Higher Productivity

20 neoantigens in a single day

The most time-consuming part of the peptide manufacturing process is the purification after synthesis. The PEC technology is a real time-saver at this end. In this case study, we demonstrate the purification of 20 neoantigen peptides with a final average purity of 91% in a single day.

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1. Introduction

The manufacturing of multiple, purified peptides generally consists of two steps: (1) the parallel solid-phase peptide synthesis (SPPS) and (2), subsequent one-by-one purification by reversed-phase high-pressure liquid chromatography (RP-HPLC). Thus, the purification step is the most time-consuming part during the production of peptide libraries such as neoantigen vaccines for cancer immunotherapy. About three to four months pass from the moment of taking a patient's blood sample until the delivery of the vaccine formulation – by far too long for an adequate time to needle in late-stage diseases.[1] Being able to purify peptides in parallel would massively increase the manufacturing speed of the typically used 20-30 peptides per cocktail.

In this case study, we show how to surpass the time-restraints using the PEC technology. 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.[2] We evaluated the level of (im)purities and the time efficiency of the process.

2. Method

Synthesis. Peptides (Table 1) were synthesized with single (≤13 aa) and double coupling (>13 aa) of amino acids (2 x 60 min with a 5-fold excess of aa) in a 10 µmol scale on Rink Amide AM Polystyrene resin with routine capping (2 M Ac_2O, 2 M pyridine in DMF 1x5 min). This capping enabled selective coupling of the reductively cleavable linker RC+ (4 eq.) to the full-length peptide as the last building block in DMF using DIPEA (6 eq.) and Oxyma (4 eq.) for 2 h. TFA cleavage was performed for 2 h using TFA/H_2O/DTT/TIS (84:8:6:2) (10 mL cleavage cocktail per 100 µmol peptide). TMSBr (10 eq.) together with EDT (19 eq.) was added after 1.45 h to all samples with peptides containing Met residues to reduce Met(Ox).

PEC purification. The crude materials were dissolved in 0.45 mL DMSO. Then, 50 µL citric acid/GdmCl buffer (0.1 M/7 M; pH 4.5) was added. The dissolved peptide was added to 0.3 mL pre-conditioned suspension of activated filter material (15 µmol aldehyde content), and the immobilization was performed for 90 min before washing off impurities. 0.5 mL 2 w% L-Cys in a citric acid buffer (pH = 4.5) was then added to block unreacted aldehydes and displace imines (15 min incubation time).

The Cys-free peptides were incubated for 15 min with 1 mL 1:1 MeCN and 50 mg DTT dissolved in 5 w% aq. $NaHCO_3$ to reduce the linker. Peptide #7 was incubated for another 45 min to remove the StBu-protecting group. The activated filter material was washed to remove excess of DTT and formed DTT(ox).

The PEC-Linker cleavage was initiated by adding TFA/H_2O (2:3). After 60 min, 0.2 mL TFA was added, and the filtrate was collected in 15 mL tubes. Each sample was washed/eluted twice with 0.2 mL 95% aq. TFA. Chilled diethyl ether was used to precipitate the peptides before lyophilization.

Table 1: Peptides used in this study

ID	sequence	crude purity	final purity*
#1	H-GWVK PIIIG HHAYG DQYRA T-NH_2	40%	91%
#2	H-TLYEQ EIEV-NH_2	72%	99%
#3	H-HGSRK NITDM VEGAK KANG-NH_2	37%	94%
#4	H-SLLNQ PKAV-NH_2	88%	99%
#5	H-EDPYL FELPV LKYLD MGTT-NH_2	71%	83%
#6	H-ALAVL SNYDA-NH_2	82%	95%
#7	H-TMEDK IYDQQ VTKQC^{StBu} LC^{StBu}F-NH_2	43%	90%
#8	H-TMEDK IYDQQ VTKQS LSF-NH_2	41%	87%
#9	H-YSYPE TPLYM QTAST SYYE-NH_2	42%	80%
#10	H-KVGYT ERQRW DFLSE ASIM-NH_2	8%	73%
#11	H-RLRMR EHMMK NVDTN QD-NH_2	46%	98%
#12	H-VYEKN GYIYF-NH_2	64%	97%
#13	H-ALVPP SKRKM WVVSP AEKA-NH_2	43%	95%
#14	H-ISTPT PTIVH PGSLP LHLG-NH_2	70%	94%
#15	H-IVQEN NTPGT YLLSV SARD-NH_2	64%	90%
#16	H-RFHMK VSVYL LAPLR EALS-NH_2	49%	89%
#17	H-ENLKQ NDISA EFTYQ TKDA-NH_2	52%	88%
#18	H-YMMPV NSEV-NH_2	63%	90%
#19	H-TNDVK TLADL NGVIE EEFT-NH_2	51%	80%
#20	H-SAWLF RMWYI FDHNY LKPL-NH_2	45%	96%

**impurities from remaining Pbf were not considered.*

Analysis. UPLC-UV chromatograms and mass spectra were recorded with an analytical Acquity H-Class UPLC-ESI-MS system from Waters on a C-18 column (1.7 µm, 2.1 x 500 mm). As the mobile phase, mixtures of water (A) and MeCN (B) with 0.1% TFA were used.

