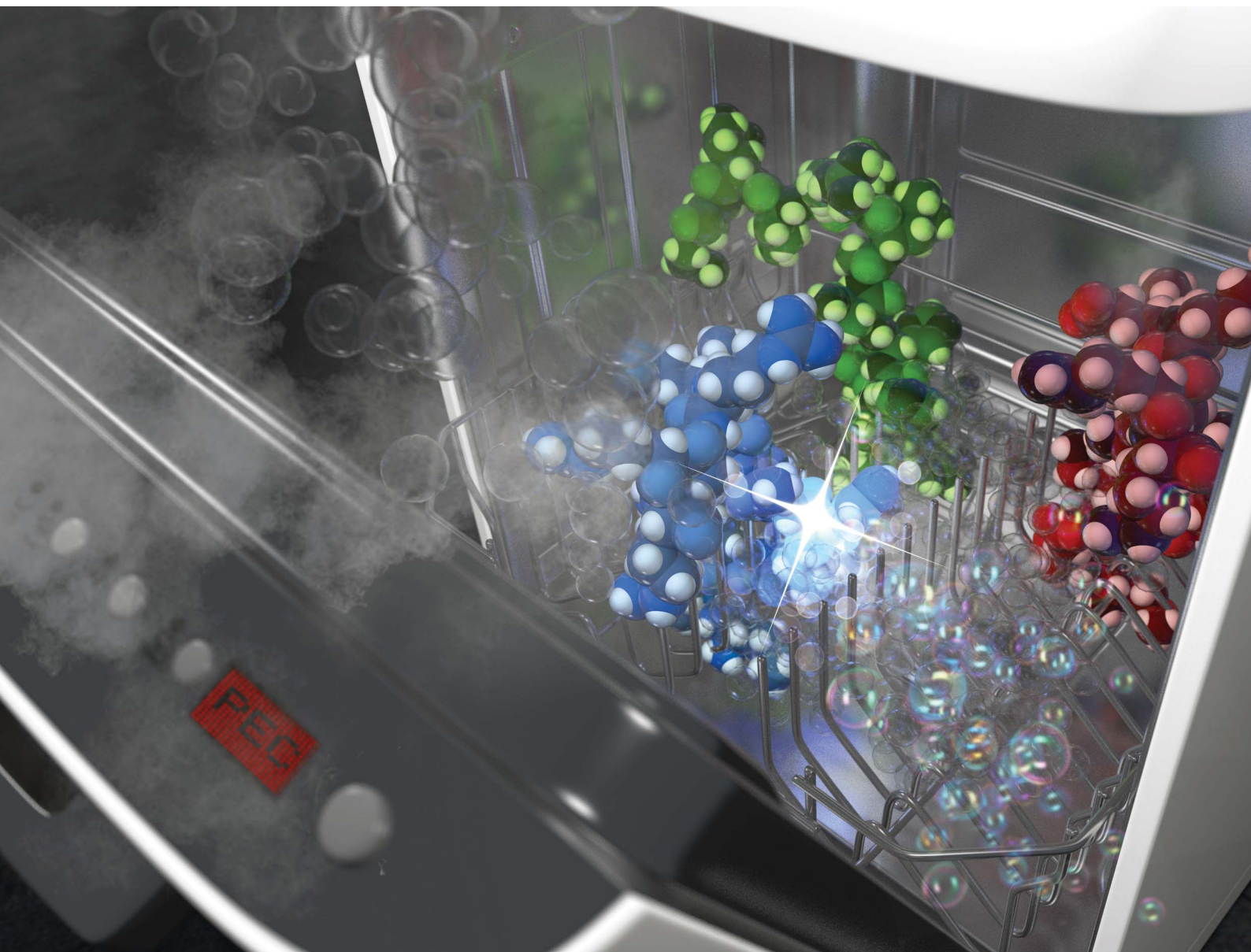


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Traceless parallel peptide purification by a first-in-class reductively cleavable linker system featuring a safety-release†

Robert Zitterbart,^{‡*} Nadja Berger,^{‡*} Oliver Reimann,^{‡*} Gavin T. Noble,^{‡*} Stephan Lüdtkke,^a Dominik Sarma^{‡*} and Oliver Seitz^{‡*}

