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Solid-phase peptide synthesis in highly loaded conditions

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ABSTRACT

The use of very highly substituted resins has been avoided for peptide synthesis due to the aggravation of chain–chain interactions within beads. To better evaluate this problem, a combined solvation–peptide synthesis approach was herein developed taking as models, several peptide-resins and with peptide contents values increasing up to near 85%. Influence of peptide sequence and loading to solvation characteristics of these compounds was observed. Moreover, chain–chain distance and chain concentration within the bead were also calculated in different loaded conditions. Of note, a severe shrinking of beads occurred during the α-amine deprotonation step only when in heavily loaded resins, thus suggesting the need for the modification of the solvent system at this step. Finally, the yields of different syntheses in low and heavily loaded conditions were comparable, thus indicating the feasibility of applying this latter "prohibitive" chemical synthesis protocol. We thought these results might be basically credited to the possibility, without the need of increasing molar excess of reactants, of carrying out the coupling reaction in higher concentration of reactants – near three to seven folds – favored by the use of smaller amount of resin. Additionally, the alteration in the solvent system at the α-amine deprotonation step might be also improving the peptide synthesis when in heavily loaded experimental protocol.

1. Introduction

Despite the huge amount of studies existing to date in the literature aiming at optimizing different aspects of the classical solid-phase peptide synthesis (SPPS) method [1–3], the exact role of the solvation process of peptide–polymer matrices in each step of the synthesis cycle which certainly affects the final yield still continues to be an elusive goal. Many types of approaches have for instance, focused not only on the evaluation of the influence of the polymeric structure, peptide sequence and loading in the solvation properties of peptide-resins [4,5] but also, in modifying some aspects of the standard experimental protocol such as the temperature [6,7], addition of chaotropic agents [8] or more efficient coupling compounds [9,10] and also the application of the microwave irradiation for optimizing different steps of the peptide synthesis cycle [11,12].

In our case and deliberately in a conceptual departure from the great majority of these approaches, we have initially interpreted the peptide-resin solvation phenomenon as a complex physicochemical example of solute–solvent interaction process. By using appropriate peptide-resins as models and solvent systems which encompassed almost entirely the polarity scale, it was possible to reveal some rules that govern the swelling of different classes of peptide-resins [13–15]. In parallel, it was also possible to introduce a dimensionless, amphoteric and more versatile solvent polarity parameter [13,16,17] which combines in 1:1 proportion, the Gutmann's [18] electron acceptor (AN) and electron donor (DN) numbers of each solvent.

Unlike most classical spectroscopic strategies already existing in the peptide-resin solvation field [19–22], we have also applied innovatively the electron paramagnetic resonance (EPR) method.

Abbreviations: Ac, acetyl; ACN, acetonitrile; AII, angiotensin II; AI, angiotensin I; AN, electron acceptor number; BHAR, benzhydrylamine resin; Boc, tert-butyloxycarbonyl; Bz, benzyl; C18, octadecyl; 2-ClZ, 2-chlorobenzyloxycarbonyl; 2BrZ, 2-chlorobenzyloxycarbonyl; For, formyl; Bu, t-butyl; DCM, dichloromethane; DIC, *N,N′*-diisopropylcarbodiimide; DIEA, diisopropylethilamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DN, electron donor number; EDT, ethanedithiol; EtOH, ethanol; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, N-[(1*H*-benzotriazol-1-yl)-(dimethylaminomethylene)]-*N*-methylmethanaminium hexafluorophosphate N-oxide; HFIP, hexafluoroisopropanol; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; LHRH, luteinizing hormone-releasing hormone; MBHAR, methylbenzhydrylamine-resina; MeOH, methanol; NMP, N-methyl-2-pyrrolidone; OcHex, cyclohexyl; PAMR, 4-(oxymethyl)-phenylacetamidomethyl-resin; PC, peptide content; Pip, piperidine; SPPS, solid phase peptide synthesis; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Tos, p-toluenesulfonyl.

