Received: 20 March 2020 | Revised: 20 April 2020 | Accepted: 24 April 2020

DOI: 10.1002/psc.3253

RESEARCH ARTICLE

Journal of PeptideScience WILEY

Improved Fmoc-solid-phase peptide synthesis of an extracellular loop of CFTR for antibody selection by the phage display technology

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Funding information
BioISI, Grant/Award Number: UID/MULTI/04046/2019; C[2]TN, Grant/Award Number: UID/Multi/04349/2019; Lisboa2020—EU FEDER; Fundação para a Ciência e Tecnologia (FCT), Grant/Award Numbers: PTDC/BTM-TEC/29256/2017, LISBOA-01-0145-FEDER-022125 from FCT and Lisboa2020—EU FEDER

Cystic fibrosis (CF), a life-shortening genetic disease, is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene that codes for the CFTR protein, the major chloride channel expressed at the apical membrane of epithelial cells. The development of an imaging probe capable of non-invasively detect CFTR at the cell surface could be of great advantage for the management of CF. With that purpose, we synthesized the first extracellular loop of CFTR protein (ECL1) through fluorenylmethyloxycarbonyl (Fmoc)-based microwave-assisted solid-phase peptide synthesis (SPPS), according to a reported methodology. However, aspartimide formation, a well-characterized side reaction in Fmoc-SPPS, prompted us to adopt a different side-chain protection strategy for aspartic acid residues present in ECL1 sequence. The peptide was subsequently modified via PEGylation and biotinylation, and cyclized through disulfide bridge formation, mimicking the native loop conformation in CFTR protein. Herein, we report improvements in the synthesis of the first extracellular loop of CFTR, including peptide modifications that can be used to improve antigen presentation in phage display for selection of novel antibodies against plasma membrane CFTR.

KEYWORDS
aspartimide, biotinylation, CFTR, cyclization, cystic fibrosis, extracellular loop, PEGylation, solid-phase peptide synthesis

1 | INTRODUCTION

Cystic fibrosis (CF) is the most common lethal genetic disease among the Caucasian population, affecting the respiratory, digestive and reproductive systems, as well as other organs. The disease is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene that codes for the CFTR protein,[1] an apical-membrane chloride and bicarbonate channel involved in the regulation of volume and

Abbreviations: Bno, 5-*n*-butyl-5-nonyl; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ECL, extracellular loop; EDT, 1,2-ethanedithiol; ESI, electrospray ionization; Fmoc, fluorenylmethyloxycarbonyl; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole hydrate; HPLC, High-performance liquid chromatography; *m*/*z*, mass/charge; MALDI, matrix-assisted laser desorption/ionization; MeOH, methanol; MS, mass spectrometry; O, oxycarbonyl; Oxyma, ethyl 2-cyano-2-(hydroxyimino) acetate; Pbf, pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl; PEG, polyethylene glycol; *p*MeOBzl, *p*-methoxybenzyl; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; QIT, quadrupole ion trap; RP, reversed-phase; R_t, retention time; rt, room temperature; SPPS, solid-phase peptide synthesis; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TOF, time-of-flight; Trt, trityl; UV/VIS, ultraviolet/visible; v/v, volume/volume.

composition of epithelial secretions. To date, more than 2000 *CFTR* variants have been identified as presumably disease-causing.[2] Most mutations result in loss of CFTR-mediated chloride secretion due to a reduction in the number of channels at the apical plasma membrane or to an impairment in channel function.

High-throughput drug screening has successfully enabled the identification of numerous small organic molecules (modulators) capable of *in vitro* correction of CFTR defects at different levels. Those efforts led to the Food and Drug Administration approval in 2012 of the first CFTR modulator for treatment of CF patients bearing a particular gating mutation, a significant milestone in the CF field.[3–7] Currently, three more treatments are approved for CF patients bearing the most common mutation, and several other molecules are in the preclinical and clinical stage.

The patient responsiveness to a modulator is assessed in terms of benefits in surrogate endpoints such as sweat chloride, nasal potential difference or lung function,[8,9] and there is still no available methodology to non-invasively detect the presence of CFTR protein at the cell surface of epithelia of CF patients after drug administration. Therefore, from the translational medicine perspective, there remains a need to improve disease biomarkers and non-invasive therapeutic endpoints used in clinical trials.

The development of a non-invasive and sensitive method to image CFTR at the epithelial cell surface of CF patients after pharmacological treatment could address this unmet clinical need. We have developed a non-invasive, small molecule-based radioprobe targeting plasma membrane CFTR for nuclear molecular imaging through the radiolabelling of a CFTR inhibitor with technetium-99m and *in vitro* assessment of binding to wild-type *versus* mutant-CFTR.[10,11] This probe showed promising results, being the first proof-of-principle validation of a molecular imaging biomarker. Nevertheless, further optimization is needed, and we are currently exploring alternative CFTR-targeting biomolecules such as antibody or antibody fragments.

