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Research Article

Evaluation of 4-*tert*-Butyl-Benzhydrylamine Resin (BUBHAR) as an Alternative Solid Support for Peptide Synthesis

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Following preliminary reports that introduced 4-*tert*-butylbenzhydrylamine resin (BUBHAR) as a novel polymer for use in solid-phase peptide chemistry (SPPS), some physical-chemical properties of its structure, certainly relevant for its application in this methodology, were compared with those of the largely used methylbenzhydrylamine resin (MBHAR). In order to rule out possible MBHAR-related commercial source effects for SPPS, we initially compared MBHAR batches acquired from three different manufacturers with homemade BUBHARs. The bead solvation properties of these two resins in solvents used in the *tert*-butyl (Boc-based) SPPS technique indicated that the mean swelling values of these solid supports (% volume of solvated bead occupied by the solvent) were 51% and 67% for MBHAR and BUBHAR, respectively. This result strongly suggests a good potential for the latter polymer in terms of application for application in SPPS. In order to move forward with this approach, the synthesis of the carboxy-terminal peptide fragment (Gln-Asn-Cys-Pro-(D-Arg)-Gly-amide) of the antidiuretic hormone, desmopressin ([3-Mpa∗-Tyr-Phe-Gln-Asn-Cys-Pro-(D-Arg)-Gly-amide], ∗1-[3-mercaptopropionic acid]), which our laboratory is producing routinely in large scale for the Health Secretary of Sao Paulo State. The comparative synthesis was conducted using these two resins with similar substitution degrees (~0.7 mmol/g). In contrast to MBHAR, surprisingly no need for a Gln⟶Asn recoupling reaction was observed when BUBHAR was used. This result might be due to improved solvation of the desmopressin C-terminal Asn-Cys-Pro-(D-Arg)-Gly-segment when bound to this latter resin as observed by microscopic swelling degrees of peptide-resin beads and also by greater mobility detected of peptide chains within the BUBHAR polymer backbone. This finding was determined by comparative electron paramagnetic resonance (EPR) of both peptide resins attaching the amino acid-type paramagnetic 2.2.6.6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (Toac) spin label early introduced by our group.

1. Introduction

Almost six decades after its inception, Merrifield's Nobel Prize solid-phase peptide synthesis (SPPS) method [1–3] continues to suffer from unresolved problems. Among these, there occur for instance great difficulties in some coupling reactions observed during the synthesis of strongly aggregating sequences. Usually site-dependent steric hindrance at the reactive N-terminal portion of a peptide segment is responsible for the impairment of coupling reactions. Alternative experimental approaches for minimizing this problem have been proposed in the literature [4, 5], most of which have focused in modifying acylating agents [6, 7] or reaction conditions [8, 9].

Another option relies on the use of alternative resins with different solvent-dependent structural and bead solvation characteristics used mainly during the critical coupling reaction [10]. By following this line of investigation, we have already examined the feasibility of using resins with a very high degree of substitution (>2 mmol/g), obtained from a

controlled experimental protocol [11, 12]. More recently, alternative amine resins for the classical methylbenzhydrylamine resin (MBHAR) [13], used for the synthesis of α-carboxamide peptide in *tert*-butyl- (Boc-) SPPS chemistry, were introduced [14]. Among these, as an alternative to MBHAR (which contains a methyl group coupled to the phenylmethylamine-functionalized copoly [styrene-divinilbenzene] structure), a larger more hydrophobic and electron-donating 4-*tert*-butyl group at this position (4-*tert*-butylbenzhydrylamine resin [BUBHAR]) was proposed. Still, in this report, other amine resins based on this same type of polymer matrix but containing chloro or dichloro groups as alternatives and known to be strong electron acceptor moieties were also proposed in order to examine the stability of peptide-resin linkage towards final acid cleavage step. In the case of BUBHAR, only preliminary tests were carried out with this solid support in which its feasibility for peptide synthesis was checked, based on the assembly of an easy-to-make sequence [14].

Thus, in order to better investigate the potential application of this alternative resin in the SPPS methodology, we decided to compare it with MBHAR to obtain α-carboxamide peptide through the Boc (*tert*-butyl) synthesis strategy. For this purpose, we decided initially to discard possible MBHAR suppliers production effects on its physical-chemical and structural properties. MBHAR batches (see Materials) were tested for comparison with those homemade BUBHARs in terms of swelling capacity of their beads accordingly to previous protocol [15–17]. In this case, the average volume of resin beads occupied by the solvent (in percentage) is calculated for estimating the solvation property of a resin in each solvent.

