UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY TECH

Escuela de Ciencias Químicas e Ingeniería

Título: Globular polymer supports assisted scaled-up Membrane Enhanced Peptide Synthesis: an overview

Trabajo de titulación presentado como requisito para la obtención del título de Químico(a)

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Urcuquí, abril 2024

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A mi madre,

Gracias

AGRADECIMIENTOS

Eterna gratitud a mis maestros por su noble acto de enseñar, sin ellos ninguno de los siguientes agradecimientos tendría lugar. A la entera guía de mi profe Hortensia, que me resaltó siempre mis aciertos por encima de los inevitables errores y me llenó de alegría y motivación en cada ocasión. Gracias a todos por hacerme saber que, si hay alguien por ahí haciéndolo, yo también puedo ser curiosa, entender, experimentar, hacer mil preguntas y encontrar felicidad en ello. No forman solo profesionales, forman personas críticas incapaces de indiferencia, con sueños y semillas de lo mejor de ustedes para perseguirlos.

Infinita gratitud a mi madre, Miryan, mi señora todo terreno y la razón de mi vida. Su confianza y positivismo me hacen saber que nada puede ir mal, que el mundo es muy gentil y que nuestros anhelos nunca son demasiado grandes. A mi segunda madre, Gladycita, quien me ha dado todo lo que soy desde que tengo uso de razón. A mis padres grandes, Humberto y Camelita, por criar mujeres fuertes, luchadoras y sembrar el deseo de superación en nuestro corazón, cada uno de mis pasos les pertenece solo a ustedes. A mis padres en el otro lado del mundo, Silvia y Bayardo, quienes me abrazaron y apoyaron las decisiones valientes en los momentos más acertados.

A las lindas amistades que me dejó la carrera, gracias por ser genuinamente personas maravillosas, por compartir lo que saben siempre con una sonrisa y enseñarme cómo luce el verdadero compañerismo. Gracias por la complicidad de estos años, las risas sin sentido, los desvelos y el mutuo apoyo emocional que nos mantuvo casi cuerdos.

Al pueblito y la gente de Urcuquí, su amabilidad y hospitalidad hicieron que mi casa no se sintiera tan lejos. Vivir en medio de montañas, comer la fruta más fresca y bailar los mejores San Juanes, sin duda son motivos de gratitud.

ABBREVIATIONS

CSPS	Classical solution peptide synthesis
SPPS	Solid-phase peptide synthesis
LPPS	Liquid-phase peptide synthesis
MEPS	Membrane enhanced peptide synthesis
OSN	Organic solvent nanofiltration
PEG	Polyethylene glycol
MWCO	Molecular weight cut-off
HBTU	N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide
DIEA	N,N-Diisopropylethylamine
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
PMDA	Pyromellitic dianhydride
HPLC	High Performance Liquid Chromatography
CuAAC	Copper-Catalyzed Azide-Alkyne Cycloaddition
ROS	Reactive oxygen species

RESUMEN

La fabricación a gran escala es un paso difícil para llevar medicamentos biológicos al mercado, y se ve significativamente afectada por los métodos utilizados en la síntesis química de péptidos. Estos métodos, derivados de la síntesis clásica de péptidos en solución (CSPS), se pueden clasificar en términos generales en síntesis de péptidos en fase sólida y en solución (SPPS y LPPS, respectivamente). Aunque los primeros péptidos comerciales se fabricaron utilizando esta última síntesis, el SPPS ha ganado rápidamente aceptación. El principal inconveniente asociado a la síntesis de péptidos en fase sólida (SPPS) corresponde a que las reacciones ocurren en un medio heterogéneo. En este contexto, las metodologías de síntesis de péptidos en fase líquida (LPPS), que emplean un polímero soluble, emergen como una alternativa prometedora. La última mejora en la preparación de ingredientes farmacéuticos basados en péptidos a escala de laboratorio, es la síntesis de péptidos mejorada por membrana (MEPS). Este enfoque innovador integra la síntesis de péptidos en fase líquida (LPPS) con la nanofiltración de solventes orgánicos (OSN), lo que ofrece diversas ventajas sobre la síntesis de péptidos en fase sólida (SPPS). MEPS ha demostrado una resistencia superior frente a las limitaciones de transferencia de masa, ya que requiere un menor exceso de reactivos y sus pasos de purificación consecutivos son confiables. Estos atributos colocan a MEPS como una metodología atractiva para propósitos de escalamiento. El presente estudio proporciona un protocolo detallado para MEPS asistido por soporte polimérico globular, en una escala superior al kilogramo. Este protocolo contempla condiciones de reacción, consideraciones de seguridad y factores de costo, todos diseñados para ser automatizables y compatibles con la química peptídica ortogonal basada en Fmoc bien establecida.

Palabras clave: Escalado, filtro de membrana, soportes poliméricos, síntesis de péptidos, nano filtrado.

ABSTRACT

Scale-up manufacturing is a bottleneck step to bringing biological drugs to market, significantly impacted by the methods used in chemical peptide synthesis. These methods, derived from classical solution peptide synthesis (CSPS), can broadly be categorized into solid-phase and solution peptide synthesis (SPPS and LPPS, respectively). Although the first commercial peptides were manufactured using the latter synthesis, SPPS has rapidly gained acceptance. The principal drawback associated with Solid-Phase Peptide Synthesis (SPPS) corresponds to reactions occurring within a heterogeneous medium. In this context, Liquid-Phase Peptide Synthesis (LPPS) methodologies, employing a soluble polymer, emerge as a promising alternative. The latest improvement in the preparation of peptide-based pharmaceutical ingredients at laboratory scale is Membrane Enhanced Peptide Synthesis (MEPS). This innovative approach integrates Liquid-Phase Peptide Synthesis (LPPS) with Organic Solvent Nanofiltration (OSN), offering diverse advantages over Classical and Solid-Phase Peptide Synthesis (CSPS and SPPS). MEPS has demonstrated superior resilience against mass-transfer limitations, requiring a smaller excess of reagents, and featuring reliable consecutive purification steps. These attributes place MEPS as an appealing methodology for scaling purposes. The present study provides a detailed protocol for Globular Polymeric Support-Assisted MEPS, at a scale beyond kilogram. This protocol contemplates reaction conditions, safety considerations, and cost factors, all designed to be automatable and compatible with the well-established orthogonal Fmoc-based peptide chemistry.

Keywords: Scale-up, membrane filtration, polymeric supports, peptide synthesis, nanofiltration.