Specific binding of antibodies to a given target with nanomolar or picomolar affinity provided the basis for the development of therapeutic antibodies as well as antibody-based imaging agents for *in vivo* disease assessment in areas as oncology, cardiology, immunology, autoimmunity and infectious diseases.[12] Major breakthroughs in antibody development such as the hybridoma technology[13] followed by the phage display technology[14–17] have been pivotal for the discovery and production of monoclonal and recombinant antibodies against a variety of antigens. Phage display represents a fast and cost-effective technique for antibody generation, allowing the selection of antibodies against a great antigen diversity, such as recombinant proteins, membrane proteins, peptides, toxins, self-antigens, deoxyribonucleic acid, among others.

Membrane proteins, as ion channels or G-protein-coupled receptors, are notoriously difficult to express *in vitro* in the soluble form, as expression often results in protein aggregation and misfolding due to the hydrophobic nature of the membrane-spanning segments. In the CF context, the production of antibodies capable of recognizing plasma membrane CFTR was reported *via* animal immunization with a peptide mimicking the first extracellular loop of CFTR (ECL1).[18–20] From the six extracellular loops of CFTR, four have less than five amino acids, and another contains two consensus *N*-linked glycosylation sites, thereby being unsuitable for antibody production. Therefore, the antigen choice fell on the first extracellular loop, an accessible epitope with 15 amino acid residues covering positions 103 to 117 of CFTR.

Taking in consideration the inherent variability of polyclonal antibodies obtained by animal immunization, as well as the slow *in vivo* blood clearance of full-length antibodies in imaging, our strategy is to develop a radioprobe based on smaller antibody fragments owing to their better pharmacokinetic properties. Those antibody fragments targeting the first extracellular loop of CFTR will be selected through the phage display technology, using the ECL1 peptide sequence as antigen. Herein, we describe improvements to the reported microwave-assisted solid-phase peptide synthesis (SPPS) of the first extracellular loop of CFTR. Side chains of aspartic acid residues were shielded with bulky protecting groups to avoid aspartimide formation, the most frequent side reaction in SPPS. On-resin peptide modifications useful for antibody selection by phage display and subsequent cyclization through the N- and C-terminal cysteine residues yielded a cyclic peptide mimicking the native loop conformation in CFTR.

2 | MATERIALS AND METHODS

2.1 | Materials

Solvents for peptide synthesis and chromatographic analysis were purchased from CARLO ERBA Reagents (Val de Reuil, France).

Fluorenylmethyloxycarbonyl (Fmoc)-L-Cys (Trt)-Wang resin (100–200 mesh) and Fmoc-NH-PEG(3)-COOH were purchased from Iris Biotech GmbH (Marktredwith, Germany). All Fmoc-amino acids, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBt), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and ethyl 2-cyano-2-(hydroxyimino) acetate (Oxyma) Pure were purchased from Novabiochem (Burlington, MA, USA). *N*,*N*-diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS) and biotin were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 1,2-Ethanedithiol (EDT) was purchased from Fluka (Honeywell Research Chemicals, Bucharest, Romania).

Fmoc-based SPPS was performed using a Liberty 12-channel automated peptide synthesizer coupled to a Discover SPS microwave platform (CEM, Matthews, NC, USA). High-performance liquid chromatography (HPLC) analyses were performed on a PerkinElmer Series 200 LC pump (PerkinElmer, Waltham, MA, USA) coupled to an ultraviolet/visible (UV/VIS) detector (PerkinElmer LC 290). Peptide purification was performed on a semipreparative HPLC instrument (Waters 2535 Quaternary Gradient Module, Waters, Milford, MA, USA) equipped with a Waters 2996 diode array detector. Supelco® HPLC columns were purchased from Sigma-Aldrich. Eluents were of HPLC grade and the water bidistilled from a Quartz distillation unit.

Electrospray ionization-mass spectrometry (ESI-MS) was performed on a Bruker HCT ESI/quadrupole ion trap (QIT) mass spectrometer (Billerica, MA, USA). Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)/TOF was performed on a 4800*plus* MALDI-TOF/TOF mass spectrometer (Applied Biosystems/SCIEX, USA) using the 4000 Series Explorer Software v.3.5.3 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

2.2 | Reversed-phase HPLC

Analytical HPLC was performed using a Supelco® Analytical Discovery BIO WidePore C18 column (250 × 4.6 mm, 5-μm particle size, 1.0-ml/min flow rate). Eluents were filtered with 0.22-μm filters (Millipore, Merck, Darmstadt, Germany) and purged with helium. Linear gradients of eluent A (0.1% volume/volume [v/v] TFA in bidistilled water) and eluent B (0.1% [v/v] TFA in acetronitrile) were applied according to method A: 0–3 min (10% B), 3–23 min (10–100% B), 23–27 min (100% B), 27–29 min (100–10% B) and 29–30 min (10% B).

Peptide purification was performed using a Supelco® Discovery BIO WidePore C18 column (250 × 10 mm, 10-μm particle size, 3.0-ml/min flow rate). Purification was performed with eluents described above and according to method B: 0–3 min (20% B), 3–33 min (20–40% B), 33–37 min (40% B), 37–38 min (40–100% B), 38–42 min (100% B) and 42–43 min (100–20% B). Peptide elution was monitored *via* absorbance at 220 nm.