Hundreds of peptides can be synthesized by automated parallel synthesizers in a single run. In contrast, the most widely used peptide purification method – high-pressure liquid chromatography (HPLC) – only allows one-by-one processing of each sample. The chromatographic purification of many peptides, therefore, remains a time-consuming and costly effort. Catch-and-release methods can be processed in parallel and potentially provide a remedy. However, no such system has yet provided a true alternative to HPLC. Herein we present the development of a side-reaction free, reductively cleavable linker. The linker is added to the target peptide as the last building block during peptide synthesis. After acidic cleavage from synthetic resin, the linker-tagged full-length peptide is caught onto an aldehyde-modified solid support by rapid oxime ligation, allowing removal of all impurities lacking the linker by washing. Reducing the aryl azide to an aniline sensitizes the linker for cleavage. However, scission does not occur at non-acidic pH enabling wash out of reducing agent. Final acidic treatment safely liberates the peptide by an acid-catalysed 1,6-elimination. We showcase this first-in-class reductively cleavable linker system in the parallel purification of a personalized neoantigen cocktail, containing 20 peptides for cancer immunotherapy within six hours.

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Introduction

Peptides have high therapeutic potential due to their pivotal role in nature. Currently, 60 approved peptide drugs are on the market and 157 active clinical trials are ongoing.^{1,2} Yet, peptide-based medicines have limitations, such as short plasma half-life and low oral bioavailability.² To enhance pharmacokinetic and pharmacodynamic properties many peptides that enter the clinic today are chemically altered.² The trend to longer and chemically modified peptides imposes increased challenges for peptide synthesis and purification. Solid-phase peptide synthesis (SPPS)³ is the preferred method of peptide production, and reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used method for peptide purification.⁴ Commercially available automated peptide synthesizers allow the parallel synthesis of several hundreds of peptides in a single run. HPLC, in contrast, is a linear process and even with the most modern set-up, requires over 15 min per

peptide rendering purification the bottleneck in peptide manufacturing.⁵ As a consequence, researchers mostly employ crude peptide libraries and, as such, accept higher risk for false-positive responses, which hampers the speed of pharmaceutical development.^{6,7}

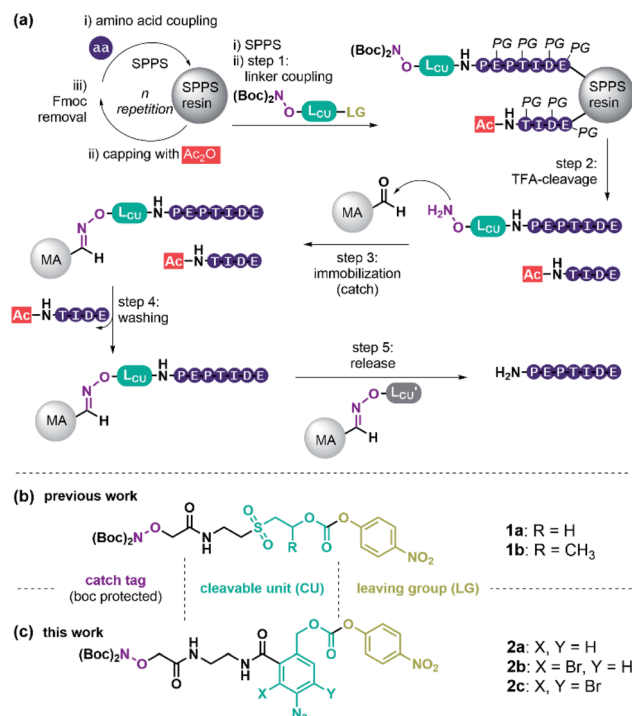
Catch and release (c&r) methods could facilitate the purification process in parallel, since precise pumps, massive columns, collector units, and high solvent volumes become obsolete. The great potential of c&r was first recognized by Krieger, Erikson and Merrifield in 1976.⁸ A key component of c&r (Scheme 1a) is a purification tag that is usually installed as the last residue at the N-terminus during SPPS. Employing capping steps for acetylation of truncations, arising from incomplete amino acid coupling, allows the selective installation of the purification tag onto the full-length product. Subsequently, the target peptide is immobilised onto a solid support (catch step) *via* interactions with the purification handle. Washing removes all non-tagged substances like capped truncations, salt burden, or protecting group debris. Finally, a chemical stimulus releases the purified peptide from the support (release step). For example, treatment with imidazole or acid has been used to liberate hexahistidine-tagged peptide from metal affinity solid supports.^{9,10} Alternatively, *O*-methoxyamine was used for releasing peptides captured by means of thiazolidine/oxazolidine¹¹ or oxime-forming reactions.¹²

^aBelyntic GmbH, Richard-Willstätter-Str. 11, 12489 Berlin, Germany. E-mail: Robert.Zitterbart@belyntic.com

^bBachem (UK) Ltd., Delph Court, Sullivans Way, St. Helens, Merseyside WA9 5GL, UK
^cHumboldt-Universität zu Berlin, Brook-Taylor-Str. 2, 12489 Berlin, Germany

† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0sc06285e

‡ Contributed equally.



