Journal of Peptide Science

Received: 17 April 2010 Revised: 1 June 2010 Accepted: 1 July 2010 Published online in Wiley Online Library: 17 September 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1282

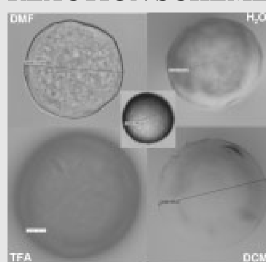
ChemMatrix® for complex peptides and combinatorial chemistry

Yésica García-Ramos,[a,b] Marta Paradís-Bas,[a,b] Judit Tulla-Puche[a,b]* and Fernando Albericio[a,b,c]*

CM resin is a totally PEG-based resin, made exclusively from primary ether bonds and therefore highly chemically stable. Compared to other PEG resins, it exhibits good loading and is user friendly because of its free-flowing form upon drying. It shows improved performance over PS resins for the preparation of hydrophobic, highly structured poly-Arg peptides. In combination with ψPros, it allows the synthesis of small proteins such as the chemokine RANTES. Like other PEG-based resins, CM resin swells well in biocompatible solvents such as water, thereby allowing on-bead screening. Furthermore, the high loading of this resin permits the use of a tiny quarter of a bead as a microreactor for HPLC and MALDI-TOF analysis, thus further extending its applications in the field of combinatorial chemistry. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-phase peptide synthesis; resin; PEG; combinatorial chemistry

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

Handling Procedure *Preliminary washings of CM resin*. CM resin is washed before its use, as follows: MeOH (2 × 1 min), DMF (2 × 1 min), CH_2Cl_2 (3 × 1 min), TFA–CH_2Cl_2 (1:99) (3 × 1 min), (DIEA)–CH_2Cl_2 (1:19) (3 × 1 min) and CH_2Cl_2 (3 × 1 min). Afterwards, it is ready for use in solid-phase peptide synthesis. *CM resin drying*. The last three washes are performed with a volatile solvent such as DCM or diethyl ether, and the resin is then dried under vacuum or inert atmosphere to prevent oxidation. *TFA cleavage*. The peptidyl-resin is cleaved with a cleavage cocktail such as TFA-H_2O (95:5) or the appropriate scavenger cocktail, and is stirred for 2 h (10 mL cocktail solution/g resin). The filtrate is collected and partially evaporated under N_2. Next, cold *tert*-butyl methyl ether is added to the acidic solution, thereby allowing the peptide precipitation. Centrifugation followed by (scavenger and ether) decantation yields the target peptide, which is dissolved in ACN–H_2O (1:1) and lyophilized. *CM resin cutting*. A single bead is isolated and placed under a microscope and then swelled in water. With the help of two needles, the bead is sliced into four pieces. Each of the four quarters is then placed in an Eppendorf tube, cleaved with TFA–H_2O (9:1) for 90 min, redissolved in 10 μL of ACN–H_2O (1:1) and then injected into the analytical HPLC for characterization.

Scope and Comments

Solid phase is the most selected method used to synthesize small and medium peptides by a stepwise strategy. In the case of large peptides, convergent strategies should be implemented because of the tendency of the peptide to form secondary structures and therefore to aggregate, resulting in the formation of incomplete sequences. These problems are more crucial when hydrophobic PS resin is used. Traditionally, the incorporation of PEG into PS has resulted in resins that partially overcome these problems [1–4]. In a seminal paper, Czarnik *et al*. compared PS resin and PS cross-linked resin with distinct amounts of PEG chains (with a 40, 70 and 70–80% PEG, respectively) [5]. Under identical conditions, faster kinetics was observed on PEG-PS resin: the greater the PEG content

* *Correspondence to: Judit Tulla-Puche, Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain. E-mail: judit.tulla@irbbarcelona.org*

Fernando Albericio, Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain. E-mail: albericio@irbbarcelona.org

a *Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain*

b *CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain*

c *Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1-11, 08028-Barcelona, Spain*

Abbreviations used: *ACH, α-cyano-4-hydroxycinnamic acid; ACP, acyl carrier protein; CM, ChemMatrix®; HCTU, 1-[bis(dimethylamino)methylene]-6-chloro-1H-benzotriazolium hexafluorophosphate 3-oxide; MASPPS, microwave-assisted solid-phase peptide synthesis; ψPro, pseudoproline; PS, polystyrene; OBOC, one bead one compound; OBOR, one bead one reactor.*

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in the resin, the faster the reaction. A possible explanation for this observation is that PEG chains interfere with solvation, dielectric properties and hydrogen bonding.