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CHAPTER 1. INTRODUCTION – JUSTIFICATION

1.1 General introduction

Over the 2017–2021, the FDA approved releasing 244 new drugs: 178 chemical entities and 56 biologics.[1]. Given the widespread recognition and acceptance of peptides as therapeutic agents, the need for refined production procedures to meet the growing demand is evident. [2,3] Along with that, the current challenge is developing methodologies that address cost-effective manufacturing on a larger scale. Three approaches, namely, classical solution peptide synthesis (CSPS), solid-phase peptide synthesis (SPPS), and liquid-phase peptide synthesis (LPPS), have played a substantial role in advancing the peptide synthesis field.[4] The very first approach used to peptide preparation was the classical solution synthesis (CSPS). [5,6] Classical solution peptide synthesis facilitates the production with minacious precision of research-scale peptides, through isolation and characterization of each intermediate in homogeneous mode. Unfortunately, this is especially demanding, time-consuming and unsuitable for medium to long-sized peptides due to its detailed level of preparation. On the contrary, the subsequent well researched solid phase strategy avoids the need of intermediate purification and simplifies the work up while reaching excellent peptides in few hours. [7]. Nevertheless, SPPS main drawback from green chemistry perspective is associated with the huge amounts of solvents required at each step. [8] However, great interest has shifted to a third synthetic route. The convergence of classical and solid peptide synthesis attributes falls in the third "wave" of peptide synthesis: liquid phase (LPPS). [4]

In liquid phase peptide synthesis, reactions are carried out similarly to CSPS while applying the "support" concept of SPPS, but simply replacing the solid support by a soluble PEG tag. [9,10] The tag is either a well-defined molecule or a soluble polymer with highly innovative and special differential properties to facilitate its removal using only physical methods. [4] Unique physicochemical characteristics of tags along with the right separation method leads to reinforcement of cyclization concepts and scaling improvements. In this context, organic solvent nanofiltration (OSN) appears as the ideal pressure-driven filtration technique capable of producing efficient molecular scale separations in

solution. [11,12]

LPPS is an efficient method that saves energy, materials, and reduces labor force.[4] When combined with the purification idea of organic solvent nanofiltration (OSN), it offers appealing improvements on a larger scale. This new converged technology platform is called Membrane Enhanced Peptide Synthesis (MEPS). [13] In previous research, our group proved that Membrane Enhanced Peptide Synthesis (MEPS) supplies a novel solution for preparing peptide-based pharmaceutical ingredients. [13,14] Compared to traditional methods driven around 50-80°C, membrane filtration operates at ordinary temperatures between 15-25°C, does not need phase transitions, and affords low energy consumption. [15–19] The MEPS strategy stands out by utilizing a single reactor for all synthetic processes, addressing the environmental concerns associated with SPPS. Besides, it is less constrained by mass-transfer limitations, requires smaller excess of reagents, and purification methods are improved by consecutive steps, yet demonstrating good purity of the final peptide. [20, 21]

For successful MEPS, both membrane and support characteristics are key. On one hand, membranes must have excellent long-term stability in organic solvents, present a low rejection of amino acids, exhibit reproducible performance and high selectivity between soluble polymeric supports, their derivatives, byproducts, and excess reagents. [22] Ceramic materials (silicium carbide, zirconium oxide, and titanium oxide) show stable performance in solvent medium, and, therefore, are excellent materials for membrane preparation. [23] On the other hand, supports morphological features help to avoid polymer loss when applying filtration pressure. The ability to deform under a flow field of branched globular tags, resulted in better rejection than the linear ones. [22, 24, 25] Consequently, the design and synthesis of new anchors PEG functionalized branched polymers are desirable globular supports with better % rejection. Addressing that necessity, our group had successfully designed three novel branched soluble polymeric supports: DPEG, DNPEG, and PyPEG, revealing excellent rejection percentage. [13]

This study provides the detailed protocol for the scaled-up membrane enhanced peptide synthesis of the model peptide: Fmoc-RADA-NH_2. Based on reaction

conditions optimization, safety and costs, PyPEG is the anchor of choice as soluble polymeric support, and Inopor 750 (MWCO 750 Da) as the ceramic membrane. Through subsequent steps of coupling, diafiltration and deprotection, successful preparation at scale beyond kilogram is achieved.

1.2 Problem Statement

Increasing demand of peptide based pharmaceutical ingredients highlights the lack of automatized processes at large scale to lead an environmentally conscious synthesis. MEPS has demonstrated its suitability to overcome the limitations of CSPS and SPPS, combining the best of them in LPPS using globular polymeric supports along with organic solvent nanofiltration technique.

In this regard, as **hypothesis**, globular polymer supports could assist membrane enhanced peptide synthesis to ease the scaling-up of biological drugs. The designed model peptide could serve as proof of concept to stablish a novel synthetic methodology due to its numerous advantages over traditional techniques.

1.3 General and specific objectives

1.3.1. General objective

To study the viability of employing globular polymer supports to assist scaled-up of the process via membrane enhanced peptide synthesis (MEPS).

1.3.2 Specific objectives

- To synthesize a model peptide using PyPEG as soluble branched support by means of MEPS.
- To study the reproducibility under similar conditions of lab-scale protocol to a scaled-up protocol.
- To develop a detailed protocol of larger scale preparation of peptides addressing cost-effective synthesis through green chemistry.

CHAPTER 2. METHODOLOGY

2.1 Materials and Methods

The OSN membrane used was ceramic Inopor® 750 (MWCO of 700 g· mol^{-1}, pore size: 1 nm, material: TiO_2) provided by Inopor company HITK (Germany). PEEK (MWCO not reported) flatsheet membranes provided by Imperial College (UK) and MET company (UK). All Fmoc-L- AA-OH and Fmoc-Rink-Amide, and HBTU were purchased from Iris Biotech. DIEA, O,O'-Bis(3-aminopropyl)polyethylene glycol, piperidine and PMDA were purchased from Sigma-Aldrich and all used without purification unless otherwise noted. All other reagents were purchased from Carlo Erba, Romil and Lonza and used without further purification.

2.2 Chromatography and Spectroscopic Equipment

IR spectra were determined in an FT-IR Nexus (Termo Nicolet 760). All analytical HPLCs were performed in two systems: HPLC PDA 2695 Alliance using two different Sunfire columns: RP-C18 column (10 mm, 4.6 nm x 100 nm reverse phase column), gradient from 100% of ACN of the ACN (0.036% TFA) into H2O (0.045% TFA) were run at 0.3mL/min flow rate over 8 min; and RP-C18 column (10mn, 4,6mm x 150nm reverse phase column), gradient from 95% of ACN of the ACN (0.5% TFA) into H2O (0.1% TFA) were run at 0.3mL/min flow rate over 25 min, and HPLC PDA Acquity 157 UPLC Binary Sol MGR Waters using a BioBasic-18 RP-C18 column (5μm, 2.1 x 150nm reverse phase column). Absorbance was detected at 220 nm. Mass spectra were recorded using an Electron Spray Ionization (ESI) technique on a Micromass ZQ, Waters SN: MAA 076; and Acquity UPLC Binary Sol MGR (Waters Corporation), linear gradients of ACN (0.1% formic acid) into H2O (0.1% formic acid) were run at 100μL/min flow rate over 9 min, mass spectra were recorded using a LCT-Premier (Waters) (TOF analyzer). NMR spectra were recorded using two systems: Bruker DPX 400 (400 MHz-1H, 75.4-13C), and Bruker DPX 500 (500 MHz-1H, 75.4-13C). Chemical shifts are given as δ values against tetramethylsilane as the internal standard.