Scheme 1 (a) General scheme of oxime-based c&r purification of peptides. (b) Molecular structure of previously reported base-labile linker molecules **1a**, **1b** and (c) of the novel reductively cleavable linker units **2a**, **2b** and **2c** described in this paper. aa: amino acid; PG: protecting group; MA: modified agarose beads; LCU: cleavable linker unit; LG: leaving group.

Instead of utilising peptide residues for immobilisation that remain in the product, purification handles which represent tracelessly cleavable linkages offer more versatility. We identified 30 independent linker-based c&r systems, that may be categorised by their cleavage stimulus. Acid-labile c&r linkers based on 4-alkyl- or 4-alkoxybenzyloxycarbonyl ethers^{13,14} were used to capture fully protected crude peptides, however, dissolution and handling of fully protected peptides can be challenging. Base-labile linkers allow processing of unprotected crude peptides obtained after standard TFA cleavage, which typically have higher solubility than their protected counterparts. Base-labile scaffolds such as β -sulfonylethoxycarbonyl^{15–22} and fluorenylmethoxycarbonyl (Fmoc)-linkers^{23–26} provided TFA-stability while allowing cleavage upon basic conditions. Both required pH > 11 for cleavage, which can be harmful to specific peptide sequences. We previously introduced a method termed Peptide Easy Clean (PEC, Scheme 1), that used a β -sulfonylethoxycarbonyl linker to purify pharmaceutically relevant peptides in high yield and purity.²⁷ However, base-induced side reactions, such as oxazolidinone-formation were observed using linker **1a** or **1b** (Scheme 1b) with certain peptides, questioning the general applicability of this c&r system.

Some c&r linkers rely on nucleophile-induced cleavage. Typical linkers included phenyl sulfonate,²⁸ dimedone-enamines (Dde)-type^{29–31} and keto-acyl linkers,^{32,33} which are

susceptible to cleavage upon treatment with internal Cys residue, hydrazine/NH₂OMe³² or *o*-phenylenediamine,³³ respectively. However, with exception of Cys residue-mediated cleavage of the phenyl sulfonate linker,²⁸ the cleavage agents remain in the product, and a subsequent purification step such as HPLC is required to obtain a “cleavage agent-free” product. Contamination with release agents also accompanies linker cleavage reactions induced by electrophiles such as CNBr,^{8,34} or oxidizing agents such as sodium periodate.^{35,36} Light is a reagent-free trigger for the release of *ortho*-nitrobenzyl-carbamate linkers and has been reported.^{37,38} However, upscaling is difficult due to the limited penetration of light through solid- or gel-based capture materials.

Surprisingly, no reductively cleavable linker has yet been described for peptide purification. Reductively cleavable linker molecules could enable widespread application of c&r within peptide manufacturing since reductive conditions are generally mild and compatible with all canonical residues.

Katzenellenbogen *et al.* introduced a 4-amidobenzyl carbamate prodrug in 1981 that liberated the model drug aniline after tryptic cleavage of the 4-amido-group.³⁹ The resulting amino-group triggered a 1,6-elimination towards azaquinone-methide, CO₂, and aniline.⁴⁰ Similar concepts with reductive stimuli followed.^{41,42} Machida *et al.* brought this concept to the realm of oligomer synthesis, by developing a C-terminal linker to screen and release a library of peptides conjugated to peptide nucleic acids (PNAs).⁴³ The PNA-peptide-PNA sequences were synthesized on a novel C-terminal *para*-azido benzyl carbamate linker on water-swallowable amino-methyl resin, whereas over 1000 peptide sequences were generated by split-and-mix.⁴⁴ After TFA-cleavage the linkage stayed intact enabling on-bead screening, where the PNA hybridization formed a hairpin structure presenting the peptides. Positive hits were analysed by azide reduction with PMe₃ of the linker leading to 1,6-elimination and release from support.