Thus, a rational move would be the use of a totally PEG-based resin. Independently, Meldal and Côté developed, respectively, Versamatrix [6] and CM [7], both PEG-based resins. CM, which is the only commercially available PEG resin, exhibits high loadings (up to 1.0 mmol/g) in comparison with other PEG and PEG-PS resins and similar loadings to those of classical PS resins.

CM resin is composed exclusively by primary ether bonds that are highly cross-linked, forming the PEG matrix [8]. This composition makes the resin mechanically and chemically stable in the presence of a wide range of solvents. CM resin is presented as free flowing powder, thereby allowing straightforward manipulation when weighed or transferred. The distinct helical arrangements with gauche interaction between the polarized bonds shown by the vicinal carbon–oxygen bonds confer CM an amphiphilic character, thus making this matrix a well-solvated resin in polar and non-polar solvents. Thus, the greatest advantage of CM resin is its superior swelling properties than PS in current solvents for solid phase (DMF and DCM), in polar solvents such as DMSO, MeOH or ACN, and in aqueous buffers (Figure 1) [9].

Lubell *et al*. performed a comparative study of various resins [10] to examine the way in which the polymeric support influences the synthesis of difficult sequences. Thus, it was found that using ACP 65–74 as a model, a crude product of superior purity was achieved with CM resin. The same study demonstrated that PEG-PS gives better results than PS, thus confirming the findings of Czarnik *et al*. [5]

In our hands, the first example was the manual stepwise synthesis of Thymosin α1, a relevant compound for the treatment of hepatitis B and C. This 28-residue peptide presents aggregation limitations as its sequence (*Ac-SDAAVDTSSEITTKDLKEKKEVVEEAEN-OH*) contains a high number of protecting groups and β-branched amino acids. These characteristics hinder stepwise synthesis [11]. In the first attempt, Thymosin α1 was synthesized following a Fmoc/*t*Bu strategy using PS resin, and during peptide assembly low yield couplings and incomplete deprotections were detected because of the peptide arrangement as a β-sheet. The *C*-terminal decapeptide, *H-KKEVVEEAEN-OH*, already showed many amino acid deletions, and termination of the peptide was observed after the incorporation of the two Val residues. After performing several syntheses using various resins and modified synthetic strategies, a major improvement was achieved with the PEG-based resin CM. Assembly of the 10-mer *C*-terminal peptide on Rink-CM resin (0.6 mmol/g) by side-chain anchoring of Fmoc-Asp-O*t*Bu, and using HCTU/DIEA as coupling system, remarkably improved the purity of this segment (Figure 2A). The noteworthy performance of this resin could be much perceived after elongating the full length Thymosin α1 and performing the final acetylation, when a purity of ~90% for the crude peptide was obtained (Figure 2B). The peptide was purified by semi-preparative C_{18} reversed-phase HPLC using a linear gradient of 10–30% ACN over 45 min, raising the purity over 99% (Figure 2C).

CM resin is often used in an automatic synthesizer for the stepwise solid-phase synthesis of difficult sequences, such as the well-known β-amyloid (1–42) peptide, which is prone to aggregation, a problem that hampered previous attempts with other resins [8]. When the final peptide was accomplished, it was dissolved in HFIP in order to characterize it by HPLC and MALDI-TOF. The results showed the achievement of the target peptide in high purity and yield, thereby removing the aggregation problems.

A clear confirmation that CM resin has a decisive role in the outcome of the synthesis was demonstrated by the automatic assembly of ^{19}F-labeled HIV-1 protease, which is a 99-residue peptide [12]. CM resin has also shown great utility for the synthesis of MAPs [13].

Another striking example of how CM resin improves the assembly of difficult sequences is the 68-amino acid chemokine RANTES, which has a high tendency to aggregate [14]. The stepwise synthesis attempted with PS-based resin failed, although an improvement was obtained when several ψPro dipeptides [15] were introduced in the assembly. However, this modification was not enough to achieve the target peptide. Finally, the target peptide was successfully obtained with the synergic collaboration of CM resin and ψPros [14]. The same combination of ψPros and CM resin has allowed the synthesis of other chemokines [16].

The compatibility of CM resin with polar solvents has been demonstrated by the synthesis of small model peptides using only ACN as a solvent [9], as well as of oligonucleotide–peptide conjugates [17].

Although most examples of synthesis using CM resin in the literature describe Fmoc-based peptide syntheses, Tiefenbrunn and Dawson used this resin with Boc chemistry for the synthesis of a 32-residue thioester peptide, which was obtained in 40% purity. In contrast, the identical synthesis on PS resin of similar loading yielded a product with only 10% purity [18].