2.3Experimental Design

In MEPS, the anchor (PyPEG-linker) first reacts with the activated Fmoc-protected amino acid. Then, diafiltration is performed to remove the excess activated amino acid and the byproduct of coupling. The deprotection reagent is then added into the system to remove the Fmoc group, after which another diafiltration is performed to remove the deprotection reagent and the byproduct of deprotection. Further, peptide elongation is achieved by coupling cycles, diafiltration, deprotection and diafiltration. Finally, the peptide is cleaved from the anchor, globally deprotected and purifiedby preparative HPLC. This procedure is illustrated in figure 1.

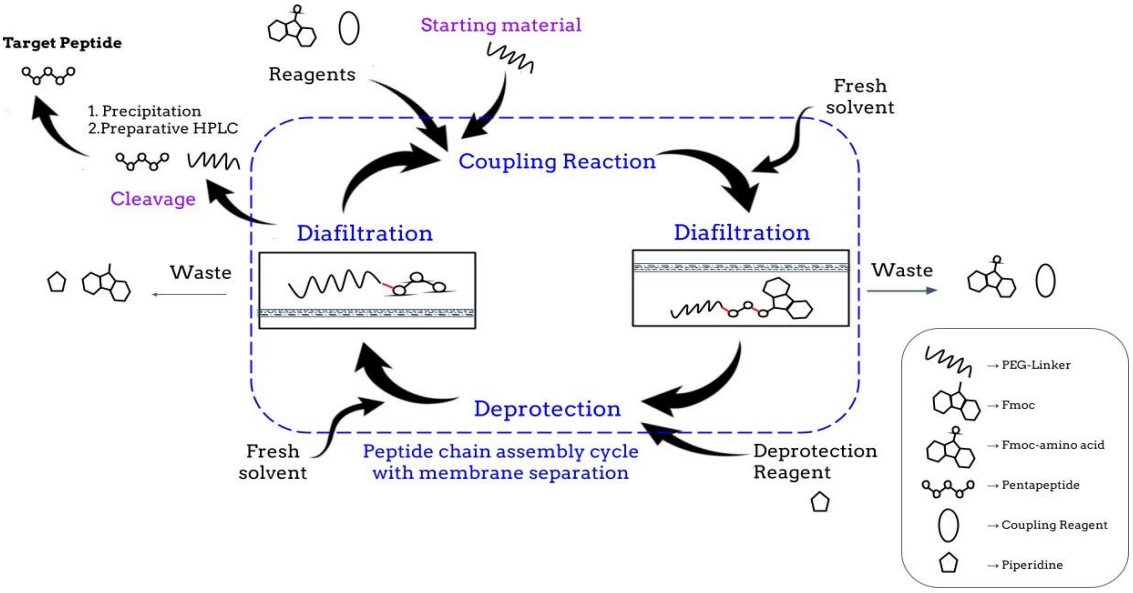


Figure 1: Membrane enhance peptide synthesis (MEPS). Adapted from Ref [26]

Rink functionalization of the soluble polymeric support is performed via amide bond formation between PyPEG and Fmoc-Rink amide-linker in the presence of HBTU, and DIEA in DCM as solvent. The obtained Fmoc-NH-Rink-PyPEG is purified through OSN with DCM solvent. Further, Fmoc deprotection is carried out using piperidine 20% in DMF for 30 min, followed by OSN purification, and H_2N-Rink-PyPEG is obtained with good purity. Next, RADA-NH_2 is obtained from H_2N-Rink-PyPEG using sequential steps of Fmoc/t-Bu strategy. Membranes used in organic solvent nanofiltration (OSN) are performed in dead-end filtration mode using hydrostatic or gaseous pressure (N_2 at 10 bar), letting the entire solvent volume pass through the membrane. The experimental setup and a representative scheme are illustrated in figure 2.

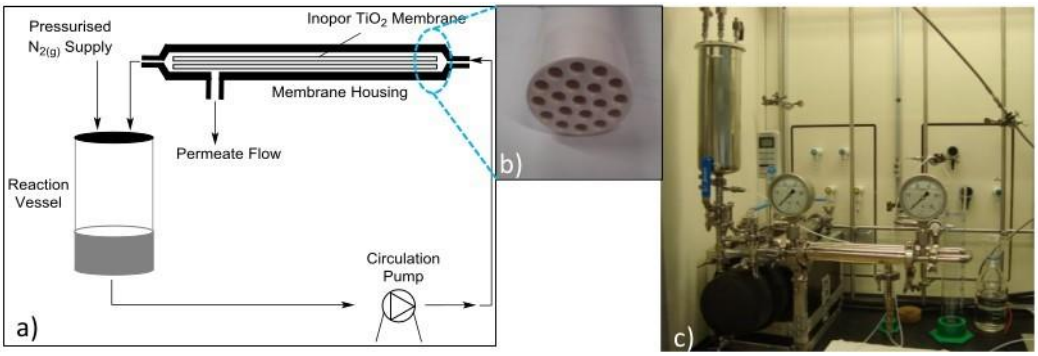
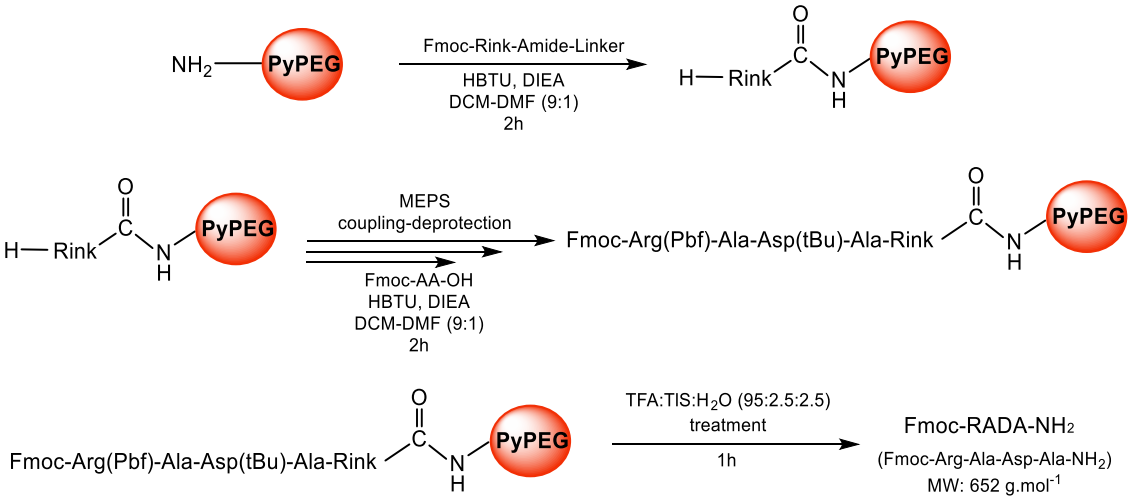


Figure 2: Ceramic membranes on OSN (a) Representative scheme (b) Ceramic membrane (c) Experimental equipment.