2.3 | Mass spectrometry

For ESI-MS analyses, samples were dissolved in water:acetonitrile and injected into the sample loop with a 150 μl/h mobile phase flow rate consisting of 50:50 (v:v) water:acetonitrile with 0.1% (v/v) formic acid. Mass spectra were collected in linear ion trap mode (positive mode).

For MALDI-TOF/TOF analyses, dry samples were dissolved in methanol or 5% (v/v) formic acid in water; 0.6 μl of sample was spotted directly onto the MALDI plate and allowed to air dry. Afterwards, an equal volume of 5 mg/ml of alpha-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 5% (v/v) formic acid was added. Data were acquired in reflectron MS mode (positive mode).

2.4 | Synthesis of ECL1 peptides

For the synthesis of ECL1 peptide variant 1 (CCGRIIASYDPDNKEERC, CFTR original sequence underlined), the following amino acid side-chain protecting groups were used: *tert*-butyl (*t*Bu) for aspartic acid, glutamic acid, serine and tyrosine; *tert*-butyloxycarbonyl (O*t*Bu) for lysine; pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) for arginine; and trityl (Trt) for asparagine and cysteine. The N-terminal cysteine was protected with the *p*-methoxybenzyl (*p* MeOBzl) group.

In the synthesis of ECL1 peptide variant 2 (CGRIIASYDPDNKEERC), the 5-*n*-butyl-5-nonyl (OBno) group was used as side-chain protecting group of aspartic acid residues, in substitution to *t*Bu group. In addition, the N-terminal cysteine containing the *p*MeOBzl group was removed. The remaining amino acids maintained the respective side-chain protecting groups.

Amino acids (5 equiv.) were coupled to the Cys (Trt)-preloaded Wang resin (loading of 0.6 mmol/g) on a scale of 0.1 mmol (~167 mg of resin), using HBTU (5 equiv.) as the activating reagent and DIPEA (10 equiv.) as base. Fmoc-protecting groups were removed with a 20% (*v*/v) piperidine/*N*,*N*-dimethylformamide (DMF) solution containing 1 M Oxyma Pure. The standard microwave program used for the couplings was 25 W at 80°C for 5 min. Asp, Cys and Glu were coupled at lower temperature with 0 W at 50°C for 6 min. For Arg coupling, a two-stage program was used: (1) 0 W, 80°C, 30 min and (2) 25 W, 80°C, 5 min. Double couplings (25 W, 80°C, 5 min) were applied to Asn, Ile and Pro. Fmoc removal was achieved with microwave irradiation (35 W) at 75°C for 3 min.

Small-scale peptide cleavage from the resin was performed for MS and HPLC analyses, whereas the remaining resin was used for polyethylene glycol (PEG) and biotin conjugation to ECL1 peptide variant 2. Cleavage was achieved through reaction with a cleavage cocktail (v/v) (94% TFA, 1% TIS, 2.5% EDT, 2.5% water) for 3 h. After resin washing with pure TFA, the solution was concentrated under nitrogen gas. The peptide was precipitated with cold Et_2O, pelleted *via* centrifugation (3000 rotations per minute [rpm], 10 min) and lyophilized.

Retention time (R_t) = 11.9 min (method A, analytical column). ESI-MS mass/charge (*m*/*z*) 657.2 $[M + 3H]^{3+}$, 493.3 $[M + 4H]^{4+}$, calcd for $C_{80}H_{130}N_{25}O_{29}S_2$ 1968.89 $[M + H]^+$.

2.5 | On-resin PEGylation and biotinylation of peptide

PEGylation of ECL1 peptide variant 2 was performed by coupling of Fmoc-NH-PEG(3)-COOH (2.5 equiv.) using HOBt (2.5 equiv.), PyBOP (2.5 equiv.) and DIPEA (10 equiv.) in DMF for 3 h with stirring. Small-scale peptide cleavage with the cocktail previously described allowed MS analysis of the Fmoc-protected PEGylated peptide. ESI-MS *m*/*z* 799.0 $[M + 3H]^{3+}$, calcd for $C_{104}H_{157}N_{26}O_{35}S_2$ 2394.07 $[M + H]^+$.

The Fmoc-protecting group of ECL1 peptide variant 2 was removed with 2 cycles of treatment (10 min) with 20% (v/v) piperidine/DMF. The resin was washed with DMF and DCM and dried under nitrogen gas. Coupling and Fmoc-cleavage were monitored by the Kaiser (or ninhydrin) test,[21] a sensitive colorimetric test for primary amines commonly used in SPPS to determine reaction completeness. The test is based on three solutions: solution A (16.6 mg of KCN in 25 ml of distilled water, 1:50 dilution in pyridine), solution B (1 g of ninhydrin in 20 ml of butanol) and solution C (40 g of phenol in 20 ml of butanol). A dark blue bead colour was observed when ninhydrin reacted with a free primary amine, whereas a colourless or faint blue bead colour confirmed the completeness of the coupling step.