MASPPS currently offers advantages over conventional SPPS, not only is the former less time consuming but it also enhances the purity of the final product. On CM resin, the β-amyloid (1–42) peptide was achieved in higher quality and in only 15 h. Microwave radiation in coupling and deprotection steps were carried at 86 °C [19,20]. Racemization-sensitive residues, such as histidine, were coupled during 60 min at room temperature. Special mention should be given to the stability of CM resin under high temperatures and microwave radiation during the assembly of a long peptide.

The last application of CM that should be highlighted is the synthesis of libraries that require a solid support compatible both with organic solvents, necessary during the synthesis, and with aqueous solution, which is essential to allow the screening [21]. The libraries were performed by the OBOC strategy through a divide–couple–recombine method, acquiring one compound per bead in an inexpensive and rapid way [22]. Although the beads can be characterized directly, they can also be sliced into two or four parts. The characterization of one portion can be performed by HPLC and MALDI-TOF-MS, whereas the remaining parts can be reserved for further analysis and/or manipulations. When the four slides were analyzed, good homogeneity of each portion was shown, as demonstrated by identical chromatographic profiles and MS [23]. This intriguing performance of CM resin has allowed the development of the OBOR concept as the peptide attached in one bead is enough to make several manipulations.

Limitations

The first limitation of CM resin is that during peptide synthesis, there is a large increase in resin volume, which can result in an overflow of the resin into the reactor. This overflow could affect the automatic synthesis of large peptides and therefore it is necessary to control the reactor.

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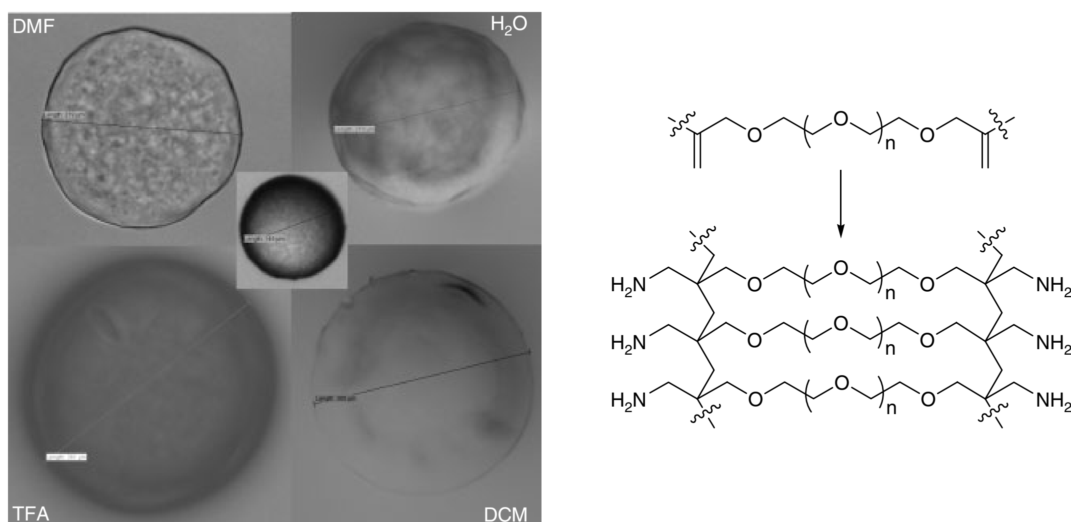

Figure 1. Left, swelling of CM beads of 150 ± 10 µm (center) in different solvents used in solid phase; and right, schematic representation of CM monomer and resin.

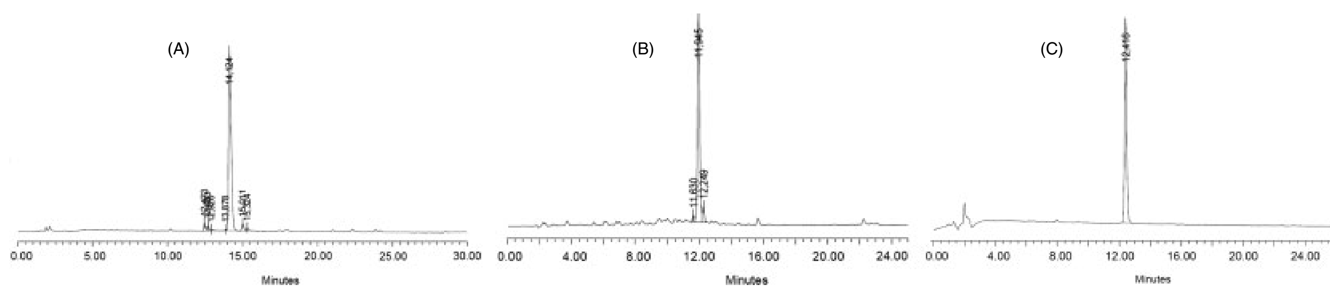

Figure 2. Analytical HPLC of (A) the decapeptide *H-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH*; (B) full Thymosin α1; and (C) pure Thymosin α1.