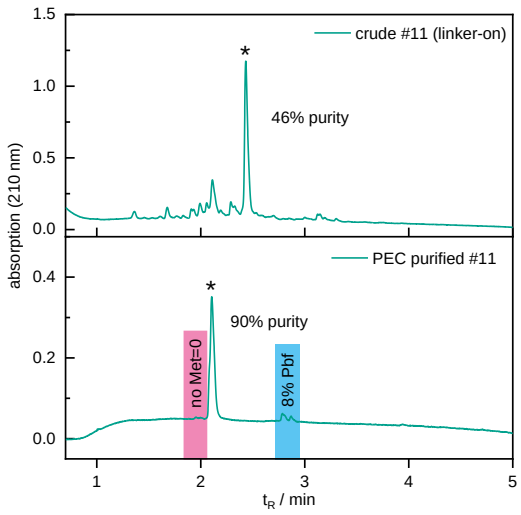
3. Discussion

Crude purity. The crude purities (Table 1) after the coupling of the PEC-Linker varied significantly, between 8% (#10) and 88% (#4), reflecting well the diversity of synthesis challenges in this set. Though the majority of peptides (14 of 20) had crude purities of 40-70% (average = 50%). The purity of 4 peptides was higher than 70%.

PEC purity. The mean final purity of all peptides was 91%. 19 out of 20 peptides had purities of above 80%. Peptide #10 was the only peptide with lower ultimate purity. However, PEC enabled a remarkable purity gain from 8% to 73%.

Eight peptides (40%) contained at least one Arg(Pbf) residue. Up to 8% of Pbf-adducts remained in the final products of these peptides (Figure 1 for an exemplary chromatogram). The odorless TFA cocktail (TFA/H_2O/DTT/TIS (84:8:6:2)) was not able to entirely remove the Pbf protecting group within two hours.

Figure 1: Chromatogram of crude and purified peptide #11



More than half of the peptides (55%) contained one or more Met, which tends to oxidize during SPPS. The addition of TMSBr and EDT at the end of the TFA cleavage successfully reduced any existing Met=O (Figure 1).[3]

Peptides with Cys typically contain tBu adducts (+56 Da) after SPPS and TFA cleavage due to the addition of released protecting groups. The utilization of Cys(StBu) is a useful tool to prevent this undesired side-reaction. DTT easily removes the protecting group during the reduction step of the PEC-Linker. In this example, we modified the original peptide #8 with two Cys(StBu) to show this feature in neoantigen manufacturing. As expected, no tBu adducts remained in the final product.

Time efficiency. To highlight the significant time savings using PEC, we recorded the processing times needed for the purification process.

Six hours passed from the dissolution of the peptides until the final precipitation, and only three hours of active time was necessary, leaving three hours passive time free for other tasks.

Table 2: Time table for PEC purification of 20 neoantigens

task	active time	passive time
Preparations and dissolution	60 min	
Immobilization (Catch)		90 min
Preparation & addition of blocking agent	15 min	
Aldehyde blocking & imine detachment		15 min
Preparation of reducing agent	5 min	
Washing off impurities	15 min	
Addition of reducing agent & reduction	5 min	15 min*
Washing excess of (oxidized) reducing agent	20 min	
Addition of acid & release		60 min
Elution & 1st ether precipitation	30 min	
Centrifugation & 2nd ether precipitation	20 min	8 min
Total	170 min	188 min

**60 min for StBu-deprotection of Cys.*

4. Results at a glance

- significantly improve manufacturing cycles in neoantigen production
- purify neoantigen peptide vaccine with final purities of >90% in a single day
- purify 20 peptides in 3 hours of active working time

References

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[3] Beck W.; Jung G. *Lett. Pept. Sci.* 1994, 1, 31.

About Belyntic

Belyntic GmbH is a chemistry-for-healthcare enterprise focusing on the purification of biopolymers, especially peptides. Belyntic offers the world's first broadly applicable peptide purification kits as well as development and implementation services of its proprietary Peptide Easy Clean (PEC) technology.

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