Biotinylation was performed using biotin (2.5 equiv.), HOBt (2.5 equiv.), PyBOP (2.5 equiv.) and DIPEA (10 equiv.) in DMF in two reaction cycles of 3h with stirring. After resin washing, full coupling was confirmed by the Kaiser test. The final PEGylated and biotinylated ECL1 peptide was fully cleaved from the resin and purified (>95%) by semipreparative HPLC (method B, semipreparative column).

R_t = 16.9 min (method B, analytical column). ESI-MS *m/z* 800.9 $[M + 3H]^{3+}$, calcd for $C_{99}H_{161}N_{28}O_{35}S_3$ 2398.08 $[M + H]^+$.

2.6 | Cyclization of peptide

ECL1 peptide variant 2 (approximately 0.5 mg) was cyclized with 20% (v/v) dimethyl sulfoxide (DMSO) in 0.1 M ammonium acetate buffer pH 7 (0.5 ml). The reaction was performed at room temperature (rt) with stirring for 3 days. Purification was carried out with semipreparative HPLC (method B, semipreparative column), giving the desired peptide with a purity > 95%.

R_t = 13.1 min (method A, analytical column). ESI-MS *m/z* 1198.9 $[M + 2H]^{2+}$, 799.8 $[M + 3H]^{3+}$, calcd for $C_{99}H_{159}N_{28}O_{35}S_3$ 2396.08 $[M + H]^+$.

3 | RESULTS AND DISCUSSION

3.1 | Synthesis of the first extracellular loop of CFTR (ECL1)

Polyclonal antibodies against plasma membrane CFTR were previously produced using the peptide sequence of the first extracellular loop of CFTR as antigen. Peters *et al*. reported the synthesis of a conformationally constrained ECL1 peptide, in which the N- and C-termini were linked by a disulfide bridge, mimicking the native CFTR loop, with the addition of a third cysteine at the N-terminal for immunization purposes.[20]

We started by synthesizing the ECL1 peptide as reported in literature through automated microwave-assisted SPPS (ECL1 variant 1). We used a Wang resin preloaded with cysteine (0.6 mmol/g), since functionalization of Wang resins can be problematic when the first amino acid residue is a cysteine due to partial amino acid epimerization. For regioselective cyclization, the N-terminal cysteine side chain was protected with the *p*MeOBzl group, whereas Trt side-chain protecting groups were used for the remaining cysteine residues.

Small-scale peptide cleavage allowed for MALDI-TOF/TOF analysis to confirm the presence of the desired peptide (Figure 1). MS analysis revealed the presence of the peptide molecular ion (*m/z* calcd 2191.96 $[M + H]^+$; *m/z* found: 2192.0 $[M + H]^+$). However, two other signals were observed: one at *m/z* minus 18 u than the *m/z* of the expected peptide and another at *m/z* plus 67 u.

These species were identified as by-products of the most frequent and serious side reaction affecting aspartic acid residues: aspartimide formation (+18 u). It results from a ring closure between the nitrogen of the α-carboxyl amide bond and the β-carboxyl side chain with loss of the carboxyl protecting group (Scheme 1). This side reaction is promoted by strong bases, such as piperidine, used for the removal of Fmoc group, and constitutes a major problem for the synthesis of long peptides and sequences containing multiple aspartic acid residues. The degree of aspartimide formation is largely dependent on the amino acid at the C-terminus of the aspartic acid residue, with the Asp-Asn motif being one of the sequences more prone to aspartimide formation.

Aspartimide epimerization and ring opening can lead to the formation of seven different by-products, including a mixture of D/L-α- and β-piperidides (+67 u) and undesired D/L-β-aspartyl peptide in a ratio of 3:1 to the α-aspartyl peptide. Separation of the D/L-β-aspartyl peptides and epimerized D-α-aspartyl peptide is almost impossible, as they will have similar retention times to the target peptide.[22]

One of the critical aspects for successful antibody selection by phage display is antigen quality. High antigen purity is essential to avoid