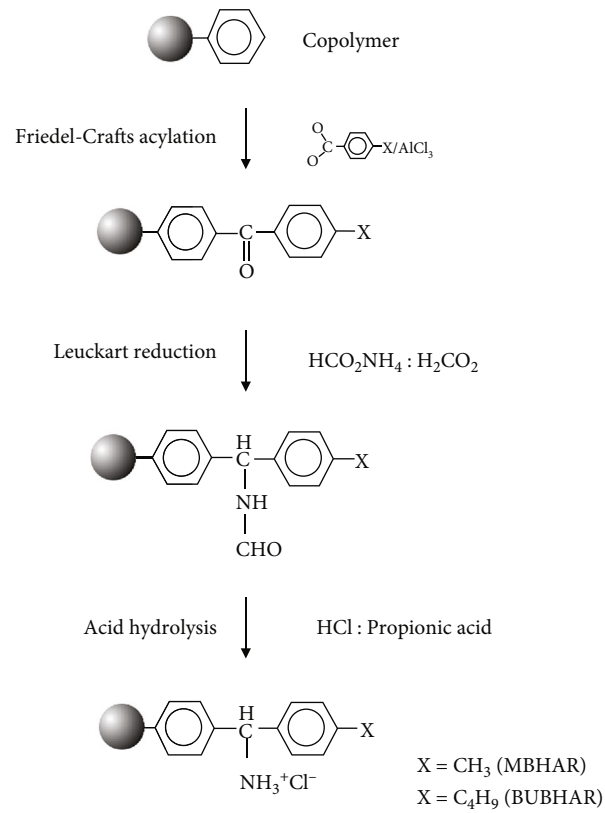
The next necessary step for this type of study involved a direct comparison between both resins with respect to overcoming a known difficult peptide synthesis step during the chain elongation of a peptide sequence. The selected hexapeptide Gln-Asn-Cys-Pro-(D-Arg)-Gly carboxy-terminal fragment of the antidiuretic hormone, desmopressin® (3-Mpa∗-Tyr-Phe-Gln-Asn-Cys-Pro-(D-Arg)-Gly-amide), ∗1-(3-mercaptoprionic acid) in which the Gln⟶Asn acylation step occurs only with a need for a recoupling reaction when MBHAR is used. Hence, the focus was now directed to this specific acylation in which solvation details of the Asn-Cys-Pro-(D-Arg)-Gly segment coupled to both resins were investigated in terms of their bead swelling capacities and also of degrees of peptide chain mobility spread throughout the polymer backbone of both resins when solvated in different solvent systems applicable for the SPPS method.

2. Experimental

2.1. Materials. Solvents and reagents were purchased from Bachem, Advanced ChemTech, Novabiochem, Sigma-Aldrich, or Fluka. Batches of MBHAR (0.49, 0.70, and 1.10 mmol/g) were acquired from Bachem, Advanced ChemTech, and Sigma-Aldrich Co.

2.2. Methods

2.2.1. Peptide Synthesis. Peptide synthesis was carried out manually in a 0.4 mmol scale accordingly to the classical



Scheme 1: Synthesis scheme of MBHAR and BUBHAR.

Boc-chemistry protocol [1, 2]. The Boc-protecting group was removed with 30% trifluoroacetic acid (TFA) in dichloromethane (DCM), and the amino group neutralization step was performed with 10% triethylamine (TEA) in DCM. Coupling reactions were carried out in a 1 : 1 DCM : N, N-dimethylformamide (DMF) mixture using 2.5 excess of Boc-amino acid/2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/diisopropylethylamine (DIEA) (1 : 1 : 2). All coupling reactions were qualitatively monitored using the ninhydrin test, and a recoupling reaction was needed at the Gln⟶Asn acylation step only when MBHAR was as the solid support. The final peptide cleavage procedure was carried out in the trifluoromethanesulfonic acid (TFMSA)/TFA solution as previously described [18].

2.2.2. Synthesis of BUBHAR. The general synthesis protocol of this type of polystyrene-divinylbenzene-based amino copolymer, initially described [13, 19] and further modified by us [11] in order to better control the degree of substitution of amino groups in the resin, was used for preparing the three batches of BUBHAR. Basically, the amino group content of the resin was controlled at the Friedel-Crafts acylation step [11] followed by the Leuckart reduction reaction, which ensured the complete conversion of carbonyl groups generated in the previous step. The general procedure for the BUBHAR synthesis is outlined using 4-tert-butylbenzoyl as the acylation agent in the Friedel-Crafts reaction. A general synthesis protocol for the synthesis of MBHAR and BUBHAR is shown in Scheme 1, below.

Table 1: Bead swelling[a] values of methylbenzhydrylamine resin (MBHAR) and 4-*tert*-butyl-benzhydrylamine resin (BUBHAR) batches in different solvent systems.