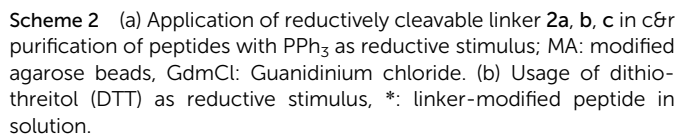
Herein we report the development of a first-in-class reductively cleavable linker molecule for peptide purification that provides unprotected peptides without contamination of cleavage agents. The linker contains a *para*-azidobenzyl carbamate core **2** (Scheme 1c). This molecular arrangement enables a safety-release reaction sequence, in which azide reduction is used to activate the linker for mild acid-induced fragmentation. Of note, reducing agents can be removed by filtration prior to release enabling a side-reaction free purification of SPPS peptides *via* a truly traceless c&r process.

Results and discussion

The first reductively cleavable linker for peptide purification

To retain the working principles of linker coupling and immobilisation from our previously published base-labile linker (Scheme 1b), we constructed the new linker with an oxyamine catch tag, a *para*-nitrophenol leaving group, and a reductively cleavable *para*-azido-benzyl unit (Scheme 1c). The reductively cleavable linker **2a** was accessible in gram-scale *via* a four-step synthesis from 6-amino-phthalide (Scheme S1†).





Next, we sought a reagent that effectively reduces the *para*-azido benzyl moiety to its aniline derivative. Triphenylphosphine (PPh₃) has frequently been used in Staudinger

A TFA cleavage with a fraction of each peptidyl resin was performed after solid phase synthesis. Ultra-performance liquid chromatography (UPLC) analyses revealed impurities, mostly from capped truncations that were present prior to the introduction of the cleavable capture tags (Fig. 1(i)). In case of **P4**, these impurities were closely or co-eluting with **P4**. After coupling of the capture tags **1a** and **2a** and TFA-cleavage, the main products were in all cases the linker-modified target peptides (Fig. 1(ii)). However, a substantial amount (~30%) of

^a Bold underlined motif underwent base-induced side reactions earlier.
^b The mass deviation to the native peptide when side product is formed.

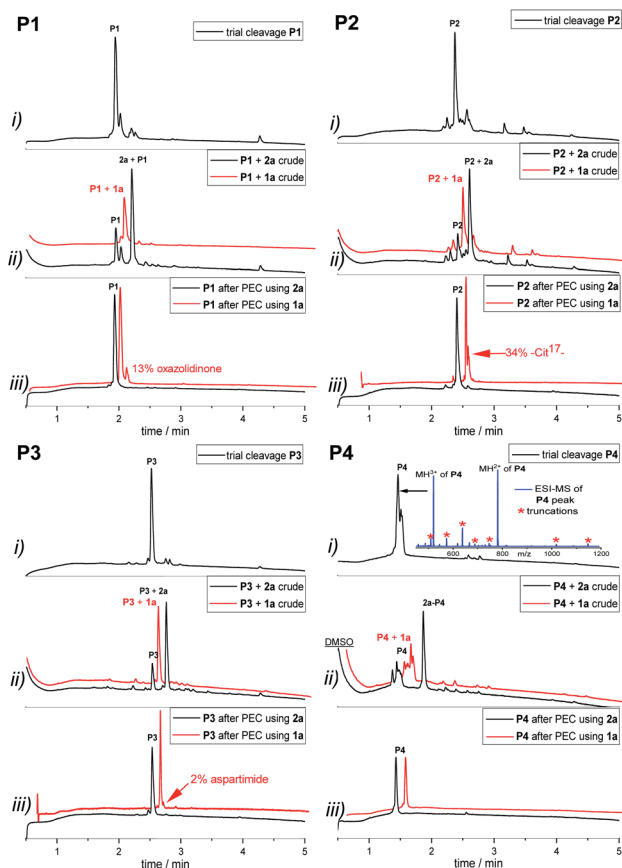


Fig. 1 PEC-purification of base-sensitive peptides **P1–3** and Histone H3 (1–15) peptide **P4**. (i) Trial cleavage of peptide before linker coupling; (ii) full cleavage after linker coupling of **1a** or **2a**, (iii) after PEC-process using **1a** or **2a**. *Assigned masses of co-eluting truncated peptides in **P4** trial cleavage; red traces using base-labile **1a** and black traces using reductively cleavable linker **2a**.

unmodified peptide remained when employing linker **2a**. Next, the target peptides were bound to the solid support (modified agarose, MA), while most impurities remained in the supernatant (Fig. S2–S5†).