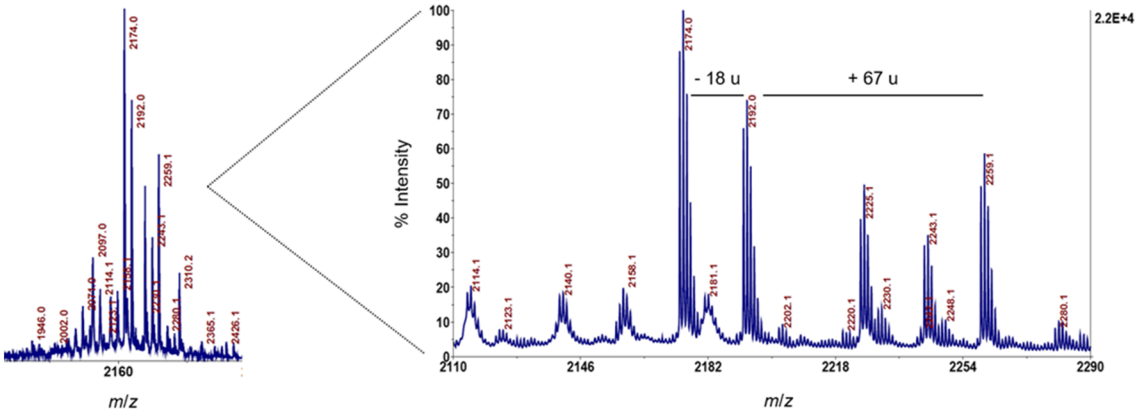
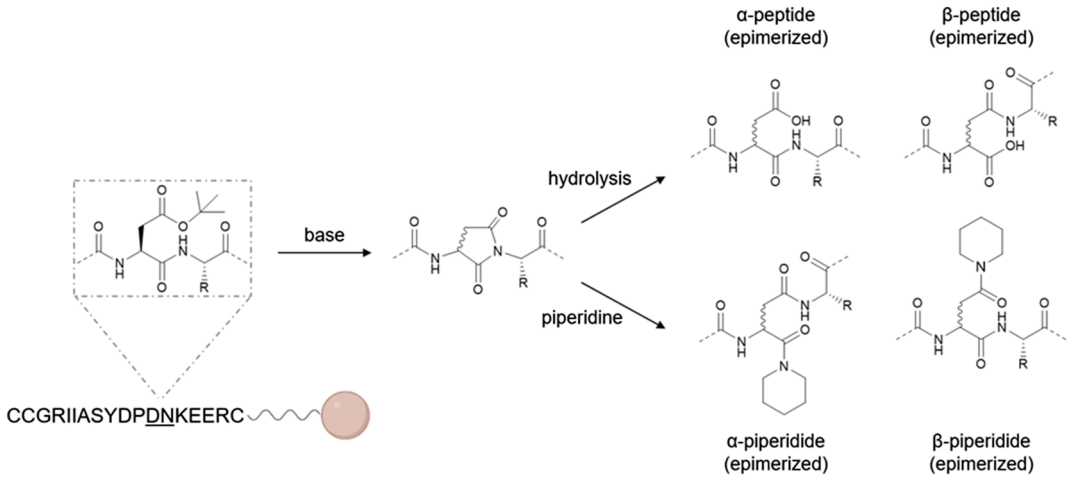


FIGURE 1 Mass spectrum of ECL1 variant 1 deprotected crude product with *p*MeOBzl-protected N-terminal cysteine obtained by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)/TOF (*m/z* calcd for $C_{91}H_{143}N_{26}O_{31}S_3$ 2191.96 $[M + H]^+$; *m/z* found: 2192.0 $[M + H]^+$). Sample dissolved in MeOH



SCHEME 1 Side reactions arising from aspartimide formation

the generation of antibodies against molecules other than the target antigen. Moreover, the antigen should be in its native conformation in order to obtain clones that bind to a functional protein not only *in vitro* but also in a cellular context.[23] Therefore, it was of great importance that ECL1 peptide presented high purity and a correct conformation.

Given the difficulties encountered in the previously described strategy for Fmoc-SPPS of ECL1, we then adopted a different side-chain protection strategy for aspartic acid residues to minimize aspartimide formation. The *t*Bu group of aspartic acid was replaced by the analogue OBno in which the methyl groups are substituted with a longer alkyl chain (butyl) to shield the aspartyl β-carboxyl group. This derivative has been shown to provide protection against aspartimide formation, for instance, in the Fmoc-based SPPS of a variant of the scorpion toxin II model peptide containing the Asp-Asn sequence.[24] The addition of 1 M of Oxyma Pure to the Fmoc-removal solution was also implemented to avoid aspartimide formation. Acid modifiers have also been shown to reduce the levels of undesired aspartimide-related by-products, presumably by reducing the ionization of the Asp-Aaa amide bond.[25] In addition, the N-terminal Cys (*p*MeOBzl) was removed from the sequence of ECL1 variant 1, since the original purpose for which it was introduced was conjugation to an immunogenic protein for animal immunization.[20]

The modifications described above were adopted in a new peptide synthesis, ECL1 variant 2, and after small-scale peptide cleavage from the resin, ESI-MS analysis confirmed successful synthesis of this peptide (Figure 2).