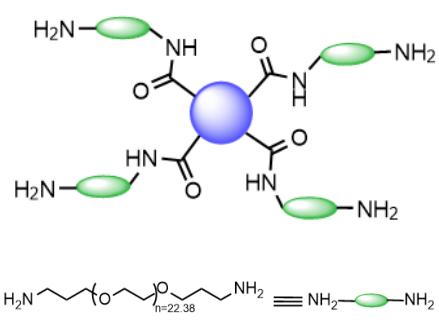
2.4 Scaled-up synthesis

We developed the experimental design for the scaled-up process based on previous lab-scale MEPS optimization using globular polymeric supports. [13]. First, soluble polymeric support preparation of globular PyPEG was performed, followed by its Rink functionalization attaching Fmoc-Rink amide- linker to prepare the anchor. Finally, MEPS is performed to obtain the peptide Fmoc-RADA-NH_2, which is cleaved from the anchor and purified successfully. A graphical representation of the general procedure is presented in scheme 1.



Scheme 1: General procedure for the synthesis of Fmoc-RADA-NH_2 from globular anchor

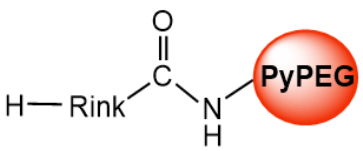
2.4.1 PyPEG polymer preparation



Poly(ethylene glycol) bis(3-aminopropyl) (30 g, 20 mol, 8 eq) was dissolved in toluene (300 mL) and pyromelliticdianhydride PMDA (0.54 g, 2.5 mol, 1 eq). The mixture was placed in 250 mL vessel equipped with a Dean-Stark and a reflux column. The reaction was stirred, heated at 122°C for 8 hours, and analyzed by HPLC and ^{1}H NMR. Solvent removal under reduced pressure was applied and purification to obtain PyPEG by OSN. The membranes were pre-conditioned with pure solvent until steady-state fluxes were achieved. Next, 500 ml of test solution PyPEG crude was charged to the cell. 500 ml were allowed to permeate through the membrane for each filtration, needing 60 diafiltrations to obtain pure PyPEG polymer. For the purification step, it was necessary to neutralize amine with diethylamine (2 g, 27 mmol, 2 eq) in DCM for 1 h. Subsequently, the solvent was removed and under reduced pressure, the solid was again dissolved in DCM (100 mL) and evaporated (10 repeats). Purified PyPEG polymer was analyzed by HPLC and ^{1}H-NMR.

- **IR(KBr):** 3427, 2871, 1653, 1456, 1351, 1299, 1250, 1106, 925, 846 cm^{-1}
- **^{1}H-NMR (500 MHz, D$_6$-DMSO):** δ = 8.30 (s, 4H, H_C), 7.49 (s, 2H, H_A and H_B), 3.65-3.25 (m, H_{PEG}), 2.77 (m, 2H, H_D), 1.72 (m, 2H, H_E)
- **^{13}C-NMR (500 MHz, D$_6$-DMSO):** δ= 28.6, 29.2, 36.5, 37.5, 60.2, 67.6, 68.1, 69.8, 72.3, 127.0, 136.8, 166.9 ppm.

2.4.2 Fmoc-Rink-PyPEG

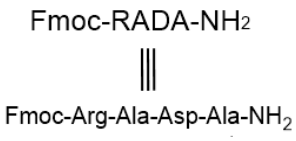


Fmoc-Rink amide Linker (18 g, 33 mol, 3 eq) was dissolved in 300 mL DCM, and DIEA (8.5 g, 66 mol, 6 eq) was added. Separately, HBTU (12.5 g, 33 mol, 3 eq) was dissolved in 120 mL DMF. Then, in a vessel containing 1 L of PyPEG polymer (17 g, 2.7 mmol. 1 eq), theoretical loading; 0.65 mmol NH_2/g was dissolved in 80 mL DCM. All solutions were combined, the reaction was stirred for 2 hours at room temperature, followed by

HPLC and the ninhydrin test. 500 ml of Fmoc-Rink-PyPEG crude were charged to the cell and purified by OSN. 500 mL were allowed to permeate through the membrane for each filtration, needing 40 diafiltrations to obtain pure Fmoc-Rink-PyPEG. Finally, purified Fmoc-Rink-PyPEG was analyzed by HPLC and the ninhydrin test.

- **HPLC:** Fmoc-Rink-PyPEG. tr=19.6 min. Gradient: from 5% to 95% ACN over 25 min.

2.4.3 Fmoc-RADA-NH_2



Fmoc-AA-OH (1.5 eq) was dissolved in 40 mL DCM and DIEA (3.3 g, 25 mmol, 3 eq) was added. HBTU (5 g, 13 mmol, 1.5 eq) was dissolved in 50 mL DMF in another vessel. In a vessel containing 1L H-Rink-PyPEG (13 g, 2 mmol. 1 eq with a theoretical loading of 0.65 mmol NH_2/g) was dissolved in DCM (50 mL), then the solution of Fmoc-AA-OH and DIEA in DCM and the HBTU solution in DMF were both added. The reaction was stirred for 2 hours at room temperature, followed by HPLC and the ninhydrin test. 500 ml of Fmoc-AA-Rink-PyPEG crude were purified by OSN. The membranes were pre-conditioned with pure solvent until steady-state fluxes were achieved. 500 ml were allowed to permeate through the membrane for each filtration, needing 10 diafiltration's to obtain pure Fmoc-AA-Rink-PyPEG. Cleaved peptide: the Fmoc-Arg(Pbf)-Ala-Asp(tBu)-Ala-Rink-PyPEG was treated with TFA-TIS-H_2O (95:2.5:2.5) for 1h and precipitated with $(C_2H_5)_2O$ to obtain the Fmoc-Arg-Ala-Asp-Ala-NH_2 crude.Purified Fmoc-protected peptide was analysed by HPLC and the Kaiser Test.

