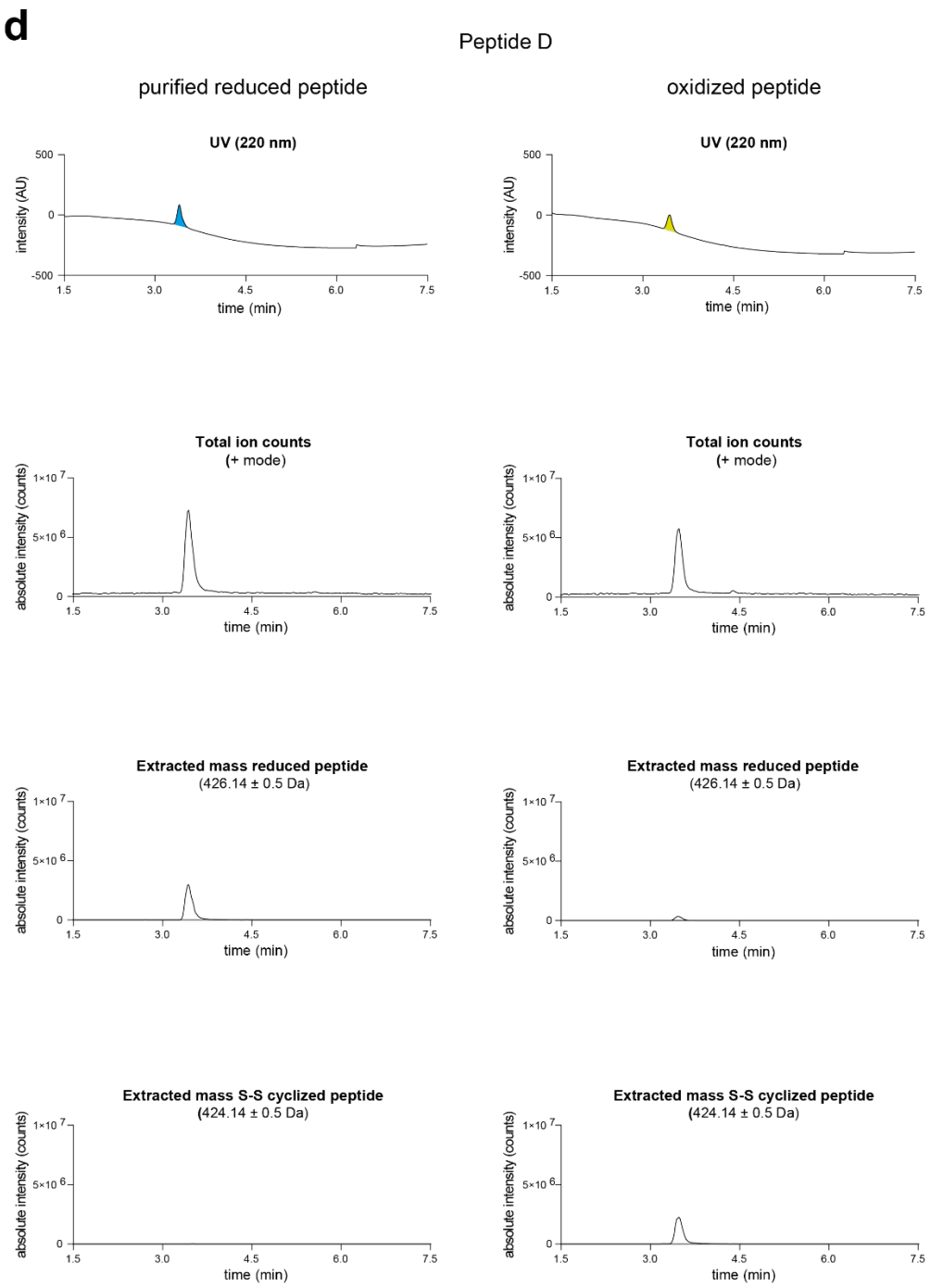

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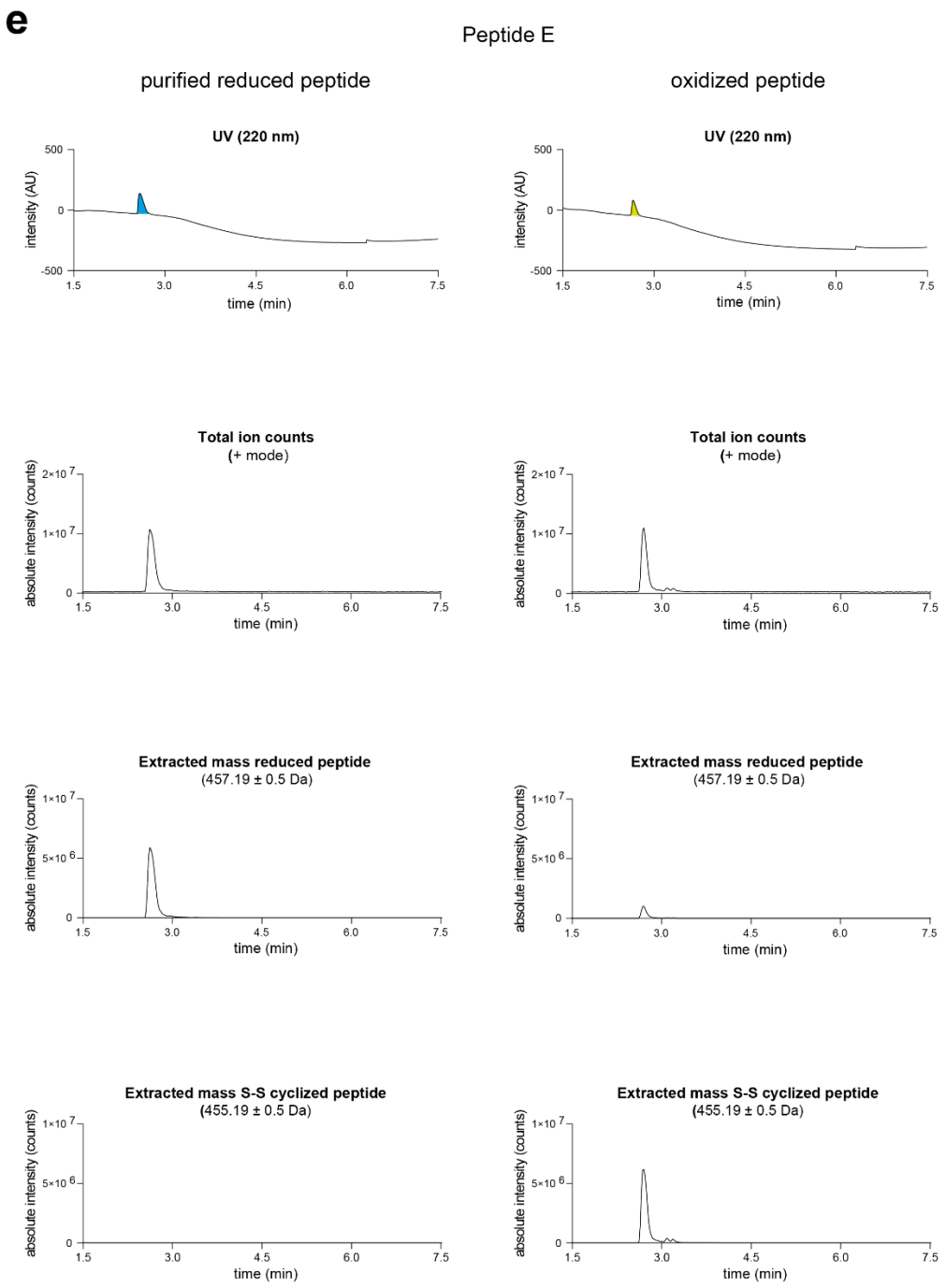

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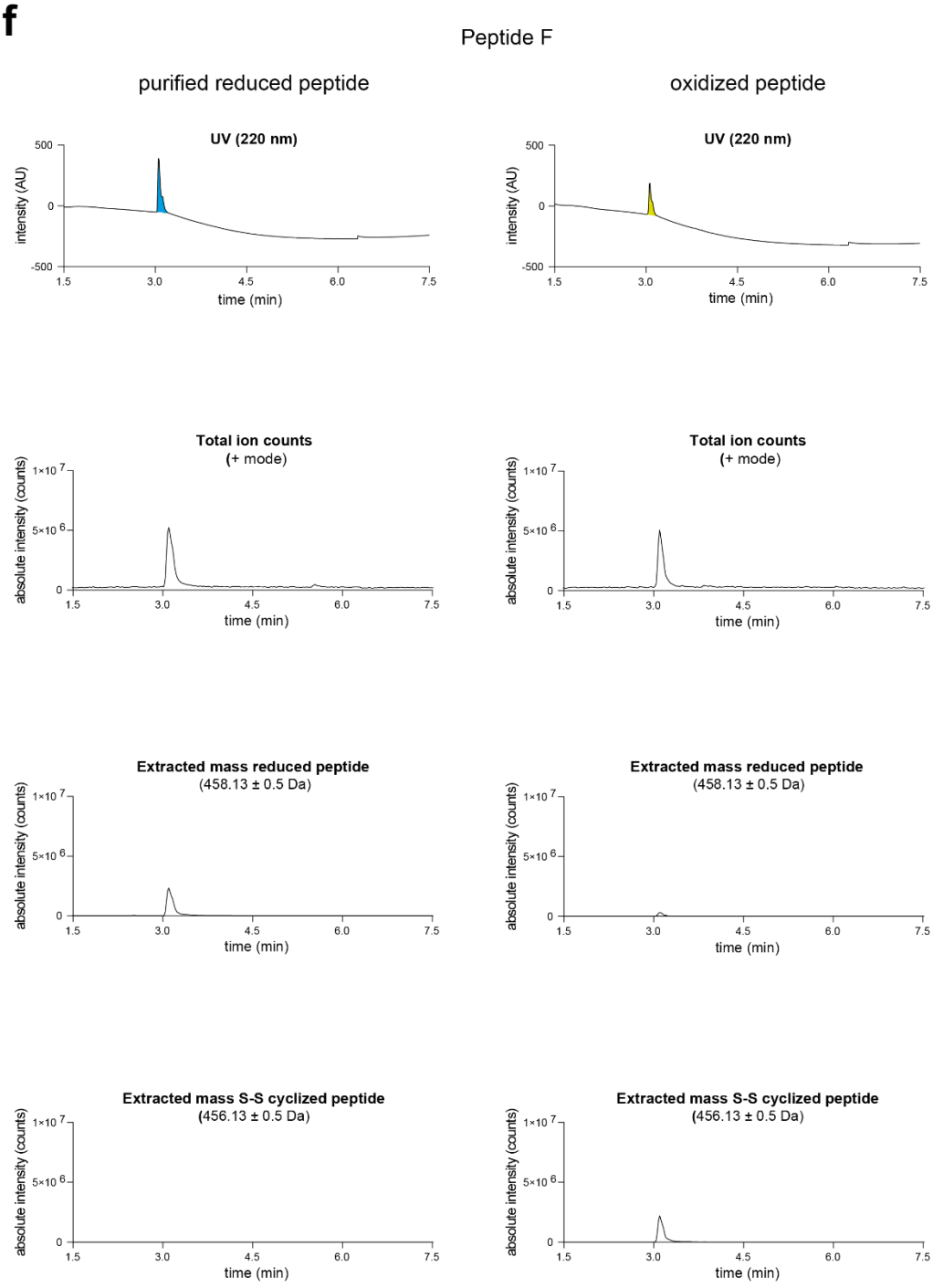