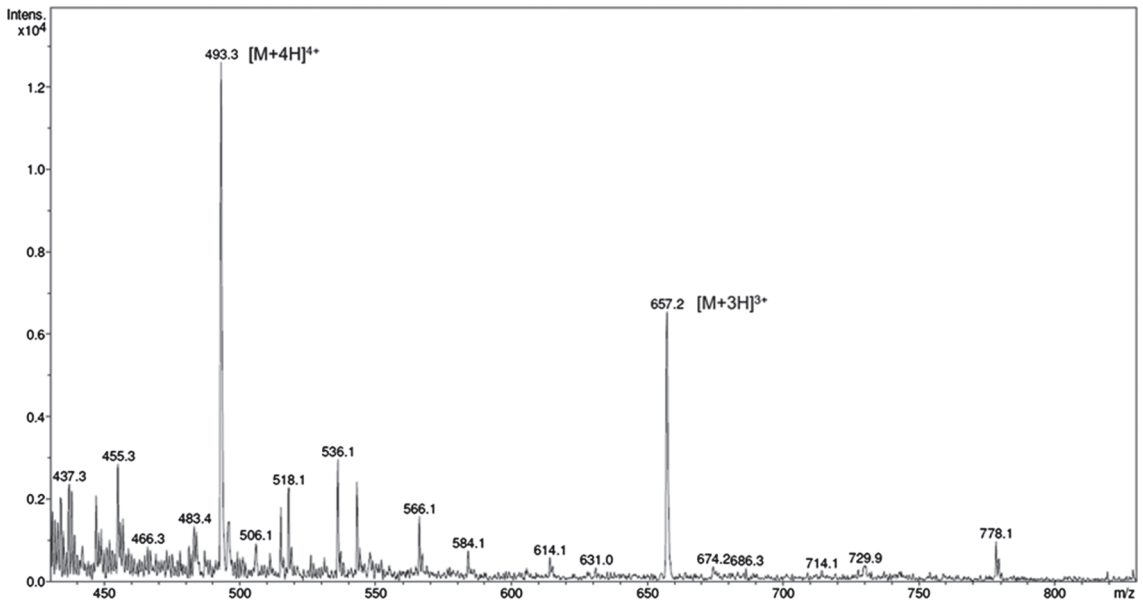


FIGURE 2 Electrospray ionization-mass spectrometry (ESI-MS) mass spectrum (positive mode) of ECL1 variant 2 deprotected product (*m/z* calcd for $C_{80}H_{130}N_{25}O_{29}S_2$: 1968.89 $[M + H]^+$; *m/z* found: 657.2 $[M + 3H]^{3+}$, 493.3 $[M + 4H]^{4+}$)

3.2 | On-resin peptide modifications for antibody selection by phage display

Presentation of a conformationally functional antigen constitutes another critical aspect during antibody selection by phage display. Most frequently, proteins are adsorbed to a plastic surface through non-covalent associations, as electrostatic and Van der Waals interactions. However, this process may cause alterations in antigen conformation, which will reduce the proportion of correctly folded antigen and can expose hydrophobic regions, diminishing the accessibility of relevant epitopes and favouring nonspecific binding of phages or antibodies to those regions.[23,26]

Affinity tags provide an excellent alternative for immobilization in a relatively uniform orientation, with minimal disruption of native conformation. There are numerous approaches, one of which the biotin-streptavidin capture system. This method allows to perform selections in solution with a subsequent pull-down step to isolate antibody-displaying phages bound to the biotinylated antigen through the biotin-streptavidin interaction. To avoid the interference of the molecule to be tagged in the biotin-streptavidin interaction, a spacer is usually inserted between biotin and the molecule. PEG spacers have been previously shown to enhance the solubility of peptides.[27,28] More recently, several studies reported the synthesis of peptide derivatives containing PEG spacers of varying length.[29–31] Therefore, on-resin PEGylation and biotinylation of ECL1 peptide variant 2 was performed to allow for antibody selection in solution through the biotin-streptavidin capture system (Scheme 2).

After successful synthesis of ECL1 peptide variant 2, the Fmoc group of the N-terminal cysteine was removed, rendering a free amino group available for conjugation to the PEG molecule functionalized with a carboxylic acid (spacer length 14.4 Å). Activation was performed with the phosphonium-based coupling reagent PyBOP in combination with HOBt, an additive to ensure faster and