- **HPLC chromatogram:** tr=8.2 min. Gradient: from 5% to 80% ACN over 9 min.
- **Calculation for $C_{31}H_{40}N_8O_8$:** 652.71 g/mol.
- **ESI-MS (M+H):** 653.31 g/mol.

CHAPTER 3. RESULTS AND DISCUSSION

PyPEG performed better at laboratory scale with a 96.8% purity from the three previously synthesized globular polymeric supports. [13] Due to the globular shape, all polymers presented in figure 3 seem to be good candidates to be used as support in MEPS.

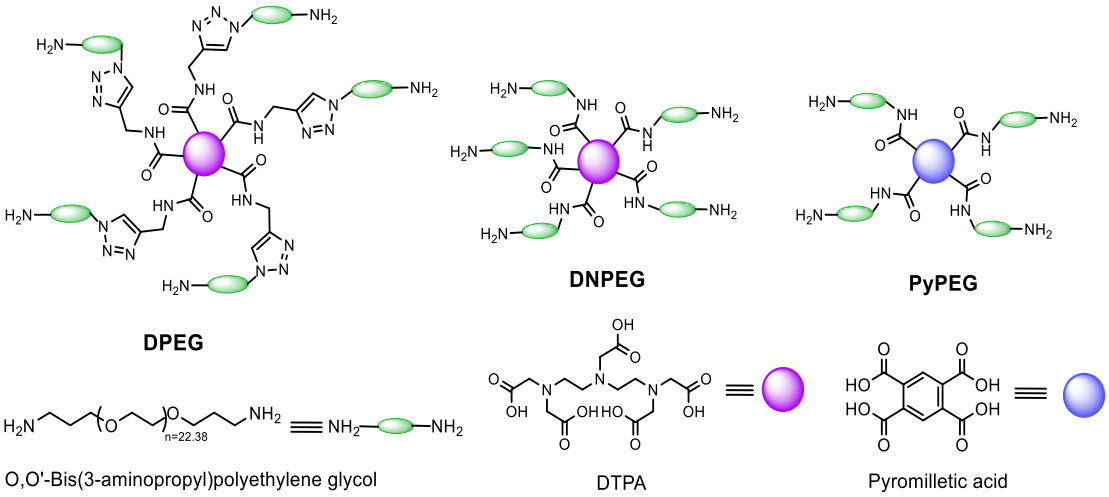
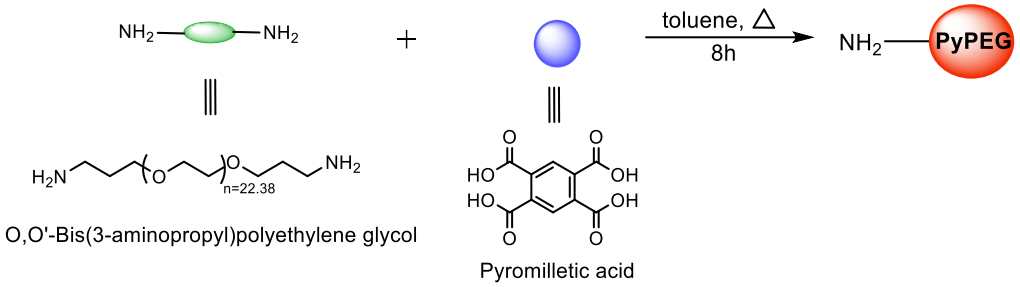


Figure 3: Structures of the three novel branched polymers.

However, in the scaled-up application context, using PyPEG facilitates the overall process since its synthesis can be driven via amide bond formation, instead of the CuAAC reaction performed for DNPEG. The major drawback limiting the use of the Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) reaction in biological systems is the copper-mediated formation of reactive oxygen species (ROS), leading to the oxidative degradation of proteins or peptides. [27] Besides, compared to the insufficient 50% purity reached for DPEG, PyPEG purity is relevantly upgraded. Also, the ease of following the reaction through routine laboratory techniques (NMR, HPLC), makes PyPEG the most suitable candidate for the scaled-up process.

3.1 PyPEG polymer synthesis

PyPEG was prepared following our previously reported lab-scale synthetic path using the same equivalences in the scaled-up process. [13] Scheme 2 summarizes the general procedure.



Scheme 2: Synthesis of PyPEG

Monitoring the progress of the process at each stage is crucial. The results confirm successful obtention of the desired outcomes. The characteristic peaks in the FTIR spectra shown in figure 4 demonstrates the conversion of initial O,O'-Bis(3-aminopropyl)polyethylene glycol into the branched PyPEG polymeric support.

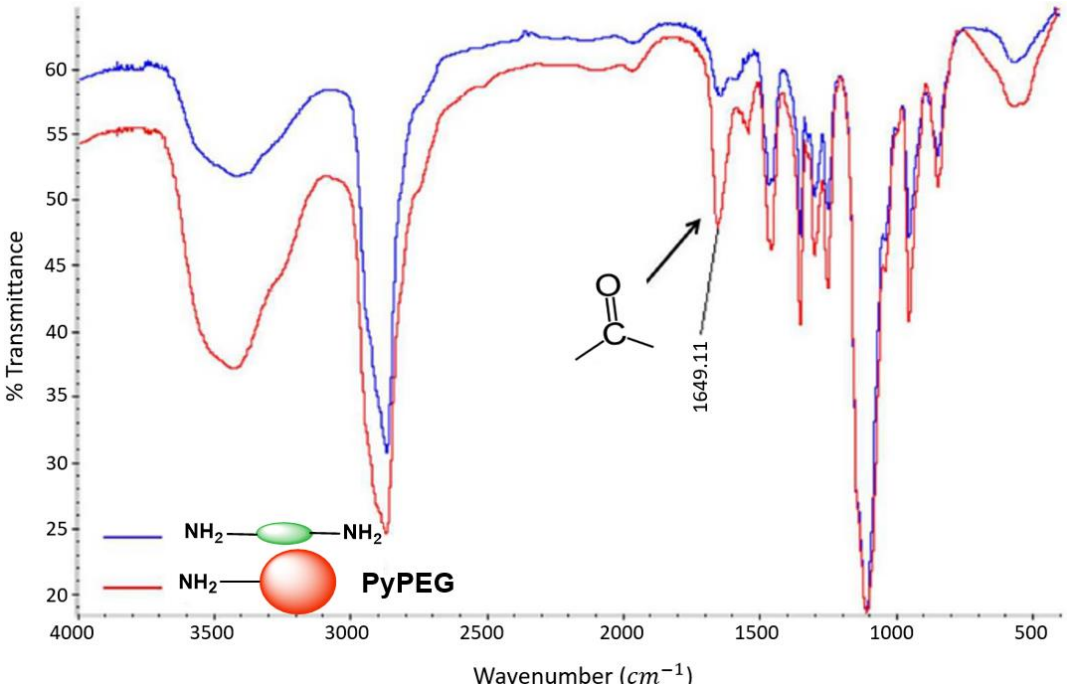


Figure 4: FT-IR specta comparison: O,O'-Bis(3-aminopropyl)polyethylene glycol (blue line), and PyPEG (red line).