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This experimental strategy applied for revealing dynamic features of peptide-resins [23–27] depends fundamentally in the use of a key and versatile amino acid-type paramagnetic probe [28] that was appropriately modified structurally for use in the solid-phase peptide synthesis methodology [29–31]. Advantageously to most of other spectroscopic methods which usually reflect the solvation of the whole peptide, the EPR technique allows the peptide chain accessibility in the N-terminal region where the extent of steric hindrance is critical for the success of the synthesis. This approach has also allowed for instance, the determination of some unusual solvation parameters of peptide chains spread throughout the polymer network such as the average distance between peptide chains and their concentration inside the bead which are dependent on the swelling degree measured in each solvent system [26,32]. In complement, this combined EPR-swelling investigation was also important for validating the novel (AN + DN) solvent polarity scale [33] and also to propose a direct time-resolved monitoring of coupling reactions within resin beads [32].

Despite all these collective efforts existing in the literature regarding the relevance of peptide resin solvation process, some limitations have been still persisted in the SPPS strategy. As an example, serious difficulties in assembling long or strongly aggregated sequences remain as a concern in this methodology. In this context, one special case refers to the challenge in synthesizing peptides in heavily loaded condition inside the polymer matrix. The application of this alternative synthesis approach has not been recommended [34] as it clearly aggravates peptide chain–chain interactions inside the beads as a consequence of the presence of much greater amount of peptide chains, thus affecting the coupling reactions and decreasing the final synthesis yield. In spite of this drawback, this synthesis strategy carries comparatively to the common experimental protocol, a very clear economical advantages due to the lesser solvent consumption and the possibility of obtaining much higher amount of peptide per each synthesis.

Thus, an improved knowledge of the influence of different physicochemical details, most of them related to the complex solvation characteristics of a polymeric matrix when attaching large amount of peptide chains, turns out to be a relevant target to be pursued in the SPPS field. The present report intended basically to verify, through a variety of strategies and synthesizing large amount of model peptide-resins, the feasibility in assembling peptide sequences even under very highly loaded conditions in the solid support.

2. Material and methods

2.1. Materials

All reagents and solvents for solid-phase peptide synthesis were analytical grade and were used from freshly opened containers without further purification. Protected Boc-amino acids were purchased from Bachem (Torrance, CA), with the following side chain protections: Asp(OcHex, Bz and Bu), Ser(Bzl), Trp(For), Tyr(2-BrZ), His and Arg (Tos).

2.2. Methods

2.2.1. Peptide synthesis

The peptides were synthesized manually accordingly to the standard Boc protocol [1,3]. In the Boc chemistry, after coupling the C-terminal amino acid to the resin, the successive α-amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and 10% TEA/DCM (10 min). The amino acids were coupled with 3-fold excess, using DIC/HOBt in DMF and, if necessary, Boc-amino acid/(N-[(1*H*-benzotriazol-1-yl)-(dimethylaminomethylene)]-*N*-methylmethanaminium hexafluorophosphate N-oxide (HBTU)/HOBt (1:1:1), in the presence of excess of diisopropylethylamine (DIEA, 5 equiv.) using 20% DMSO/NMP as the solvent system. After a 3-h coupling period, the qualitative ninhydrin test was performed to estimate the completeness of the reaction. To check the purity of the synthesized peptide sequence attached to the resin, cleavage reactions with small aliquots of resin were carried out in anhydrous HF, at 0 °C for 2 h. Analytical HPLC, as well as LC/MS (electrospray) mass spectrometry (Micromass, Manchester, UK) and amino-acid analysis (Biochrom 20 Plus, Amersham Biosciences, Uppsala, Sweden), were used to check the homogeneity of each synthesized resin-bound peptide sequence.