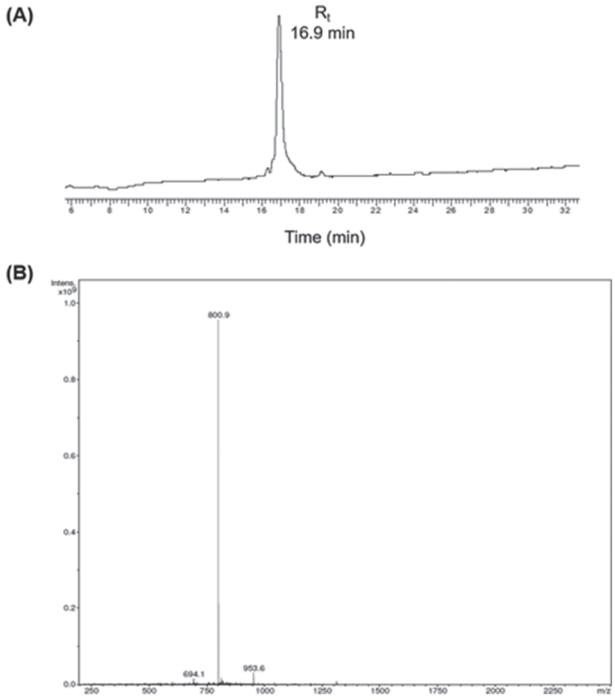
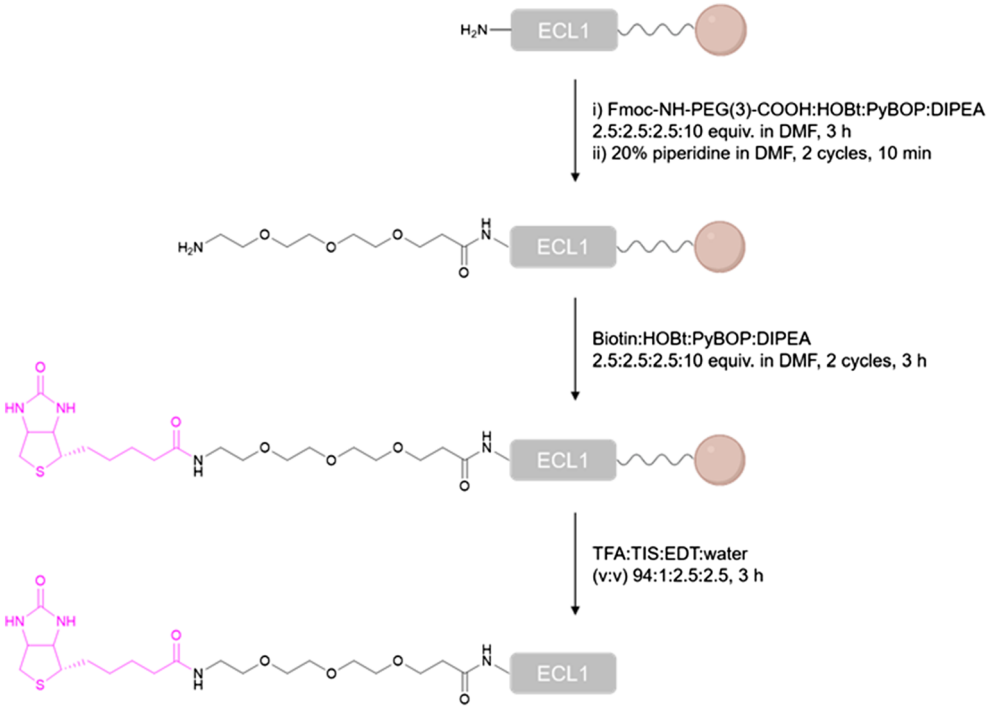


FIGURE 3 (A) Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram of PEGylated and biotinylated ECL1 peptide variant 2. R_t = 16.9 min (method B, analytical column). (B) Electrospray ionization-mass spectrometry (ESI-MS) mass spectrum (positive mode) of PEGylated and biotinylated ECL1 peptide variant 2 (m/z calcd for $C_{99}H_{161}N_{28}O_{35}S_3$: 2398.08 $[M + H]^+$; m/z found: 800.9 $[M + 3H]^{3+}$)



SCHEME 2 On-resin peptide modifications of ECL1 variant 2. Biotin moiety is shown in pink

more efficient couplings. After a reaction cycle of 3 h, the Kaiser test[21] revealed complete coupling of the PEG spacer to ECL1, confirmed by ESI-MS analysis (*m/z* calcd 2394.07 $[M + H]^+$.; m/z found 799.0 $[M + 3H]^{3+}$). The Fmoc group of the spacer was then removed and, once again, deprotection confirmed by the Kaiser test.

Biotinylation was performed in the same conditions used for PEG coupling, with PyBOP/HOBt as activating reagents and DIPEA as base. Complete biotin coupling to the PEGylated peptide was observed after two reaction cycles of 3 h. The PEGylated and biotinylated peptide was fully cleaved from the resin and the crude product purified by reverse-phase HPLC (RP-HPLC) (Figure 3A, purity > 95%). Analysis of the purified peptide by ESI-MS (Figure 3B) confirmed successful PEGylation and biotinylation of ECL1 peptide variant 2.

3.3 | ECL1 cyclization

To mimic the extracellular loop of CFTR, cyclization of ECL1 peptide variant 2 was accomplished through formation of a disulfide bridge between the two cysteines at the N- and C-termini. The PEGylated and biotinylated ECL1 was cyclized with 20% (v/v) DMSO in 0.1 M ammonium acetate buffer (pH 7). The reaction was performed with stirring and monitored by RP-HPLC at different time points.