The second limitation is the appearance of some negative false results in the ninhydrin test, a procedure used to verify coupling performance. This limitation can be overcome by using a greater amount of resin. Furthermore, it is advisable to check the result of ninhydrin test with the help of a microscope. Conversely, the NF-31 assay, a more sensitive test that is especially suitable with sterically hindered secondary and aromatic amines, could give rise to false positive results as a result of the reactive residual alcohols originated by resin elaboration [24].

A further limitation of CM is also related to the increase in resin volume during TFA cleavage. This increase is greater than that observed during the synthesis, because CM resin has the best swelling properties in TFA. This phenomenon is not an obstacle at the research level, but may be more problematic for the industrial use of the resin.

Finally, and for unknown reasons, the recovery of the peptide after the cleavage is often lower than the theoretical value. After TFA cleavage and regular washings, additional washings with HOAc have been observed to increase the recovery of the final peptide.

CM resin is considered a breakthrough in solid-phase peptide synthesis. It provides the conditions necessary to obtain difficult peptides through simple synthesis, something that is impossible to achieve with the traditional PS resin. The combination of CM resins with ψPros should be the strategy of choice for the preparation of complex peptides and small proteins.

Experimental Procedure

Manual synthesis of thymosin α1 using CM resin

The solid-phase synthesis of Thymosin α1 with CM resin (5 g, 0.45 mmol/g) was carried out in a glass funnel fitted with a filter. The solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine–DMF (1 : 4, v/v) (2×1 min, 2×10 min, 1×5 min). Washings, after deprotection and coupling steps, were carried out with DMF (5×0.5 min) and CH_2Cl_2 (5×0.5 min) using 10 mL solvent/g resin each time. All the stages of the peptide synthesis were performed at 25 °C. The linker Rink-Amide (2.5 equiv.) was anchored to the resin with HCTU (2.4 equiv.)/DIEA (5 equiv.) in DMF for 12 h. The remaining residues (5 equiv.) were coupled with HCTU (4.8 equiv.) and DIEA (10 equiv.). The three components were first mixed in DMF, then shaken for 1 min in order to allow pre-activation, and finally added onto the resin. The peptidyl-resin mixture was mechanically stirred for 2 h. After each coupling, the ninhydrin test was run to check the coupling yield. When the test did not show a negative result then recoupling was performed, applying the same coupling conditions. Also, the synthesis was controlled by HPLC, taking a representative sample of peptidyl-resin that was cleaved by means of the mixture TFA–H_2O (95 : 5) for 1 h. The intermediate was then analyzed. After deprotection of the last serine residue, acetylation was performed with DIEA (10 equiv.) and Ac_2O (10 equiv.). The resin was split in order to cleave the peptide. This was achieved using a glass funnel fitted with a filter, employing the cleavage mixture TFA–H_2O (95 : 5) for 2 h and 10 mL of cleavage cocktail/g

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resin. The acidic solution was poured on cold *tert*-butyl methyl ether, thereby precipitating the target peptide. The product was isolated by centrifuging the mixture and decanting the solution. This procedure was repeated twice. The white solid was dissolved in H_2O–ACN (1 : 1) and lyophilized. The peptide was characterized using an Analytical HPLC apparatus from Waters, an instrument involving two solvent delivery pumps (Waters 1525) and automatic injector (Waters 717 autosampler) and a dual wavelength UV/Vis detector (Waters 2487). We used a Symmetry™ C_{18} 4.6 × 150 mm, 5 μm column and the system solvent was ACN (0.036% TFA) and H_2O (0.045% TFA). The white solid was also analyzed by MALDI-TOF by crystallizing the peptide with ACH matrix and running the sample in a PerSeptive Biosystems Voyager DE RP: calcd 3106.50, found *m/z* 3107.57 $[M + H]^+$.

Acknowledgements

This work was partially supported by CICYT (CTQ2009-07758), the *Generalitat de Catalunya* (2009SGR 1024), the Institute for Research in Biomedicine, and the Barcelona Science Park. J.T.-P. is a Juan de la Cierva fellow (Micinn). We thank Simon Côté and Rock Terrien from Matrix Innovation (Québec, Canada) for generously providing the ChemMatrix® resin.

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