Solvent	(AN+DN)[b]	Swelling values of resins (%)							
		Batches of MBHAR (mmol/g)				Batches of BUBHAR (mmol/g)			
		0.49	0.70	1.20	Average values	0.35	0.75	1.10	Average values
DCM	21.4	70	54	64	63	68	72	81	74
DMF	42.6	46	48	46	47	76	75	72	74
50% DCM/DMF	32.0	62	51	51	55	72	71	82	75
20% DMSO/NMP	42.3	62	58	63	61	58	70	82	70
MeOH	71.3	29	29	24	27	41	56	31	43
		Average values			51	Average values			67

[ab]([swollen volume – dry volume]/swollen volume) x 100; polarity values of solvent according to ref. [17].

2.2.3. Amino Acid Analysis. All peptide-resins were hydrolyzed in a mixture of 12 mol L^{-1} HCl/propionic acid at 130°C with appropriate reaction times in Pyrex tubes with Teflon-coated plastic screw caps (13 × 1 cm). The amino acid analyses were performed in a Biochrom 20 plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England) in order to determine the amount of each amino acid of the sequence and therefore the peptide content the resin. The analyses were done in duplicate.

2.2.4. Analytical RP-HPLC. Reverse phase-high-performance liquid chromatography (RP-HPLC) analyzes were carried out using a TFA/acetonitrile gradient on a Waters Associates HPLC system consisting of two 510 HPLC pumps, an automated gradient controller, a Rheodyne manual injector, a 486 UV detector, and a 746 data module. The selected column was a Vydac C18 column (0.46 x 15 cm, 5 μm particle size, 300 Å pore size), and detection was done at $\lambda = 210$ nm using the solvent systems A: 0.1% TFA/H_2O and B: 60% acetonitrile/0.1% TFA/H_2O. A gradient of 5%–95% B at a flow rate of 1.5 mL/min for 30 min was used. The analyses were done in duplicate.

2.2.5. Liquid Chromatography-Electrospray Mass Spectrometry (LC/ESI-MS). The liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS) experiments were performed on a system consisting of a Waters Alliance model 2690 separations module and model 996 photodiode array detector (Waters, Eschborn, Germany) controlled with a Compaq AP200 workstation coupled to a Micromass model ZMD mass detector (Micromass, Altrincham, UK). The samples were automatically injected into a Waters narrow bore Nova-Pak column C_{18} (2.1 × 150 mm, 60 Å pore size, and 3.5 μm particle size). The elution was carried out with solvents A (0.1% TFA/H_2O) and B (60% acetonitrile/0.1% TFA/H_2O) at a flow rate of 0.4 mL/min using a linear gradient from 5% to 95% B for 30 min. The condition used for mass spectrometry measurements was positive electrospray ionization (ESI). The analyses were done in duplicate.

2.2.6. Resin Bead Swelling Study. Before the use in peptide synthesis or microscopic measurement of bead sizes, resin or peptide-resin batches were sized by sifting through metal sieves to lower the standard deviation of resin diameters to approximately 4%. Swelling studies of these narrowly sized populations of beads have previously been conducted [15, 17]. In short, 150-200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and the diameters of beads measured directly with an Olympus model SZ11 microscope coupled with Image-Pro Plus version 3.0.01.00 software. Since the size distributions in a sample of beads are log-normal rather than normal, the more accurate geometric mean values and geometric standard deviations were used in order to estimate the central value and the distribution of the particle diameters [20]. The measurements were carried out in duplicate. The resins and peptide-resins were measured with their amino groups in the deprotonated form, obtained by three 5 min washes in TEA/DCM/DMF (1 : 4.5 : 4.5, *v*/*v*/*v*), followed by five 2 min washes in DCM/DMF (1 : 1, *v*/*v*) and five 2 min washes in DCM. Resins were dried *in vacuum* using an Abderhalden-type apparatus with MeOH reflux.

2.2.7. EPR Studies. Electron paramagnetic resonance (EPR) measurements were carried out at 9.5 GHz on a Bruker ER 200SRC spectrometer at room temperature (22 ± 2°C) in flat quartz cells. Fmoc-Toac-labeled peptidyl-resins were preswollen overnight in the study solvent before the spectra were run, as previously introduced by us [21, 22]. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW in order to prevent saturation effects. The analyses were done in duplicate.