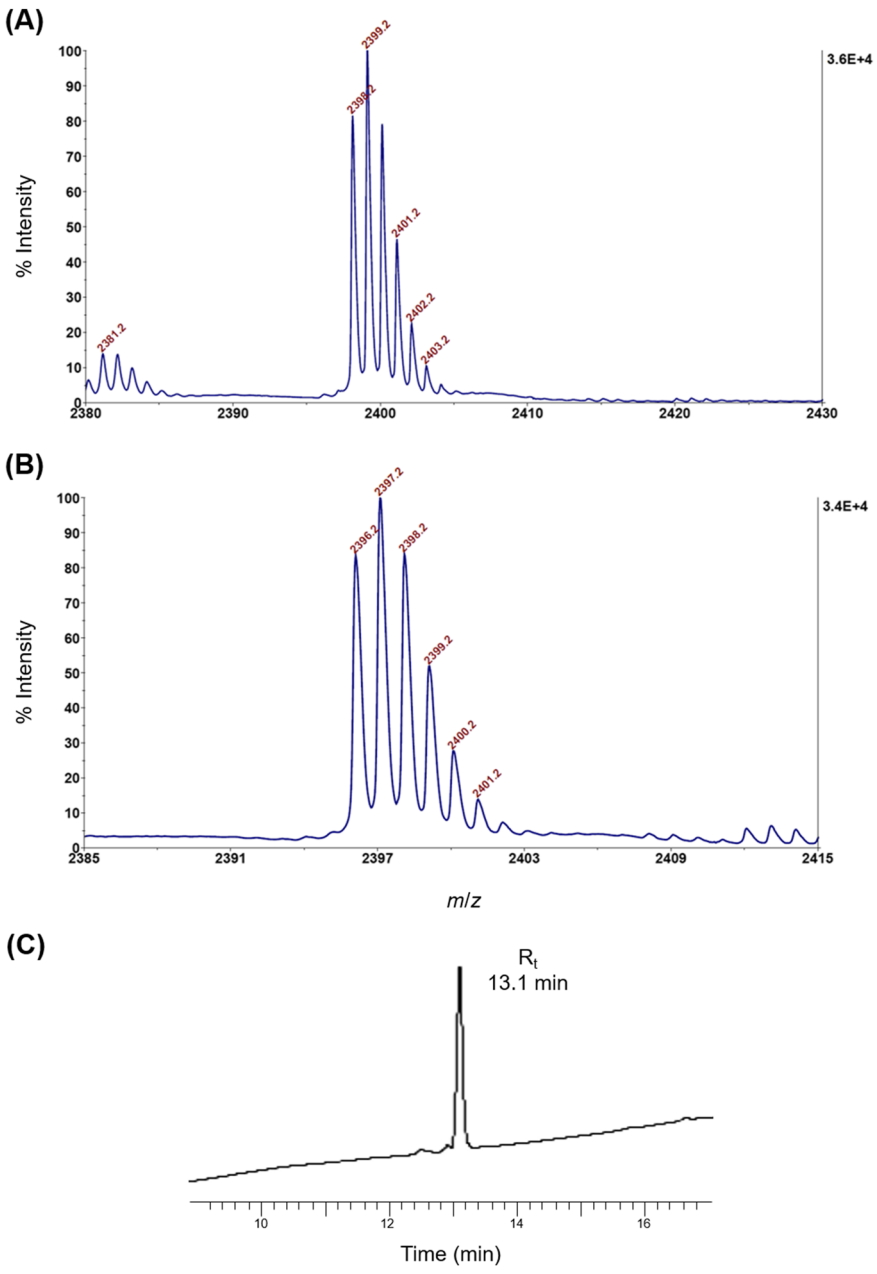


FIGURE 4 Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF)/TOF mass spectra of (A) linear and (B) cyclic ECL1 peptide variant 2 (*m/z* calcd for $C_{99}H_{159}N_{28}O_{35}S_3$: 2396.08 $[M + H]^+$; *m/z* found: 2396.2 $[M + H]^+$). Samples dissolved in 5% (v/v) formic acid in water. (C) RP-HPLC chromatogram of cyclic ECL1 peptide variant 2. R_t = 13.1 min (method A, analytical column)

Formation of the cyclic ECL1 was observed after 3 days of reaction. Analysis by MS confirmed cyclization of ECL1 peptide variant 2 (Figure 4A,B). The disadvantage of disulfide bridge formation with DMSO rests on the need to remove DMSO at the end of the reaction, which was successfully accomplished by semipreparative HPLC, obtaining the final peptide with >95% purity (Figure 4C).

4 | CONCLUSIONS

Non-invasive imaging of CFTR at the cell surface remains a challenging task in the CF field. Hence, with the goal of developing an antibody-based radioprobe against an extracellular region of CFTR, we synthesized the first extracellular loop of CFTR (ECL1) by Fmoc-based microwave-assisted SPPS using a bulky side-chain protecting group for aspartic acid residues present in the ECL1 sequence to avoid aspartimide formation. The peptide was biotinylated and a PEG spacer was introduced between the peptide and biotin to avoid steric interferences. These modifications will allow improved isolation of phage-antibody-antigen complexes during antibody selection by phage display.

ACKNOWLEDGEMENTS

This work was funded by Grant PTDC/BTM-TEC/29256/2017 from Fundação para a Ciência e Tecnologia (FCT), Portugal, cofunded by Lisboa2020—EU FEDER. FCT is also acknowledged for a PhD fellowship to V.F.C. Ferreira (SFRH/BD/108623/2015) and for Research Unit grants to C^2TN (UID/Multi/04349/2019) and to BioISI (UID/MULTI/04046/2019). ESI-MS analyses were carried out on a QITMS instrument acquired with the support of RNEM—Portuguese Mass Spectrometry Network, ref. LISBOA-01-0145-FEDER-022125, financed by FCT and Lisboa2020—EU FEDER.

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How to cite this article: Ferreira VFC, Correia JD, Farinha CM, Mendes F. Improved Fmoc-solid-phase peptide synthesis of an extracellular loop of CFTR for antibody selection by the phage display technology. *J Pep Sci*. 2020;e3253. https://doi.org/10.1002/psc.3253