After washing the purified peptides were released by basic treatment with MeNH₂ (pH 11) when **1a** or by reduction with PPh₃ when linker **2a** was used. Base induced side reactions, such as oxazolidinone formation (13%) at the N-terminal threonine of **P1**, citrulline formation (34%) at arginine in **P2**, and aspartimide formation (2%) in **P3**, were confirmed. Gratifyingly, the formation of side products did not occur when the peptides were detached *via* PPh₃ reduction in combination with linker **2a** (black traces of products in **P1–3** Fig. 1(iii)). Independent of the linker system used, for **P4**, no side-products or co-eluting impurities remained after purification.

Moreover, the comparative study showed that the new reductively cleavable linker **2a** yields higher peptide purity than the base-labile linker **1a** (Table 2). However, in contrast to the purities, recoveries of peptides purified with **2a** were lower (22–40% compared with 32–74% with **1a**). As mentioned above, UPLC traces of crudes obtained after introduction of linker **2a**, showed around 30% unmodified peptide (Fig. 1(ii)) even though

Table 2 Results of PEC purification of peptides **P1–4**

No.	Crude purity ^a	Purity ^a after PEC using linker				Recovery ^b using linker			
		1a	2a	2b	2c	1a	2a	2b	2c
P1	67%	77%	91%	91%	91%	74%	40%	74%	44%
P2	42%	67%	83%	89%	92%	60%	40%	70%	42%
P3	75%	84%	88%	87%	88%	46%	23%	56%	32%
P4	36%	92%	89%	96%	91% ^c	32%	22%	58%	29% ^c

^a Purity determined by integration of UPLC-MS traces at 210 nm. ^b (Final weight × purity after PEC)/(molecular weight × synthetic scale × crude purity) see S3.6. ^c Purity and recovery with dithiothreitol as reducing agent.

coupling of the linker proceeded quantitatively according to a negative chloranil test.⁴⁷ This pointed to a loss of linker during TFA cleavage.

To test the assumption, we analysed the linker stability in solution. The linker was coupled to *n*-propylamine and the resulting conjugate **9a** (Fig. 2a) was subjected to TFA cleavage conditions. UPLC analysis (Fig. 2b, black lines) exposed a vulnerability of the benzyl carbamate structure.

We assume that a cleavage mechanism⁴⁸ similar to the acidic deprotection of the carboxybenzyl (Cbz) protecting group (Fig. 2a) takes place.⁴⁹ In that case, protonation of the urethan moiety **9** facilitates a nucleophilic attack at the benzylic position, inducing the detachment of *N*-propyl-carbamic acid, subsequently decomposing to CO₂ and propylamine. The *ortho*-amide may provide anchimeric assistance and promotes the scission of the benzylic carbamate.

2nd generation reductively cleavable linker molecules

We expected the acid stability of the linker to correlate with the electron density of the benzylic carbon atom. Using Hammett-values, we reasoned that the electron-withdrawing properties of the *ortho*-amide (σ_{op} : 0.35)⁵⁰ and *para*-azide (σ_p : 0.08)⁵⁰ did not sufficiently destabilize the transition state **10a** (Fig. 2a) to hinder decomposition in TFA.

An additional electron-withdrawing substituent was therefore required to increase the TFA stability. From the rich portfolio of listed Hammett values, we chose halogenation due to the simplicity of introduction and its relatively high electron-withdrawing effect (*e.g.* Br(σ_m): 0.39).⁵⁰ The synthesis of 2-bromo (**2b**) and 2,4-di-bromo (**2c**) linker followed the same scheme as linker **2a**, except starting with an additional bromination step of 6-amino phthalide using either one or two equivalents of *N*-bromosuccinimide (NBS, Schemes S2 and S3†). The stability test with linker-coupled *n*-propylamine confirmed an increased TFA-stability for the mono- **9b** and di-bromo carbamate **9c** (Fig. 2b). After 2 hours of treatment with TFA cocktail reagent K,⁵¹ 92% of **9b** and 97% of **9c** remained intact (Fig. S10†). The stability for all linker conjugates was higher in TFA/TIS/H₂O cleavage cocktails (Fig. 2b).