3. Results and Discussion

3.1. Comparative Swelling Studies of Resins. By also considering the solvent polarity values accordingly to the amphoteric and dimensionless scale proposed previously by us [17], solvent systems with (AN+DN) parameter values ranging from about 20 to 70 were selected for the comparative swelling evaluation of MBHAR and BUBHAR batches of rather equivalent substitution degrees (from about 0.3 to 1.1 mmol/g). Table 1 displays the swelling degrees of resin beads measured in DCM, DMF, 50% DCM/DMF, 20% dimethylsulfoxide (DMSO)/N-methylpyrrolidinone (NMP),

and methanol (MeOH). The results displayed in this table initially indicate that regardless of MBHARs' manufacturers' and amine group content values, BUBHAR's beads solvated better than those of MBHAR. Measured mean swelling values in this solvent system were 67% and 51% for BUBHAR and MBHAR, respectively, thus indicating better solvation properties for the *tert*-butyl instead of methyl group-attaching resin at position 4 of the phenylmethylamine moiety of the structure of both polymers.

3.2. Comparative Synthesis Study of a Model Peptide Segment in Both Resins. The next step of the present study compared the efficiencies of the two resins in problems specifically related to the peptide synthesis methodology. In order to develop this second topic, as early explained, we decided to choose a difficult-to-couple amino acid reaction observed during the synthesis of the C-terminal hexapeptide Gln-Asn-Cys-Pro-(D-Arg)-Gly sequence of the antidiuretic desmopressin. In this peptide segment, we observed that irrespective of the source of MBHAR batch, its degree of substitution or acylation protocol used the Gln⟶Asn coupling reaction always needs to undergo the recoupling process. Thus, this critical coupling step in the desmopressin hormone was selected to more thoroughly evaluate the physical-chemical details of solvated BUBHAR and MBHAR solid supports attaching Asn-Cys-Pro-(D-Arg)-Gly sequence.

Batches of commercial MBHAR (0.7 mmol/g—Advanced ChemTech) and the homemade BUBHAR (0.75 mmol/g) were chosen as solid supports for synthesizing the Gln-Asn-Cys-Pro-(D-Arg)-Gly segment in 0.4 mmol synthesis scale. No synthesis problems were observed until the Asn residue incorporation, using standard coupling protocol which comprised the use of 2.5 molar excess of acylating components (amino acid and N,N`-diisopropylcarbodiimide (DIC)/4-hidroxybenzotriazole [HOBt]) in 50% DCM/DMF for 2 h at room temperature.

3.2.1. Solvation Studies of Asn-Cys-Pro-(D-Arg)-Gly-Resins

(1) Microscopic Measurement of Peptide-Resin Batches. The comparative swelling values (percentage of peptide-resin bead occupied by solvent) measured for the Asn-Cys-Pro-(D-Arg)-Gly-resin beads in DCM, DMF, 50% DCM/DMF, NMP, and DMSO were determined and are shown in Figure 1.

By using this approach, we detected a better solvation degree for the peptide-BUBHAR than for peptide-MBHAR support for all of the examined solvents. Moreover, the yield from both resins at this peptide sequence position, the swelling degree of peptide-resin beads in the routinely used 50% DCM : DMF solution for the acylation step was much greater for peptide-BUBHAR (83%) than for the peptide-MBHAR (46%), which is a relevant finding for the interpretation of the coupling reaction. Noteworthy and in accordance with these resin swelling data, there was no need for a Gln⟶Asn recoupling reaction in BUBHAR as systematically occurred with MBHAR. This result strongly supports the importance of the resin solvation property as previously demonstrated

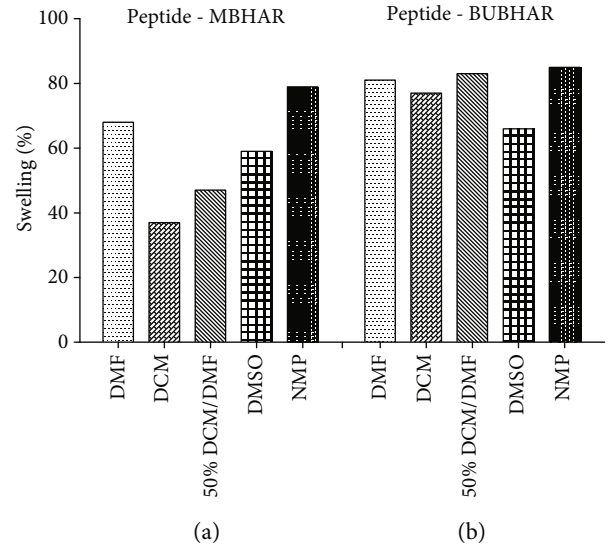


Figure 1: Swelling degree values (in percentage) of Asn-Cys-Pro-(D-Arg)-Gly fragment bound to methylbenzhydrylamine resin (MBHAR) (a) and to 4-*tert*-Butyl-benzhydrylamine resin (BUBHAR) (b) in different solvents.

in critical acylation reactions within beads in other peptide-resins [23–25].