2.2.2. Analytical HPLC

Analysis was performed in a system consisting of two model 510 HPLC pumps (Waters, Milford, MA, USA), an automated gradient controller, Rheodyne manual injector, 486 detector and 746 data module. Unless otherwise stated, peptides were analyzed on a 4.6 × 150 mm column with a 300-Å pore size and a 5-μm particle size (C18; Vydac, Hesperia, CA, USA) using the solvent systems: A (H_2O containing 0.1% TFA) and B (60% ACN in H_2O containing 0.1% TFA). A linear gradient of 10–90% B in 30 min was applied at a flow rate of 1.5 mL/min and detection at 220 nm.

2.2.3. Amino-acid analysis

Peptide composition was monitored using amino-acid analysis which was performed on a Biochrom 20 Plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England) equipped with an analytical cation-exchange column. The peptides were hydrolyzed with 6 M HCl in sealed tubes under nitrogen atmosphere at 110 °C for 72 h. The samples were concentrated under high vacuum conditions, suspended in 0.2 M sodium citrate buffer, adjusted to pH 2.2 and automatically injected into the analyzer.

2.2.4. Mass spectrometry

LC/ESI-MS experiments were performed on a system consisting of a Waters Alliance model 2690 separation 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, Cheshire, UK). The samples were automatically injected on a Waters narrow bore Nova-Pak column C_{18} (2.1 × 150 mm, 60 Å pore size, 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 in 30 min. The condition used for mass spectrometry measurements was a positive ESI.

2.2.5. Measurements of bead swellings

Before use in peptide synthesis or microscopic measurement of bead sizes, most resin batches were sized by sifting through metal sieves to lower the standard deviation of resin diameters to about 4%. Swelling studies of these narrowly sized populations of beads have been previously conducted [13,25]. In short, 150–200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly with an Olympus model SZ11 microscope coupled with Image-Pro Plus version 3.0.01.00 software. Since the sizes in a sample of beads are log-normally rather than normally distributed, the more accurate geometric mean values and geometric standard deviations were used to estimate the central value and the distribution of the particle diameters [35]. The resins were measured with their amino groups in the deprotonated form, obtained by 3 × 5 min washes in TEA/DCM/DMF (1:4.5:4.5, v/v/v), followed by 5 × 2 min washes in DCM/DMF (1:1, v/v) and 5 × 2 min DCM

washings. Resins were dried *in vacuum* using an Abderhalden-type apparatus with MeOH reflux.

3. Results

As an essential prerequisite for the development of the present study, different resins routinely used in the classical *tert*-butyloxycarbonyl (Boc) – chemistry strategy [1,3] but with much higher substitution degrees than those existing in the market were initially synthesized following our earlier works [36]. Thus, batches of benzhydrylamine-resin (BHAR) [37] and methylbenzhydrylamine-resin (MBHAR) [38] needed for the synthesis of α-carboxamide peptides and containing substitution degrees ranging from 0.3 mmol/g to 2.6 mmol/g were obtained in controlled experimental conditions [36]. Otherwise, for the synthesis of free α-carboxylate peptides, differently substituted batches of the 4-(oxymethyl)-phenylacetamidomethyl-resin (PAMR) [39] were also synthesized, starting from highly polystyrene-type aminomethylated resin (AMR), followed by the inception of the 4-(oxymethyl)-phenylacetamido-group linker and lastly by desired C-terminal Boc-amino acid. In the case of the 1.4 mmol/g Boc-Leu- or Boc-Ile-PAMR, these solid supports were obtained starting from a 3.3 mmol/g AMR batch synthesized accordingly to previous detailed procedure [40].