The vibrational mode at 1649 cm^{-1} corresponds to the carbonyl group (υ C=O) of the amide bonds in PyPEG, highlighting a crucial distinction between globular polymeric supports and linear polymers. As previously introduced, branched PyPEG offers a high rejection percentage when used in ceramic membranes, and therefore, is suitable support candidate for MEPS.

^{1}H-NMR spectra of PyPEG at 500 MHz in DMSO-d_6 without and with previous presaturation of methylene's signals corresponding to the PEG fragments, are

displayed in figure 5. At δ = 8.30 ppm and 7.49 ppm two single peaks appear, corresponding to 4H (H_C) and 2H (H_A and H_B), respectively. A broad multiplete signals between 3.65-3.25 ppm corresponding to the methylene's H from PEG units could also be observed. Two peaks integrating 2H´s each are detected at δ = 2.77 ppm and 1.72 ppm, which are associated to methylene H´s (H_D and H_E). These assignations were corroborated by COSY experiments (figure 6).

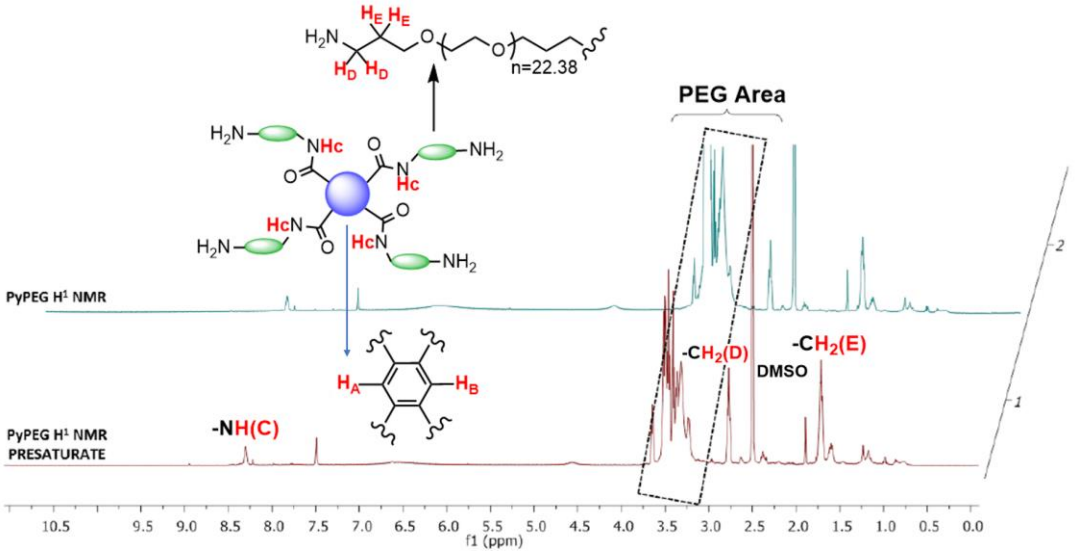


Figure 5: [1]H-NMR spectra comparison: PyPEG spectrum without and with presaturation of PEG signal (Spectrum 1), in DMSO-d_6.

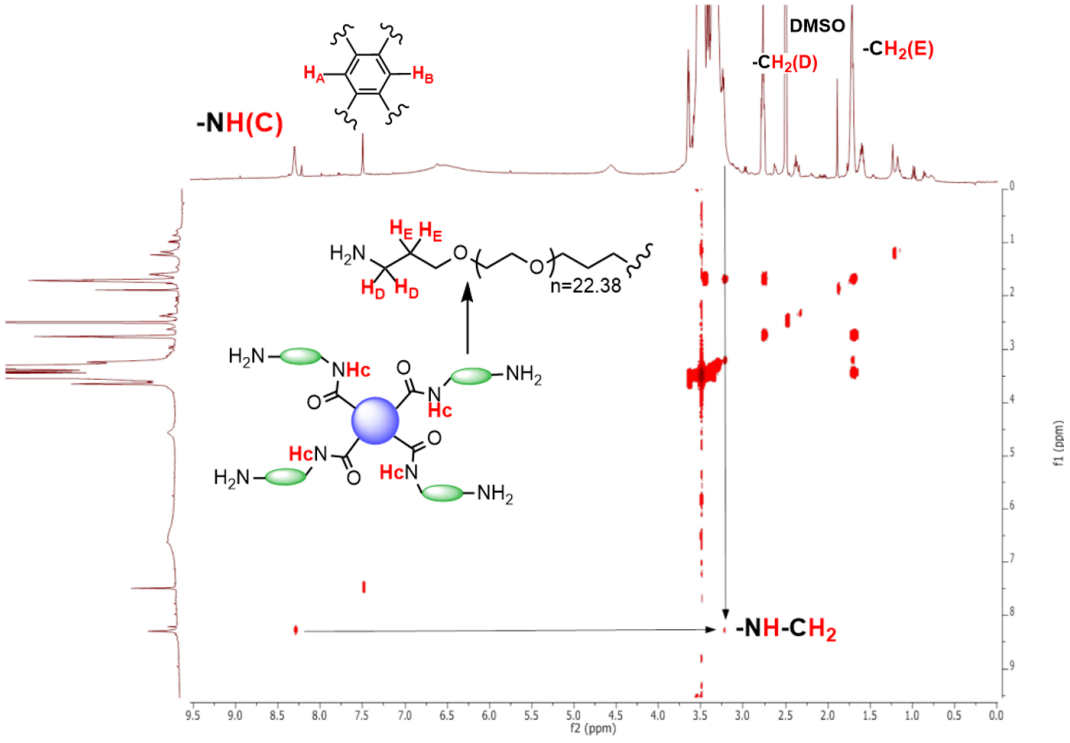


Figure 6. COSY spectrum of PyPEG in DMSO-d_6.

For ^{13}C-NMR (500 MHz, d_6-DMSO) the characteristic peaks appear at δ= 28.6, 29.2, 36.5, 37.5, 60.2, 67.6, 68.1, 69.8, 72.3, 127.0, 136.8, 166.9 ppm.

Additionally, high performance liquid chromatography (HPLC) exhibiting a retention time of 10.1 min, supports the adequate formation of PyPEG.