(2) EPR Experiments with Spin-Labeled Peptide-Resins. In order to complement the above-described peptide-resin solvation, the degree of motion and steric hindrance neighboring of the peptide chains spread throughout the resin beads were now estimated by EPR experiments. Small portion of the N-terminal groups of peptide chains in both resins were labeled with the amino acid-type spin probe Toac (2,2,6,6-tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid) introduced by us for peptide and polymer labeling [26–28] as previously described for evaluating, for instance, the solvation properties of model peptide-polymers [21–25].

Figure 2 shows the EPR spectra of both peptide-resins in the solvent system (50% DCM: DMF) used for the coupling step of peptide synthesis in the present study, and Table 2 displays the EPR central peak line widths (W_0) values (in Gauss) that are known to be sensitive to the level of molecular mobility of labeled sites within resin network [16, 22].

As a rule [16, 25], there is an inverse correlation between the W_0 value and the rate of labeled molecular motion. Comparatively smaller W_0 values were obtained for peptide-BUBHAR than for peptide-MBHAR in all solvent systems, which were in close agreement with the swelling data of both peptide-resins.

Lastly, the analytical HPLC profiles of the crude Gln-Asn-Cys-Pro-(D-Arg)-Gly-peptide fragment assembled in both resins are shown in Figure 3. Rather similar profiles are observed but an interesting finding consisted of the presence of small contaminants near 13.26 min in the crude peptide cleaved only from MBHAR and after the Gln recoupling reaction in this resin (Figure 3(a)). To complement these

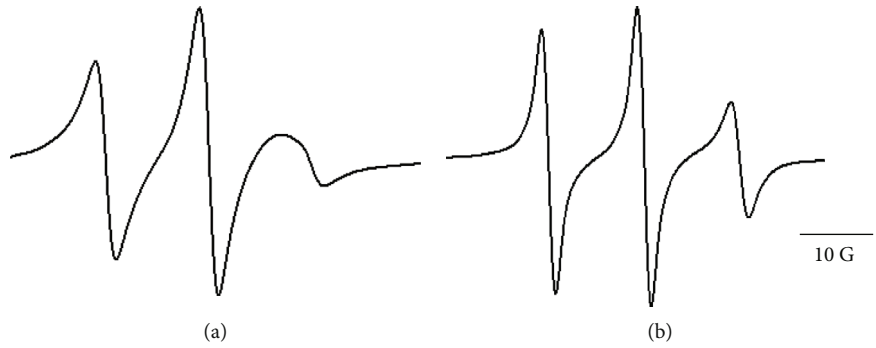


Figure 2: Electron paramagnetic resonance (EPR) spectra of Toac-labeled Asn-Cys-Pro-(D-Arg)-Gly segment bound to MBHAR (a) and to BUBHAR (b) in 50% DCM/DMF.

Table 2: EPR W_0 values determined in different organic solvents for Asn-Cys-Pro-(D-Arg)-Gly peptide segment coupled to MBHAR and to BUBHAR and labeled with Toac spin label.

Resins	W_0 (Gauss)				
	DCM	DMF	DCM/DMF	DMSO	NMP
MBHAR	3.7	2.3	3.4	2.8	2.3
BUBHAR	2.4	2.1	2.1	2.5	2.1

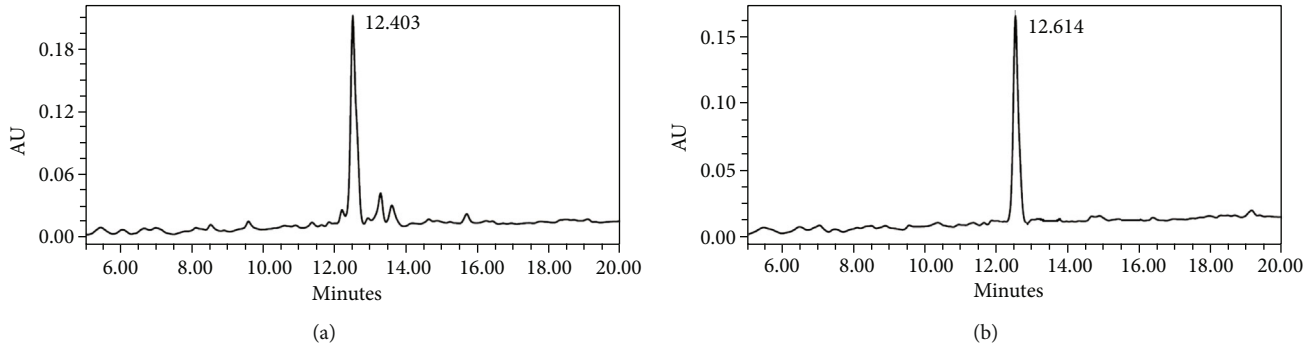


Figure 3: High-performance liquid chromatography (HPLC) profiles of crude Gln-Asn-Cys-Pro-(D-Arg)-Gly peptide amide synthesized in MBHAR (a) and in BUBHAR (b).