Batches of near 10 g of BHAR (0.2 mmol/g and 1.4 mmol/g), MBHAR (0.3 and 2.6 mmol/g) and Phe- and Leu-PAMR (0.5 and 1.4 mmol/g substitution degrees of these amino acids) were prepared for the development of the present solvation–synthesis yield investigation. Complementarily, the following set of peptide sequences were selected for assembly in these resins but with different loading degrees for further comparative approaches: (i) the very polar $(NANP)_{(1-4)}$ sequence found in the antigenic and immunodominant epitope of the sporozoite of *Plasmodium falciparum* involved in malaria transmission [41] and its more hydrophobic $Bz(DADP)_{(1-4)}$ and $Bu(DADP)_{(1-4)}$ analog sequences. These three sequences were synthesized starting from a 0.2 mmol/g or 1.4 mmol/g BHAR; (ii) the ING (72–74)-acyl carrier protein aggregating fragment [42] was deliberately assembled in a lysyl-branched $(K)_2$-K core linked to the 2.6 mmol/g MBHAR [$(ING)_4$-K_2-K-resin]; (iii) the vasoactive peptides DRVYIHPF (angiotensin II or AII) and DRVYIHPFHL (angiotensin I or AI) [43] assembled in low (0.5 mmol/g) and high (1.4 mmol/g) Phe- and Leu-PAMR supports, respectively; (iv) the salmon luteinizing hormone-releasing hormone analog pGlu-HWSYGLRPG-amide (LHRH) [44] in 0.3 and 2.6 mmol/g MBHAR. The final calculated peptide contents (PC = weight/weight) but always considering the presence of the side chain protecting groups in all these peptide-resins ranged up to near 85% in the present work.

3.1. Solvation studies of peptide-resins

3.1.1. Coupling step

3.1.1.1. Loading and sequence effects.

3.1.1.1.1. $(NANP)_{(1-4)}$, $Bz(DADP)_{(1-4)}$ and $Bu(DADP)_{(1-4)}$–BHAR (1.4 mmol/g). Table 1 and Fig. 1 show the variation of swelling degree of beads of the $(NANP)_{(1-4)}$-, $Bz(DADP)_{(1-4)}$- and $Bu(DADP)_{(1-4)}$-BHAR (1.4 mmol/g) in DCM, DMF and DMSO as a function of the peptide chain length. The PC values of these resins ranged from near 40% to 75% (Table 1).

The results displayed in this table revealed, in addition to the expected influence of the strong hydrophobic character of the own polystyrene–divinilbenzene matrix of the resin, a simultaneous influence of the effect of the amount of peptide chains in the bead and the nature of the sequence upon the final swelling property of each peptide-resin [4,6,13]. Due to the polar character of a peptide bond, the swelling of beads in the polar aprotic solvents DMF and DMSO increased progressively in the three peptide-resins as a function of the chain elongation. These solvation data thus indicated a clear effect of the peptide loading in the resin.

Otherwise, the influence of the nature of the peptide sequence could be detected by verifying the pronounced decrease in swelling of beads in the apolar DCM, observed only with the more hydrophilic $(NANP)_{(1-4)}$-attached resin (Fig. 1A). In contrast with this polar sequence, the swelling behavior of the more hydrophobic $Bz(DADP)_{(1-4)}$- and $Bu(DADP)_{(1-4)}$-peptide-resins (Fig. 1B and C) remained constant in DCM during their chains growth, thus revealing their greater hydrophobic characters, given mainly by the presence of apolar Bz and Bu protecting groups in the peptide backbone.

3.1.1.1.2. $(ING)_4$-K_2-K-MBHAR (2.6 mmol/g). Variations in the solvation profile of this Lys-branched peptide sequence bound to a highly substituted solid support (2.6 mmol/g MBHAR) were monitored during its chain assembly (Fig. 2). Due to the pronounced increase in the peptide loading as the chain elongation proceeded – the PC value reached 83% by the end of the synthesis. The swelling in the more apolar solvent DCM reduced drastically, with the percentage of volume of resin bead occupied by this apolar solvent varying from near 85% to 20%. This resin swelling behavior in DCM paralleled that observed with the $(NANP)_{(1-4)}$-BHAR in this same solvent (last item), thus indicating that in the case of the Lys-branched peptide-attached resin, there also occurred the dominant influence of the great amount of polar peptide bonds as a consequence of the absence of any type of hydrophobic side chain protecting groups and of the reduced contribution of apolar resin backbone as 83% of the total weight of the peptide-resin are given by the peptide chains. These findings thus depicted the inadequacy of DCM as the solvent system for the critical coupling step of this type of peptide sequence, especially in heavily peptide loaded condition.