Encouraged by these results, we repeated the purification of peptide **P1–4** with the stability-enhanced linkers **2b** and **2c**. The



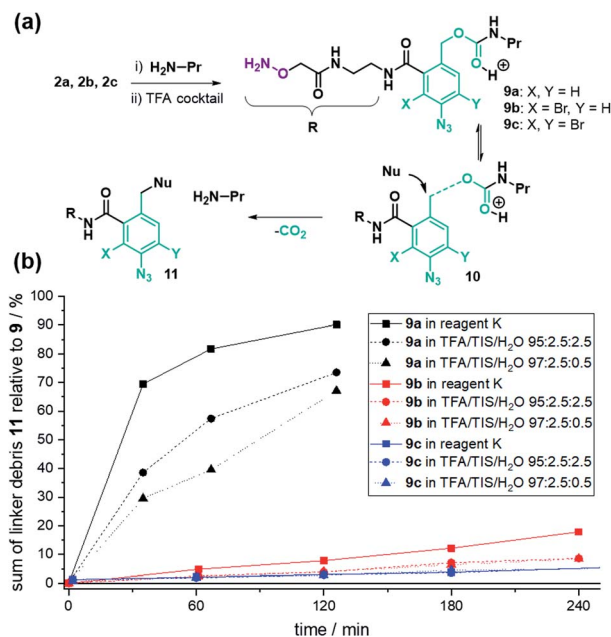


Fig. 2 (a) Generation of propylamine carbamates **9a–c** from **2a–c**. (b) TFA-stability analysis of carbamates **9a–c** relative to the sum of UV-UPLC integrals of TFA-cleavage products **11**. reagent K: TFA/H₂O/PhOH/PhSMe/ethanedithiol 82.5 : 5 : 5 : 5 : 2.4.

absence of unmodified peptides after global TFA deprotection provided testimony for the increased TFA-stability using the brominated linkers **2b** and **2c** (Fig. S2–S5†). Accordingly, after PEC purification, the amounts of obtained peptides were twice as high on average when using mono-bromo linker **2b** compared with linker **2a** (Table 2). Surprisingly, with di-bromo linker **2c** the recoveries of the PEC-process were as low as with linker **2a**. We assumed that di-bromination might kinetically hamper the reductive release (Scheme 2).

Consequently, we investigated the azide reduction, imino-phosphorane hydrolysis, and 1,6-elimination of linker-coupled model sequences in solution (from **3a–c** to **8**, Schemes 2 and S7†). First, we found that 20 eq. PPh₃ in MeCN/AcOH/H₂O is sufficient for a quantitative reduction of **3a**, **3b** and **3c** after 15 min (Fig. S11, Tables S8 and S9†). For all cases, the imino-phosphorane intermediate **5*a–c** was absent in UPLC-MS analysis indicating that hydrolysis is as fast as described,⁵² and the elimination is the rate-limiting step. Interestingly, we found significant differences between linker-derivates, judged by the amount of aniline-linker-modified peptides **6*a–c**. The linker peptide conjugate **6a*** without bromination was only present in traces, while the brominated aniline derivates **6b*** and **6c*** were the main products after reduction. Quantitative release of free peptide **8** could be triggered for **6b*** by addition of 10 vol% TFA (Table S8†). The di-bromo linker conjugate **6c*** gave only 88% free peptide **8** after treatment with TFA amounts as high as 75% (Table S9†).

In parallel, we investigated dithiothreitol (DTT) as a non-hazardous reducing reagent alternative to PPh₃. The model sequence (**P5**: AKADEVSLHKWYG) was modified with all linker-types **2a–c**. Analysis of the reduction of linker modified peptides

3a–c in solution showed that a 15 min treatment with 0.32 M DTT at pH 8 induced the quantitative reduction for all linker types (Fig. S12†).

In contrast to the reduction with PPh₃ at acidic pH, aniline linker peptide conjugates **6*a–c** showed higher stability at pH 8 (Fig. S12D†). Thus, a safety-release became conceivable, where the stability of brominated linker conjugates at neutral to basic pH is allowing reductive agent wash-out with subsequent acidic release of peptide.

Brominated linker **2b** enables safety-release with DTT

To test the safety-release hypothesis, we quantified the stability of linker-conjugates **6a–c** on solid support. Peptide **P5** was linker modified to **3a–c** and immobilized. Reduction with DTT at pH 8 (Scheme 2b) was carried out as in solution yielding **6a–c**. Monitoring the UV-integrals of the free peptide **8** (**P5**) in reduction supernatant (Fig. 3, violet lines) and during acidic treatment (green lines) was used to quantify the lability of **6a–c** during these steps.

The brominated linker conjugates **6b** and **6c** remained stable during the reduction of the azide at pH 8, while **6a** decomposed gradually, releasing 90% **8** (**P5**) after 4 h. Removal of excess reducing agent and oxidized by-products is, therefore, only feasible for **6b** and **6c**.