results, Figure 4 displays the LC/ESI-MS spectra of crude peptides cleaved from MBHAR and BUBHAR (4a and 4b, respectively), revealing the expected molecular weight of the Gln-Asn-Cys-Pro-(D-Arg)-Gly sequence (673.80 Da), whereas Figure 4(c) displays the LC/ESI-MS spectrum of the above mentioned small contaminant that appeared near 13.5 min in the crude peptide synthesized in MBHAR. In this case, the molecular weight found was 544.36 Da, which corresponds to the entire peptide sequence but lacking the Gln residue even after a double coupling of this residue.

4. Conclusions

The present report suggests that BUBHAR can be considered an efficient solid support for use in Boc-synthetic chemistry, and that it would be an alternative to the standard MBHAR. The better synthesis yields resulting from the use of BUBHAR seems to be due to the improved solvation characteristics of its peptide-bearing polymeric matrix as demonstrated by experiments carried out not only with microscopic swelling measurements of peptide-resin beads but also to the greater peptide chain mobility that occurs exactly at the Gln-acylation step in this alternative solid support as verified with EPR experiments. In fact, these results seemed to be in close agreement with previous observations [14] in which much easier handling (swelling and washing of beads), regardless of the step of the SPPS synthetic cycle, was observed using BUBHAR than MBHAR. In order to deeper evaluate this comparative investigation between both resins, synthesis of the same peptide fragment is currently in progress, but using resin batches with a lower (~0.2 mmol/g) and higher (2.0–3.0 mmol/g) degree of amine group contents. Although designed to be used for the Boc-chemistry approach, the novel BUBHAR polymer might also be tested in the 9-fluorenylmethyloxycarbonyl- (Fmoc-) chemistry method [3, 29] after previous coupling of appropriate spacer