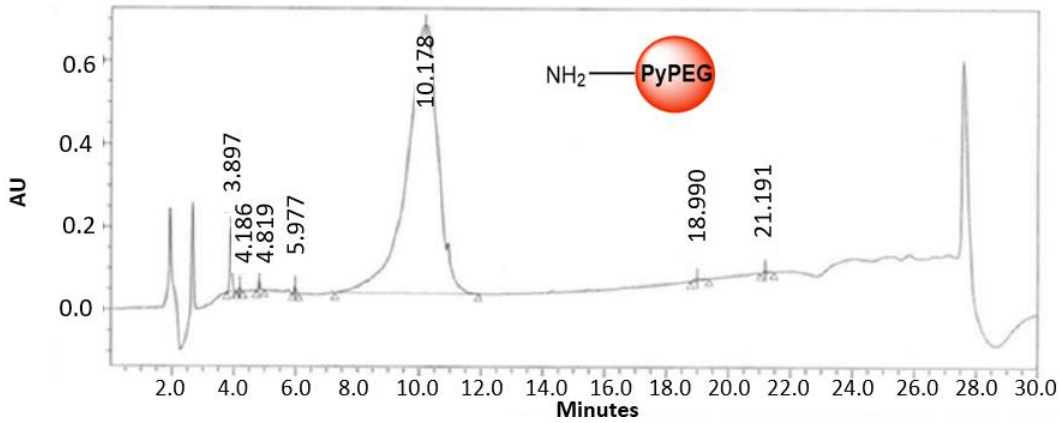
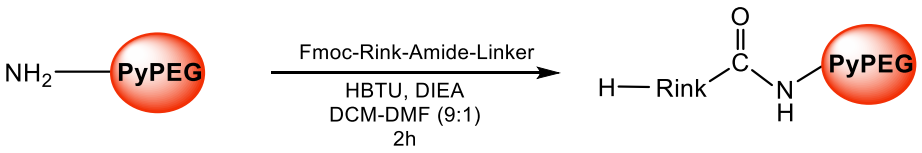


Figure 7. HPLC chromatogram PyPEG polymer, tr = 10.1 min. Gradient: from 5% to 95% ACN over 25 min.

FT-IR along with 1H-NMR, 13C-NMR spectroscopy and HPLC corroborate the proposed structure for PyPEG with the aromatic core, including its four NH_2-PEG-functionalized branches. Then, with PyPEG in hand, attaching the Fmoc-Rink amide-linker to the polymeric support is the next step in the scale-up before growing the amino acid sequence.

3.2 Fmoc-Rink-PyPEG

Fmoc-Rink linker was coupled to NH_2-PyPEG via amide bond formation in presence of *N*-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluoro-phosphate *N*-oxide (HBTU) and DIEA as coupling reagents, and DCM-DMF (9:1) as solvent for two hours, as illustrated in scheme 3.



Scheme 3: Fmoc-Rink-PyPEG formation

The coupling time was established using the Kaiser test, which was carried out on previously precipitated PyPEG derivative samples with diethyl ether, presenting a clear negative result after two hours of reaction and demonstrating successful

polymer coupling with Fmoc-Rink amide linker. Kaiser test results are presented in figure 8.

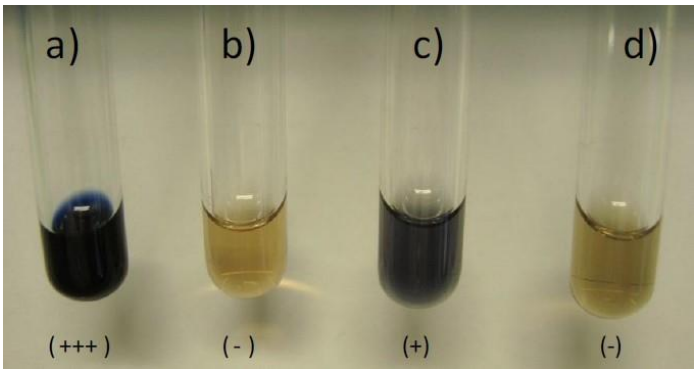


Figure 8: a) Kaiser test for positive control (PyPEG alone), b) Negative control (coupling reagents), c) 1h post reaction and d) 2 h post reaction. The quantity of (+) is the intensity of blue color, where (+++) is high and (+) is low intensity.

Then, the purification of Fmoc-Rink-PyPEG driven by OSN in a ceramic membrane (Inopor 750) was confirmed with adequate purity by HPLC monitoring as presented in figure 9a. Finally, Fmoc deprotection was achieved similarly to Fmoc-Rink-PyPEG with piperidine 20% in DMF, and after 40 diafiltrations results remained constant. Thus, high purity obtention of H-Rink-PyPEG, as shown in figure 9b.

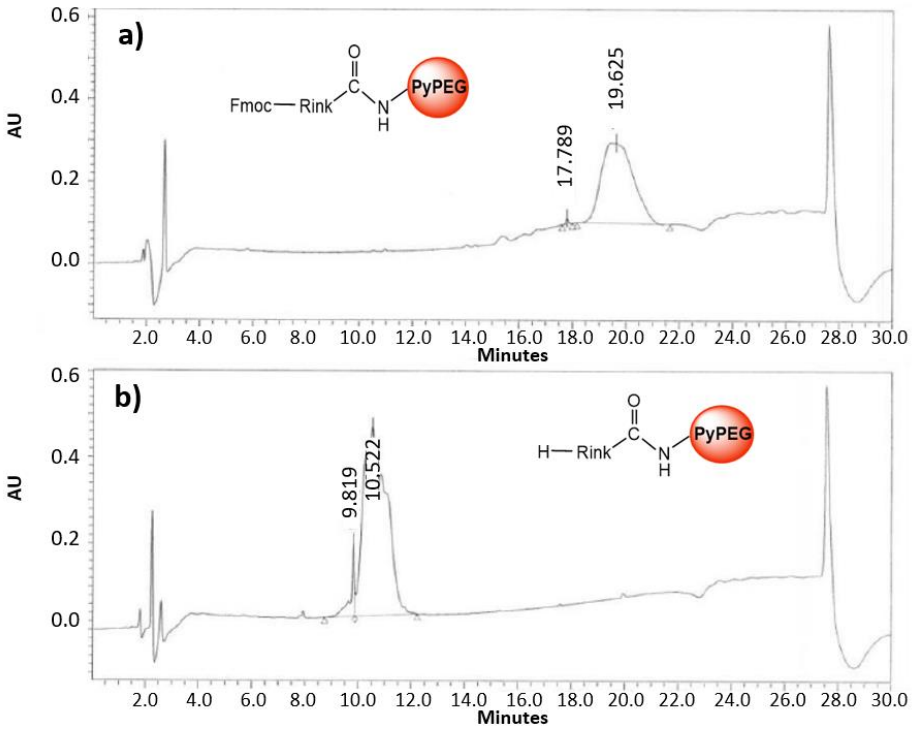
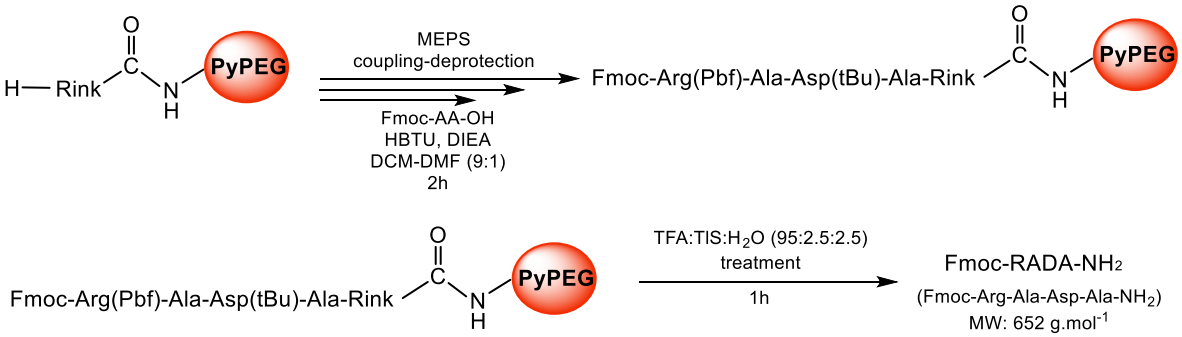