After washing away DTT/DTT(Ox.) with water and acetonitrile, the resin was treated with 5% TFA in water (pH 0.41). UPLC analysis of the supernatant indicated that 79% of free peptide **8** was released from **6b**, and only 10% from **6c**, after 4 h. Hence, the mono-bromo linker **2b** provides sufficient stability of the aniline species for wash-out of DTT/DTT(Ox.) while allowing the peptide release by a final acid treatment.

Density functional theory (DFT) calculations helped to understand the effect of bromination on linker stability in the various states of the PEC process. We calculated proton affinities and partial charges of the linker-propylamine conjugate **9a–c** and its reduced aniline derivates **12a–c** (Fig. S18, S19 and

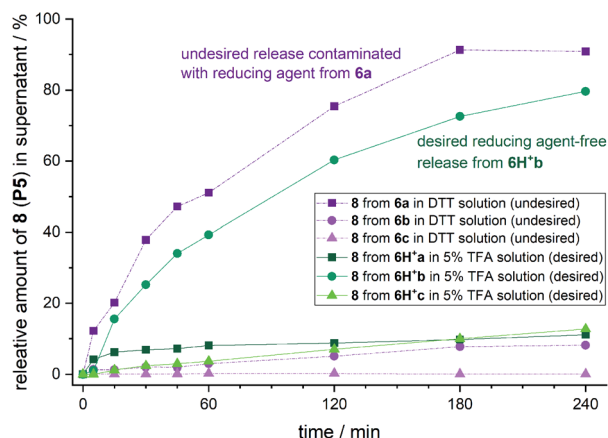


Fig. 3 Amount of released peptide **P5** in DTT supernatant (undesired) and after DTT wash-out in 5% aq. TFA supernatant (desired) calculated by the UV(210 nm) integral of product peak relative to the sum of DTT and TFA released peptide from **6a**.

Table 3 Sequences and results of 20 peptides (P6–P25) intended for neoantigen cancer vaccination purified by PEC within 6 hours

No.	Sequence ^a	Crude purity ^b	Final purity	Recovery ^c
P6	H-GWVKPIIIGHHAYGDQYRAT-NH ₂	56%	93%	45%
P7	H-TLYEQEIEV-NH ₂	79%	94%	56%
P8	H-HGSRKNITDMVEGAKKANG-NH ₂	42%	91%	52%
P9	H-SLLNQPKAV-NH ₂	77%	99%	100%
P10	H-EDPYLFELPVLKYLDMGTT-NH ₂	52%	66%	73%
P11	H-ALAVLSNYDA-NH ₂	76%	92%	93%
P12	H-TMEDKIYDQVTKQC ^d LC ^d F-NH ₂	71%	98%	69%
P13	H-YSYPETPLYMQTASTSYIE-NH ₂	38%	69%	69%
P14	H-KVGYTERQRWDFLSEASIM-NH ₂	6%	99%	81%
P15	H-RLRMREHMMKNVDNTNQD-NH ₂	35%	99%	76%
P16	H-VYEKNGYIF-NH ₂	48%	98%	90%
P17	H-ALAVLC ^d NYDA-NH ₂	73%	85%	81%
P18	H-ALVPPSKRKMWVSPAIEKA-NH ₂	56%	99%	72%
P19	H-ISTPTPTIVHPGSLPLHLG-NH ₂	57%	90%	60%
P20	H-IVQENNTPGTYLLSVSARD-NH ₂	49%	81%	69%
P21	H-RFHMKVSVYLLAPLREALS-NH ₂	45%	99%	57%
P22	H-ENLKQNDISAEFTYQTKDA-NH ₂	45%	90%	82%
P23	H-YMMPVNSEV-NH ₂	71%	90%	100%
P24	H-TNDVKTLADLNGVIEEFT-NH ₂	43%	75%	63%
P25	H-SAWLFRMWYIFDHNYLKPL-NH ₂	35%	66%	49%

^a Fmoc-Lys(Trt)-OH has been used as building block for Lys residues. ^b UV-purity ($A_{210\text{ nm}}$) on UPLC-MS of crude linker-modified peptides after SPPS and TFA-cleavage. ^c (final weight × purity after PEC)/(molecular weight × synthetic scale × crude purity) see S3.6. ^d Fmoc-Cys(S_tBu)-OH has been used as building block for Cys residues.

S22†). Partial charge analysis suggests, that the brominated aniline species **12b,c** are stable towards a spontaneous 1,6-elimination due to reduced electron density of the benzylic carbon. Protonation of the carbamate oxygen pulls electron density from the aromatic core towards the benzylic carbon enabling 1,6-elimination of the brominated linkers and thus release of the peptide under acidic conditions.