3.1.1.1.3. pGlu-HWSYGLRPG-amide (LHRH, salmon sequence). The swelling profiles of this peptide sequence during its chain growth when bound either to a low (0.3 mmol/g, PC values of 31%) or highly (2.6 mmol/g, PC value of 79%) MBHAR in DCM, DMF or DMSO are comparatively displayed in Fig. 3. Noteworthy, rather similar swelling properties were observed for these peptide-resins, irrespective of the solvent system used or of the peptide loading. These results thus underscore again the relevance of the balance existing amongst the overall polarity of the LHRH sequence bearing hydrophobic Tos, For, Bz, 2BrZ side chain protecting groups, of the solid support backbone itself and the amount of polar peptide bonds for the final swelling degree of each peptide-resin in different loaded conditions.

In the present case, one might thus conclude, based on the constancy of swelling degrees observed (around 60–80%), that the overall polarity of the LHRH-resin remained rather constant during the peptide chain elongation. As a consequence, the swelling strengths of DCM, DMF and DMSO seemed to be equivalent for solvating this peptide-resin, regardless of amount of attached peptide chains in the beads.

3.1.1.2. Application of the (AN + DN) polarity term. In this topic, the relationship between the polarity of the solvent system estimated by the amphoteric (AN + DN) scale [13,16] and the solvation characteristics of highly peptide loaded resins was examined through a more complete bead swelling study of the vasoactive AII and AI sequences attached to a highly 1.4 mmol/g substituted Phe-PAMR and Leu-PAMR compounds. The PC values of both resins reached 68% and 71%, respectively.

By applying earlier reported comparative swelling approach [13,14,45], diameters of peptide-resin beads of both peptide-resins were measured in 18 solvents systems which roughly cover the polarity scale (Table 2). The Fig. 4 reveals the swelling data measured of each peptide-resin in each of these solvent systems

Table 1
Swelling degrees of BHAR (1.4 mmol/g), $(NANP)_{(1-4)}$, $Bz(DADP)_{(1-4)}$ and $tBu(DADP))_{(1-4)}$ bound to BHAR (1.4 mmol/g) in DCM, DMF and DMSO.

Resin	PC[a] (%)	Diam. dry bead (μm)	DCM		DMF		DMSO	
			Diam. swollen bead (μm)	Solvent within bead (%)[b]	Diam. swollen bead (μm)	Solvent within bead (%)[b]	Diam. swollen bead (μm)	Solvent within bead (%)[b]
BHAR	0	58	99	85	79	61	66	46
$(NANP)_1$–BHAR	37	60	80	58	102	80	103	81
$(NANP)_2$–BHAR	53	61	81	56	104	79	114	84
$(NANP)_2$–BHAR	63	63	80	50	110	81	120	85
$(NANP)_4$–BHAR	69	68	82	42	110	76	129	85
$Bz(DADP)_1$–BHAR	46	64	111	80	98	72	98	71
$Bz(DADP)_2$–BHAR	62	71	118	78	106	69	110	73
$Bz(DADP)_3$–BHAR	71	75	119	75	119	75	120	76
$Bz(DADP)_4$–BHAR	77	79	117	69	140	82	142	83
$tBu(DADP)_1$–BHAR	43	61	112	84	110	83	102	79
$tBu(DADP)_2$–BHAR	59	66	120	83	115	81	111	79
$tBu(DADP)_3$–BHAR	69	73	126	81	123	79	115	75
$tBu(DADP)_4$–BHAR	74	77	124	76	125	77	130	79

[a] Peptide content including the weight of side chain protecting groups.
[b] [(Swollen volume − dry volume)/swollen volume] × 100.