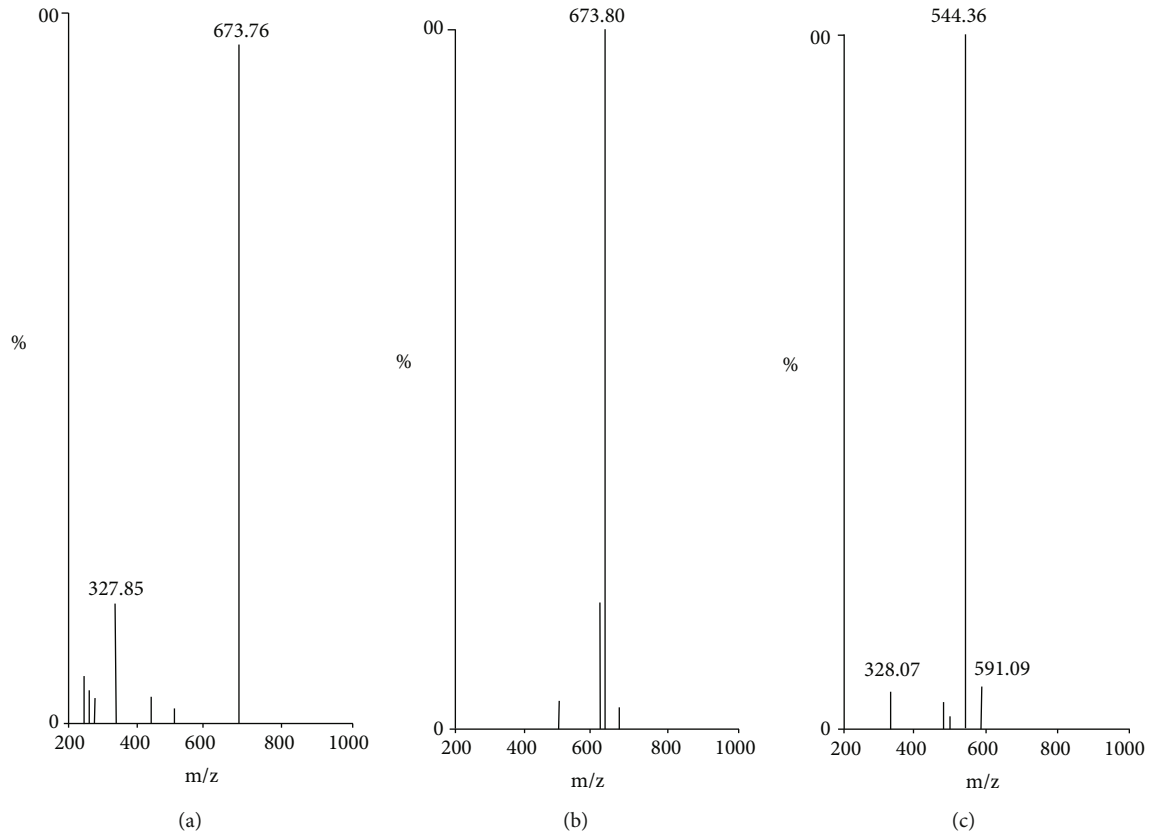


Figure 4: LC/ESI-MS spectra of the main peaks of crude peptides Gln-Asn-Cys-Pro-(D-Arg)-Gly cleaved from MBHAR (a), BUBHAR (b), and of the contaminant present in the peptide cleaved from MBHAR but lacking the Gln residue (c).

groups (linkers) necessary for peptide segment assembly. In this way, similarly to what was observed with other types of resins early proposed for use in SPPS [30, 31], BUBHAR can be considered in the future, one more potentially usable for the SPPS methodology.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors of this manuscript declare no conflict of interest, either financial or otherwise.

Acknowledgments

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References

[1] G. Barany and R. B. Merrifield, "The Peptides In: Gross E," in *Meienhofer J (eds)*, vol. 2, pp. 1–284, Academic Press Incorporated, New York, 1980.

[2] J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford III, 1984.

[3] G. B. Fields and R. L. Noble, "Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids," *International Journal of Peptide and Protein Research*, vol. 35, no. 3, pp. 161–214, 1990.

[4] S. Chandrudu, P. Simerska, and I. Toth, "Chemical methods for peptide and protein production," *Molecules*, vol. 18, no. 4, pp. 4373–4388, 2013.

[5] L. Raibaut, O. El Mahdi, and O. Melnyk, "Solid phase protein chemical synthesis," *Topics in Current Chemistry*, vol. 363, pp. 103–154, 2015.

[6] L. A. Carpino, A. El-Faham, and F. Albericio, "Racemization studies during solid-phase peptide synthesis using azabenzotriazole-based coupling reagents," *Tetrahedron Letters*, vol. 35, no. 15, pp. 2279–2282, 1994.

[7] C. U. Hjørringgaard, A. Brust, and P. F. Alewood, "Evaluation of COMU as a coupling reagent for in situ neutralization Boc solid phase peptide synthesis," *Journal of Peptide Science*, vol. 18, no. 3, pp. 199–207, 2012.

[8] L. M. Varanda and M. T. M. Miranda, "Solid-phase peptide synthesis at elevated temperatures: a search for and optimized synthesis condition of unsulfated cholecystokinin-12," *The Journal of Peptide Research*, vol. 50, no. 2, pp. 102–108, 1997.

[9] C. O. Kappe and D. Dallinger, "The impact of microwave synthesis on drug discovery," *Nature Reviews Drug Discovery*, vol. 5, no. 1, pp. 51–63, 2006.

[10] C. G. Fields and G. B. Fields, "Solvents for solid-phase peptide synthesis," in *Peptide Synthesis Protocols*, vol. 35, pp. 29–40, 1994.

[11] R. Marchetto, A. Etchegaray Jr., and C. R. Nakaie, "Kinetics of synthesis and swelling studies of highly substituted benzhydrylamine-resins: implications for peptide synthesis and perspectives for use as anion exchanger resin," *Journal of the Brazilian Chemical Society*, vol. 3, no. 1 e 2, pp. 30–37, 1992.

[12] C. R. Nakaie, E. Oliveira, E. F. Vicente et al., "Solid-phase peptide synthesis in highly loaded conditions," *Bioorganic Chemistry*, vol. 39, no. 2, pp. 101–109, 2011.

[13] G. R. Matsueda and J. M. Stewart, "A p-methylbenzhydrylamine resin for improved solid-phase synthesis of peptide amides," *Peptides*, vol. 2, no. 1, pp. 45–50, 1981.

[14] S. E. G. Souza, L. Malavolta, E. M. Cilli, S. Schreier, G. N. Jubilut, and C. R. Nakaie, "Novel copoly (styrene-divinylbenzene)-resins with different phenylmethylamine groups for use in peptide synthesis method," *Protein & Peptide Letters*, vol. 22, no. 5, pp. 392–401, 2015.

[15] E. M. Cilli, E. Oliveira, R. Marchetto, and C. R. Nakaie, "Correlation between solvation of peptide-resins and solvent properties," *The Journal of Organic Chemistry*, vol. 61, no. 25, pp. 8992–9000, 1996.