Purification of a personalized peptide vaccine set of 20 peptides within a single day

As for conventional peptide synthesis, the manufacturing of peptidic neoantigen vaccines for cancer immunotherapy consists of two main steps: (1) parallel SPPS and (2) subsequent one-by-one purification by RP-HPLC. The purification step is usually the most time-consuming production step of such peptide libraries. Timely delivery of neoantigen sets in an affordable manner is critical for immunotherapy success. Currently, three to four months pass from the moment of tumour biopsy until the delivery of the vaccine formulation.⁵³ The ability to purify peptides in parallel would massively increase the manufacturing speed of the 20–30 neoantigen peptides typically used per cocktail. Therefore, we aimed to show that PEC could accelerate peptide purification compared with linear HPLC purification. We chose 20 peptides from a previously reported vaccination cocktail, which has been tested on 16 patients in phase I clinical trials for glioblastoma immunotherapy (Table 3).⁵⁴

We used optimized TFA cleavage cocktails (Fig. S14†) together with Fmoc building blocks Fmoc-Lys(Trt)-OH (Fig. S15†) and Fmoc-Cys(S_tBu)-OH (Fig. S16†) to minimize side products in the full-length chain. The peptides were

synthesized in parallel on an automated peptide synthesiser followed by automated coupling of linker **2b**. TFA-cleavage and PEC-purification were performed in parallel within 6 hours.

The purities (determined by analysis of UPLC and detection at 210 nm) of the 20 crude peptides with an average length of 17 residues after SPPS and coupling of linker **2b** varied significantly, ranging from 6% for **P14** to 79% to for **P7**, with a mean purity of 53% (Fig. 4a). After the PEC purification, the mean purity was 89%, ranging from 66% to 99%, with an average recovery of 72% (45–100%). Impressively, the purity of peptide **P14** improved from 6% to 99%, which would have been difficult to achieve by HPLC due to closely eluting impurities (Fig. 4b).

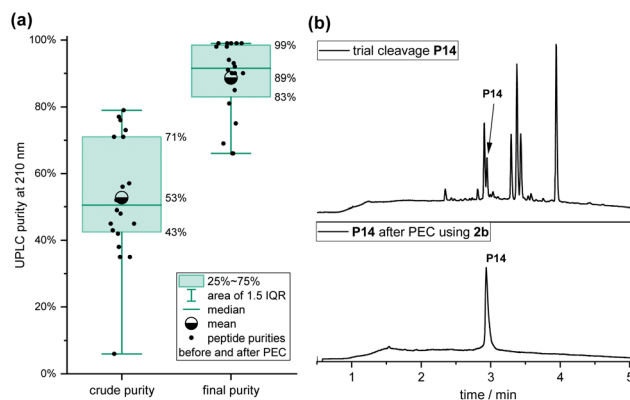


Fig. 4 (a) Box-diagram of crude vs. final purities of the 20 neoantigen peptides. IQR: interquartile range. (b) UPLC chromatograms before (i) and after the PEC process (ii) of peptide **P14** from a personalized peptide set of 20 peptides purified in parallel.



The majority of the purified peptides (14 of 20) had a final purity of over 90%.

Conclusions

In this report we have described the synthesis and development of a first-in-class reductively cleavable linker system for peptide purification and applied it successfully to parallel peptide purification. Bromination on the *para*-azido-benzyl core led to increased TFA-stability and enabled a safety-release mechanism. The linker **2b** enabled a contamination-free traceless release, where the reductive agent could be washed out prior to acid-catalysed liberation of desired peptides by 1,6-elimination (safety-release). The utility of the method was demonstrated by the rapid purification (within 6 hours) of a neoantigen peptide cocktail of 20 peptides for personalized cancer immunotherapy. The results presented demonstrate the great potential of PEC as part of the rapid production of peptide sets. Furthermore, we anticipate that the presented PEC technology will be useful for purification of peptide libraries or for accelerating the peptide manufacturing process in general.

Conflicts of interest

The authors declare competing financial interests: the technology described in the manuscript is part of pending patent applications. Kit products containing linker molecule and aldehyde-modified agarose beads as presented here can be purchased at <http://belyntic.com>.

Acknowledgements

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Notes and references

σ_p are considered to be a good approximation for the effect of *ortho*-substituents. The electron-withdrawing effect of the *o*-amide may be mitigated by the asymmetric assistance in the acidic cleavage of the linker.

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