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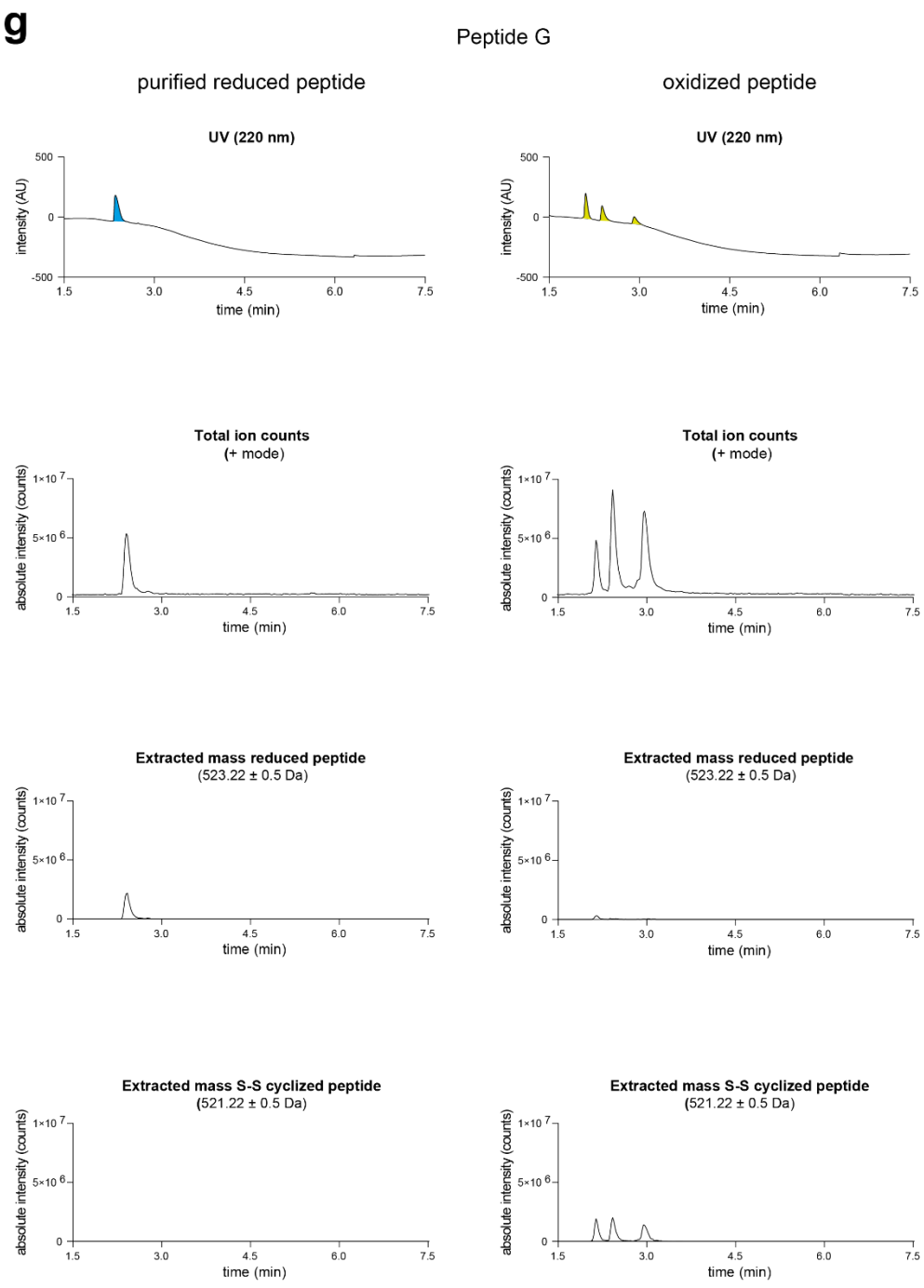

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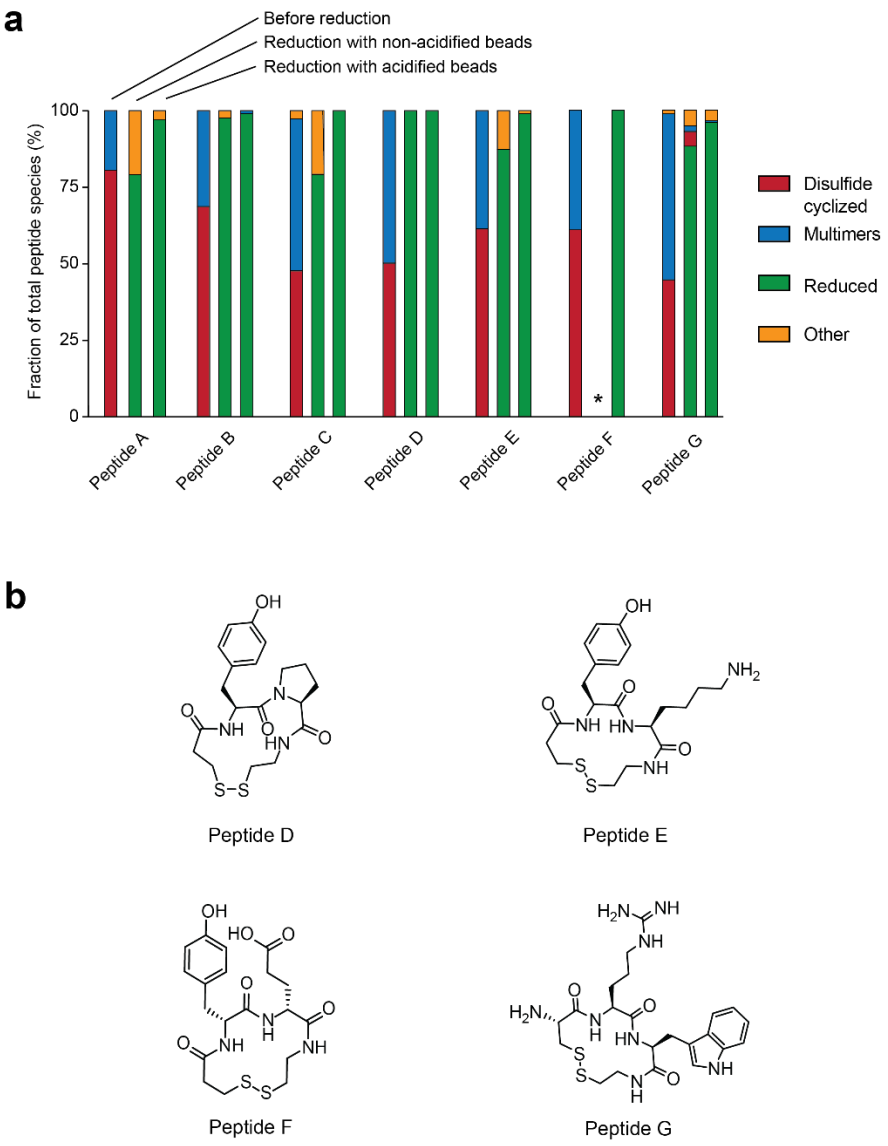
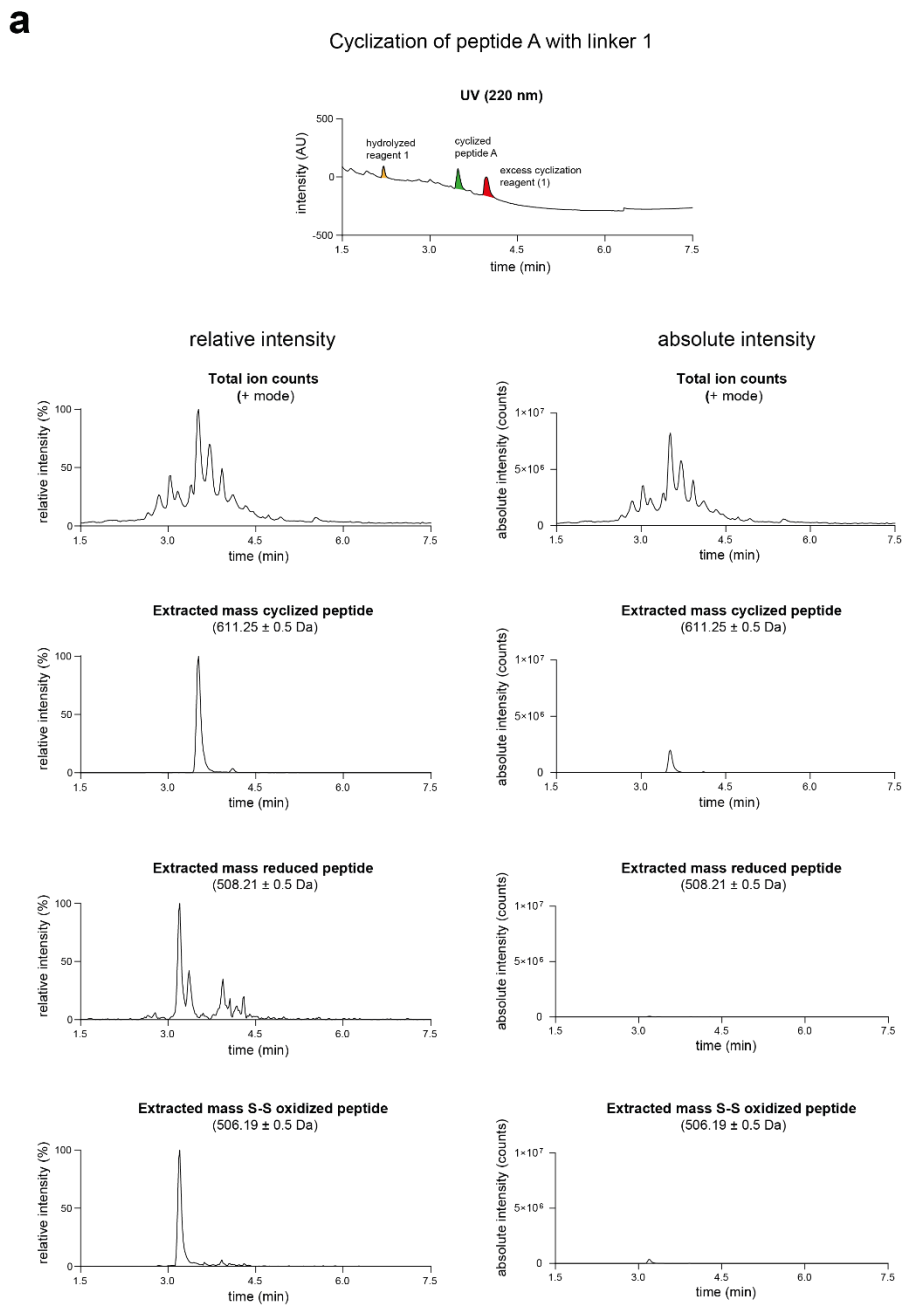
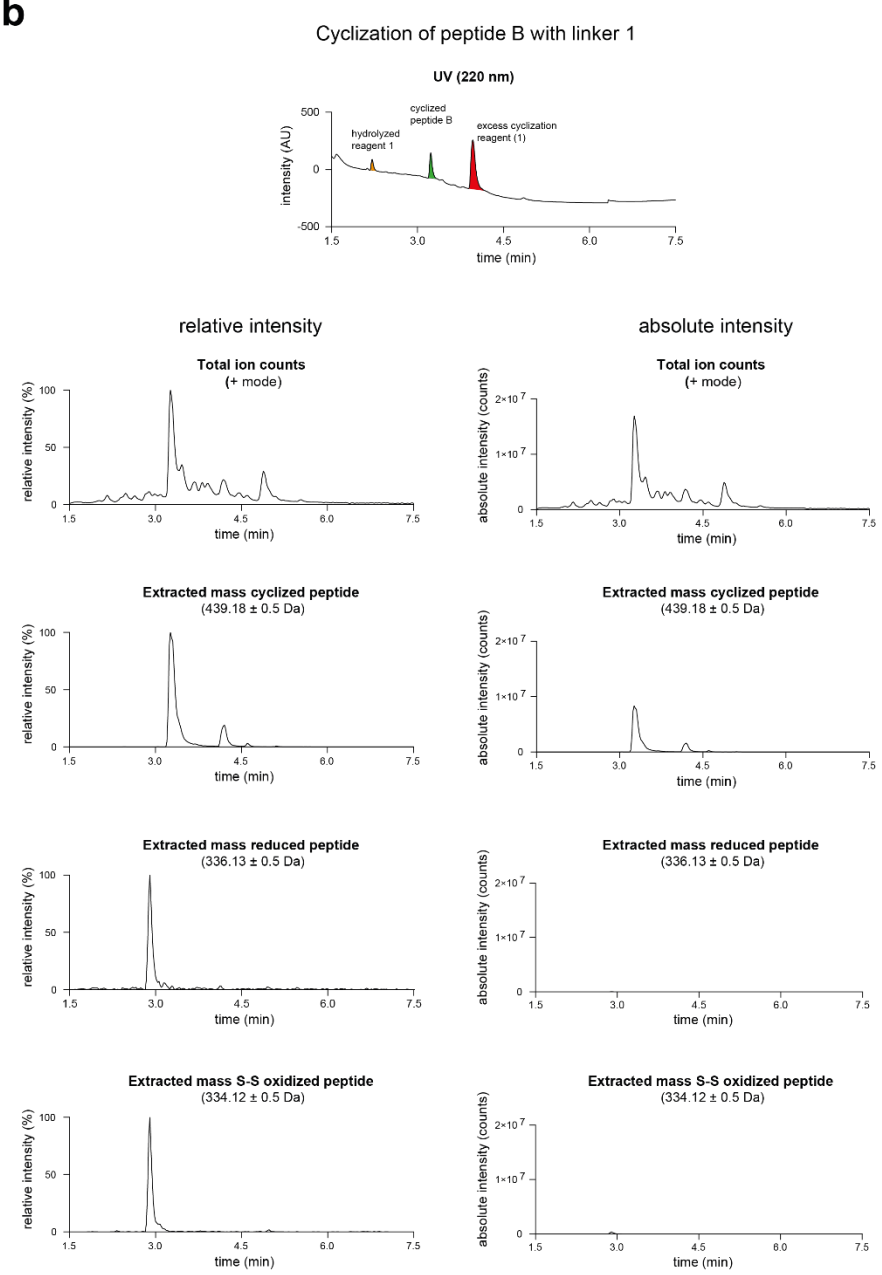


Supplementary Figure 2. Acidification of silica gel-TCEP. (a) Disulfide bridges of seven model peptides (mixtures of monomeric and multimeric species) were reduced using either non-acidified TCEP beads or acidified TCEP beads (40 μl samples, 10 mM peptide, 4 equiv. TCEP beads, 3 h, RT). The products were analysed by UHPLC-MS and quantified by the peak area at 220 nm. *) This peptide got stuck to the non-acidified beads, likely due to its negative charge. (b) Chemical structures of model peptides are shown in main figures.



Supplementary Figure 3. LC-MS analysis of cyclization reactions of peptides A to C (panels a-c) with cyclization reagent **1**. The top panel shows the UV traces recorded at 220 nm. The panels show the ion counts detected during the UHPLC runs as relative values (left; intensity relative to most intensive peak) and as absolute values (right; ion counts). The top MS panels show the total ion counts and those below the extracted masses for the cyclized, reduced and S-S oxidized peptide.