Figure 9: HPLC chromatogram a) Fmoc-Rink-PyPEG, tr = 19.6 min and b) H-Rink-PyPEG, tr = 10.5 min. Gradient: from 5% to 95% ACN over 25 min.

With the H-Rink-PyPEG support in hand, Fmoc-RADA-NH_2 peptide synthesis was carried out, as a proof of concept that the scale-up of our globular polymeric support is able to MEPS.

3.3 Fmoc-RADA-NH_2

Fmoc-RADA-NH_2 was used as the standard sequence to test the scale-up of MEPS using our globular support H-Rink-PyPEG. Each amino acid was coupled using Fmoc/t-Bu strategy in similar conditions used for SPPS. [13] HBTU and DIEA were used as coupling reagents and DCM:DMF (9:1) was the solvent system reacting for two hours. This process is presented in scheme 4.



Scheme 4: Fmoc-RADA-NH_2 formation

After the cleavage procedure, the peptide purified by semipreparative HPLC, exhibits a peak corresponding to Fmoc-RADA-NH_2, which is collected and lyophilized. The peptide characterization by HPLC and HPLC-MS is shown in figure 10. Calculation Fmoc-Arg-Ala-Asp-Ala-NH_2 crude gives $C_{31}H_{40}N_8O_8$: 652.71 g/mol and ESI-MS (M +H): 653.31 g/mol

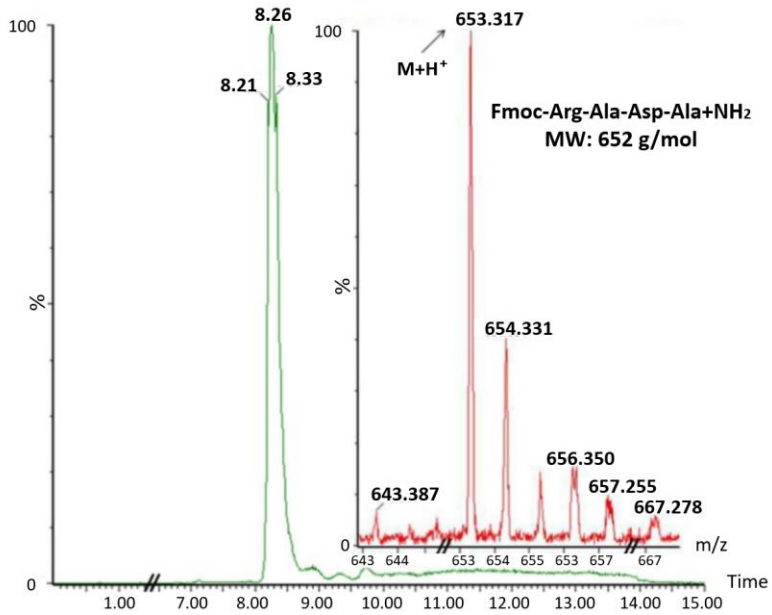


Figure 10: HPLC chromatogram (green line) and MS spectrum (red line) of Fmoc-Arg-Ala-Asp-Ala-NH_2. tr = 8.2 min. Gradient: from 5% to 80% ACN over 9 min.

Compared to laboratory scale, the results showed a more efficient purification at a higher amount of polymer. This could be related to the vessel reactor's volume (minimum: 500 mL and maximum: 1 L). Purifications were carried out for both cases with the minimum volume (500mL x 60), regardless of polymer quantity. This implies a cost-effective solvent use.

Before cleavage and global deprotection, a purity of 98.5 % and an overall yield of 78.6 % was obtained when MEPS was applied at a large scale, compared with the previously reported 84% purity achieved at smaller laboratory scale using MEPS. [13] This shows that integrating organic solvent nanofiltration into LPPS is technically feasible for obtaining high purity and decent yield of the anchored peptide. Also, it is beneficial to perform MEPS at the highest concentration of anchor in the starting solution to minimize the material cost and process time.

CONCLUSIONS

Globular and branched polymeric supports have proved to be suitable anchor alternative to effective MEPS due to its ease of performing in pressure driven filtration methodologies. However, the star-shape of PyPEG plays a more important role than the arm length of DPEG or DNPEG in the rejection of the molecule and its preparation requires less effort. Thus, PyPEG stands out above the others for scaling purposes. Besides, for the convenience of reaction following and safety, PyPEG is the most suitable and cost-effective soluble polymeric support candidate for industrial production of peptides. This work, successfully synthesized H-Rink-PyPEG anchor with adequate purity and verified its performance during organic solvent nanofiltration. Also, the resulting Fmoc-Arg-Ala-Asp-Ala-NH_2 model peptide was effectively obtained with 98.5% purity at scaling conditions, compared to the 84% purity achieved at lab-scale MEPS.

Incrementing the mass of polymer in MEPS methodology is translated into yield and purity improvements, meaning better performance at larger-scale compared to laboratory scale. Consequently, scaling up the utilization of MEPS procedures not only ensures the accomplishment of high purity in the final peptide, but also presents a crucial novel technology platform that can be extended for industrial-level production.

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