[16] E. Oliveira, E. M. Cilli, A. Miranda et al., "Monitoring the chemical assembly of a transmembrane bradykinin receptor fragment: correlation between resin solvation, peptide chain mobility, and rate of coupling," *European Journal of Organic Chemistry*, vol. 2002, no. 21, pp. 3686–3694, 2002.

[17] L. Malavolta, E. Oliveira, E. M. Cilli, and C. R. Nakaie, "Solvation of polymers as model for solvent effect investigation: proposition of a novel polarity scale," *Tetrahedron*, vol. 58, no. 22, pp. 4383–4394, 2002.

[18] G. N. Jubilut, E. M. Cilli, M. Tominaga, A. Miranda, Y. Okada, and C. R. Nakaie, "Evaluation of the trifluoromethanosulfonic acid/trifluoroacetic acid/thioanisole cleavage procedure for application in solid-phase peptide synthesis," *Chemical & Pharmaceutical Bulletin*, vol. 49, no. 9, pp. 1089–1092, 2001.

[19] P. G. Pietta, P. F. Cavallo, K. Takahashi, and G. R. Marshall, "Preparation and use of benzhydrylamine polymers in peptide synthesis. II. Syntheses of thyrotropin releasing hormone, thyrocalcitonin 26-32, and eledoisin," *The Journal of Organic Chemistry*, vol. 39, no. 1, pp. 44–48, 1974.

[20] R. R. Irani and C. F. Callis, *Particle Size: Measurement, Interpretation And Application*, John Wiley & Sons, New York, 1967.

[21] E. M. Cilli, R. Marchetto, S. Schreier, and C. R. Nakaie, "Use of spin label EPR spectra to monitor peptide chain aggregation inside resin beads," *Tetrahedron Letters*, vol. 38, no. 4, pp. 517–520, 1997.

[22] E. M. Cilli, R. Marchetto, S. Schreier, and C. R. Nakaie, "Correlation between the mobility of spin-labeled peptide chains and resin solvation: an approach to optimize the synthesis of aggregating sequences1," *The Journal of Organic Chemistry*, vol. 64, no. 25, pp. 9118–9123, 1999.

[23] C. R. Nakaie, L. Malavolta, S. Schreier, E. Trovatti, and R. Marchetto, "Direct electron paramagnetic resonance monitoring of the peptide synthesis coupling reaction in polymeric support," *Polymer*, vol. 47, no. 13, pp. 4531–4536, 2006.

[24] R. Marchetto, E. M. Cilli, G. N. Jubilut, S. Schreier, and C. R. Nakaie, "Determination of site-site distance and site concentration within polymer beads: a combined swelling-electron paramagnetic resonance study," *The Journal of Organic Chemistry*, vol. 70, no. 12, pp. 4561–4568, 2005.

[25] D. D. Lopes, J. H. Cuvero, M. M. L. Ferreira et al., "Fragments of the second transmembrane helix of three G-protein-coupled receptors: comparative synthetic, structural and conformational studies," *Amino Acids*, vol. 51, no. 2, pp. 273–282, 2019.

[26] C. R. Nakaie, G. Goissis, S. Schreier, and A. C. M. Paiva, "pH dependence of EPR spectra of nitroxides containing ionizable groups," *Brazilian Journal of Medical and Biological Research*, vol. 14, no. 2-3, pp. 173–180, 1981.

[27] C. R. Nakaie, S. Schreier, and A. C. M. Paiva, "Synthesis and properties of spin-labeled angiotensin derivatives," *Biochimica et Biophysica Acta*, vol. 742, no. 1, pp. 63–71, 1983.

[28] R. Marchetto, S. Schreier, and C. R. Nakaie, "A novel spin-labeled amino acid derivative for use in peptide synthesis: (9-fluorenylmethyloxycarbonyl)-2,2,6,6-tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid," *Journal of the American Chemical Society*, vol. 115, no. 23, pp. 11042-11043, 1993.

[29] R. Behrendt, P. White, and J. Offer, "Advances in Fmoc solid-phase peptide synthesis," *Journal of Peptide Science*, vol. 22, no. 1, pp. 4–27, 2016.

[30] M. A. Siyad, A. S. V. Nair, and G. S. V. Kumar, "Solid-phase peptide synthesis of endothelin receptor antagonists on novel flexible, styrene-acryloyloxyhydroxypropyl methacrylate-tripropyleneglycol diacrylate [SAT] resin," *Journal of Combinatorial Chemistry*, vol. 12, no. 2, pp. 298–305, 2010.

[31] R. Pipkorn, S. Rawer, M. Wiessler et al., "SPPS resins impact the PNA-syntheses' improvement," *International Journal of Medical Sciences*, vol. 10, no. 3, pp. 331–337, 2013.