and correlated with their corresponding polarity (AN + DN) values. The displayed contour solvation plot indicated that both peptide-resins beads revealed maximum swelling in solvents characterized with (AN + DN) polarity values near 40, which for instance, correspond approximately to the single solvents DMF, NMP or the mixed 20% DMSO/NMP and 50% DMSO/THF solutions (Table 2). Of note, a lower solvation capacity was observed for AI-PAMR in comparison with AII-PAMR. In the former sample, the observed maximum swelling values observed in the contour solvation plot curve were not higher than near 60% (Fig. 4B) whereas in the AII-PAMR, this value increased to about 80%. In a subsequent Section 3.2 the importance of these solvation results will be evaluated in the light of the final synthesis yield observed for both peptide-resins.

3.1.1.3. Determination of site–site distance and effective concentration of chains within beads. The salmon LHRH sequence was now selected in this topic for examining the differences existing between low and highly peptide loaded resins in terms of average chain–chain distance and peptide concentration values inside the resin beads, accordingly to previous reports [26,32]. Thus, taking into account different peptide resin bead parameters shown in Table 3 such as their swelling values (in dry and in swollen state), the degree of substitution of each resin (0.3 and 2.6 MBHAR) and also the molecular weight of attached protected peptide segment it was possible to determine some unusual bead structural parameter following a sequential calculation strategy previously described [26,32]. Amongst these parameters, one can mention the average values of the distance existing between peptide chains spread throughout the bead matrices (column 9) and the chain concentration within the bead (column 10) found for both peptide-resins when swollen in DCM, DMF and DMSO (Table 3).

For the case of the low peptide loaded resin (0.3 mmol/g MBHAR and the PC value of 31%), the data shown in this table indicated that, irrespective of the solvent used, the values of the distances between peptide chains and those of chain concentrations were near 1×10^6 Å and 0.02 M, respectively. As expected, significant differences were found for these parameters when the resin is in heavily peptide loaded condition (2.6 mmol/g MBHAR and PC value of 79%). Due to the greater amount of peptide chains within each bead, the site-site distance decreased to near 0.6×10^6 Å and the peptide chain concentration increased almost four times (~0.8 M) in the three solvents tested.

3.1.2. α-Amine neutralization and deprotection steps

To date, the solvation studies involving peptide-resins beads have only focused on the evaluation of different factors affecting the efficacy of the critical coupling reaction step. Thus, sizes of resin beads attaching peptide chains but with their α-amine groups in deprotonated form (after treatment with organic base and washings) are usually examined in a microscope aiming at searching for the solvent systems with greater potentials for optimization of the aminoacylation reaction during the synthesis cycle.

By differing conceptually from this type of strategy, the present topic investigated the solvation behavior of peptide-resin but just at the α-amine group deprotonation and deprotection reaction existing in the Boc-chemistry synthesis cycle. The main focus lied on the special case of highly peptide loaded synthesis procedure.

The reason for developing this different approach was due to our previous observation and where a significant shrinking process of resin beads seemed to occur mainly in the 10% TEA/DCM neutralization solution when a peptide segment had to be synthesized in very highly substituted resins. If true, this lack of solvation might induce incomplete deprotonation of the N-terminal portion of the peptide, with serious consequence for the incoming coupling step.

Thus, the proposed investigation examined initially the bead swelling degree at the α-amine group neutralization reaction of the model peptide-resin $(NANP)_{(1-4)}$-BHAR in low (0.2 mmol/g) and highly (1.4 mmol/g, Table 1 and Fig. 1A) loaded conditions. The swelling capacities of 10% TEA solution in DCM and in DMF were verified. By using the same model peptide-resin, a similar strategy was also applied but during the α-amine group deprotection reaction (removal of the Boc group), with the use of 30% TFA solution either in DCM or in DMF.