b



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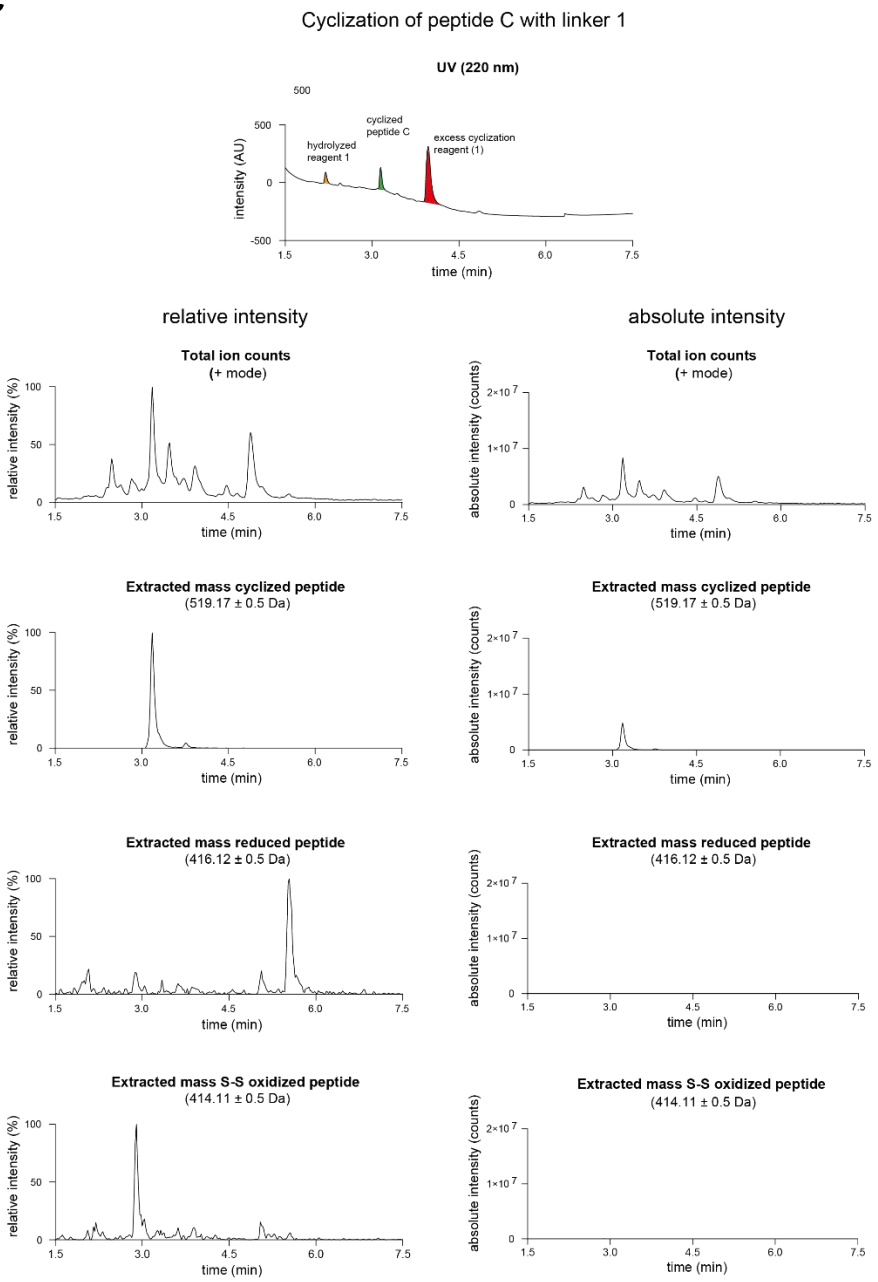

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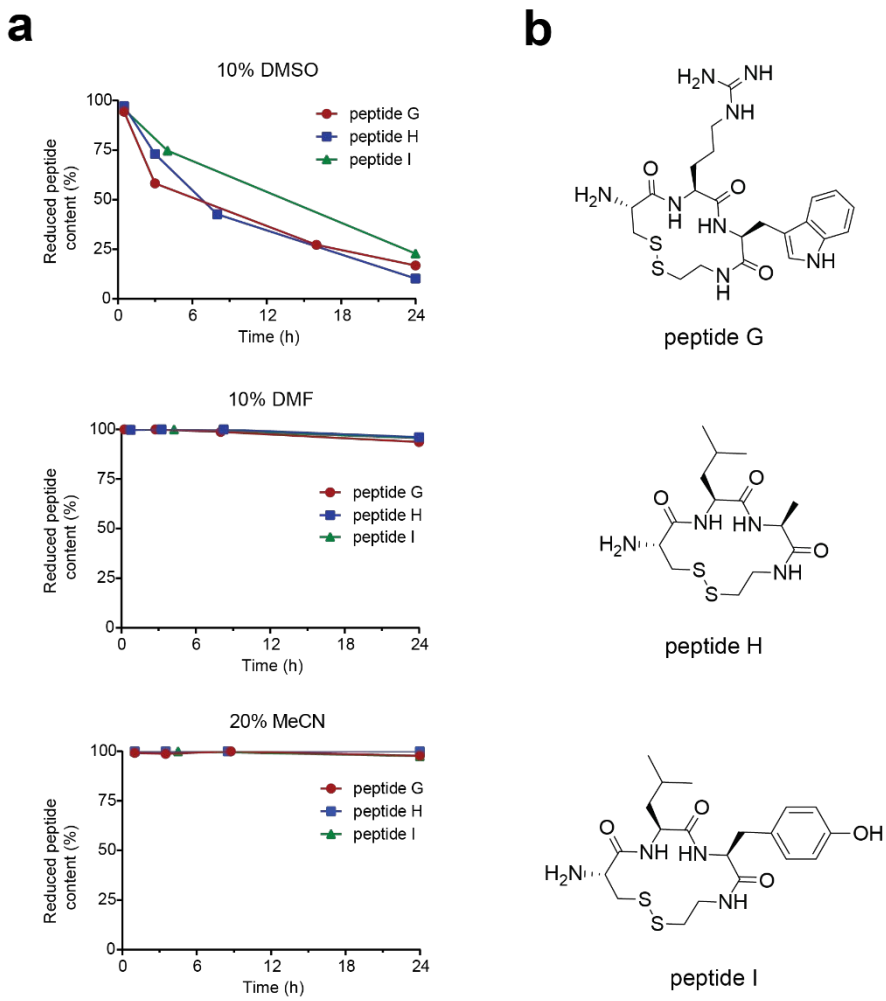
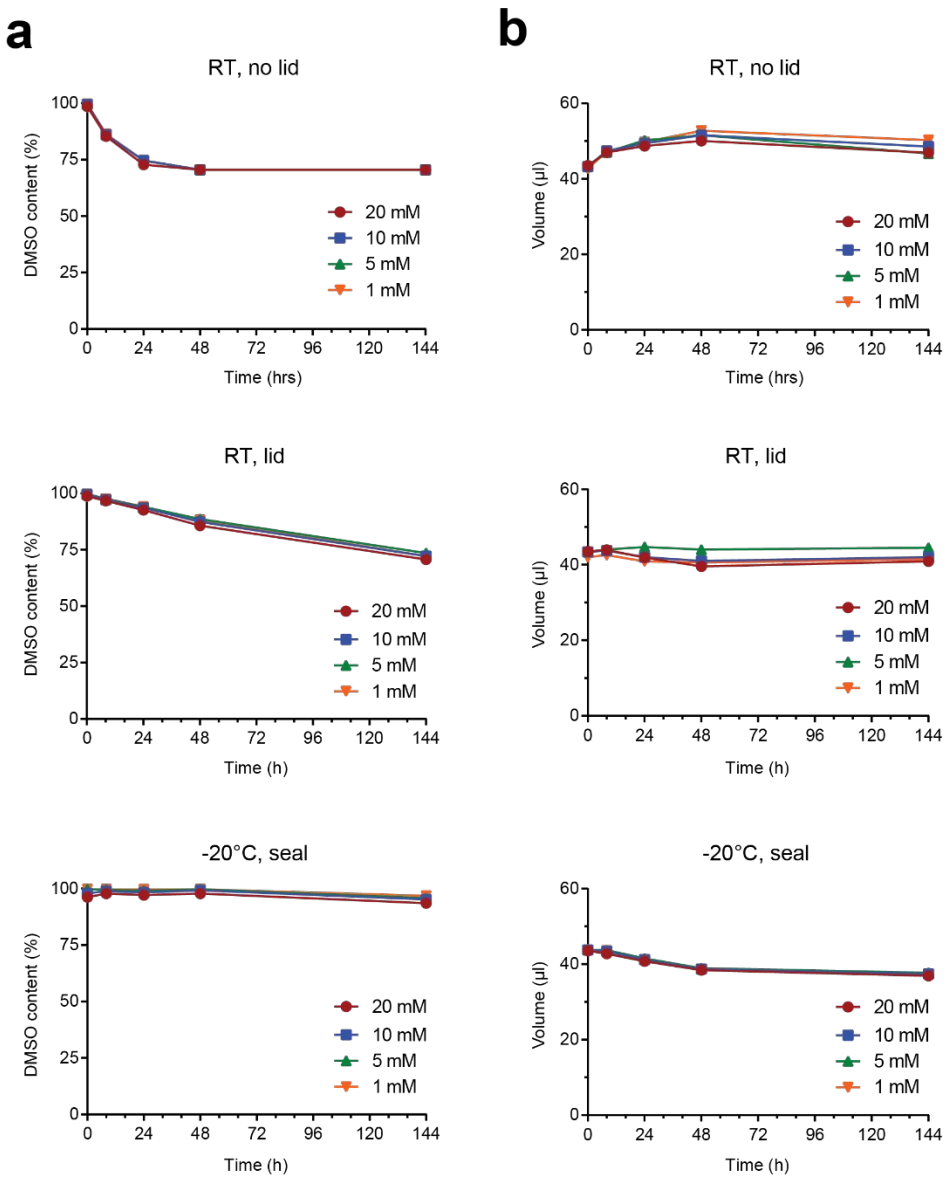


Supplementary Figure 4. Back-oxidation of peptides in different solvents and different pH. (a) Model peptides were incubated in mixtures of water and the indicated organic solvents, at the indicated pH, and monitored over 24 hours. (b) Chemical structures of model peptides applied.



Supplementary Figure 5. DMSO content and sample volumes of model peptides stored at different concentrations in DMSO and at different conditions. During storage at the indicated temperatures in microwell plates covered with the indicated lids, the samples took up water (DMSO content decrease, hygroscopic), which was measured over a time period of 7 days using acoustic waves. (a) DMSO content. (b) Sample volume.