3.1.2.1. Neutralization Step. The most significant observation detected in the swelling data shown in Fig. 5 is the strong resin bead shrinkage observed specifically with the $(NANP)_{(1-4)}$-BHAR (1.4 mmol/g) when solvated in 10% TEA/DCM. A sharp decrease (80% to near 25%) in the swelling percentage of this highly loaded peptide-resin was verified as long as the peptide chain elongated, thus suggesting the necessity of replacing this standard mixed solvent for the alternative 10% TEA/DMF. In this latter solvent system, the swelling degree of this peptide-resin was maintained constant and high (about 75%) during the peptide chains growth.

Conversely, lesser pronounced decrease in swelling in the case of low peptide loaded resins (0.2 mmol/g BHAR) was verified with

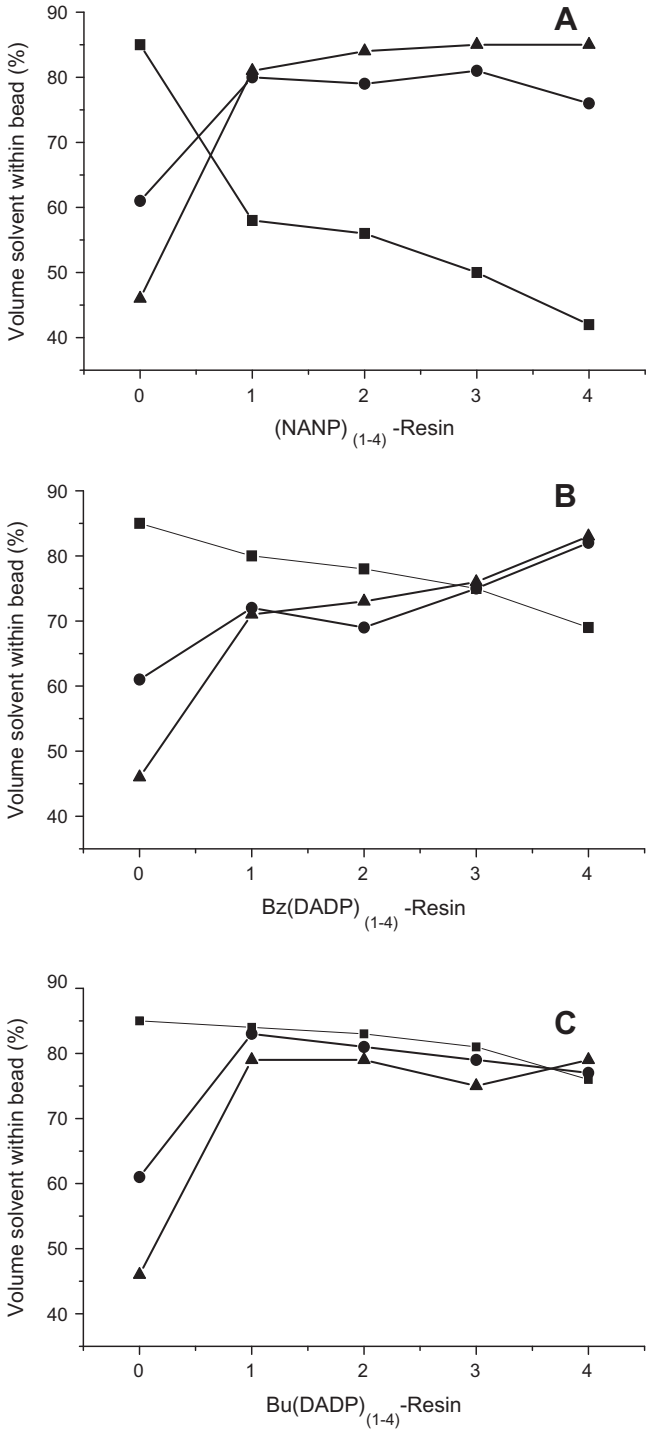

Fig. 1. Swelling degrees of $(NANP)_{(1-4)}$ (A), $Bz(DADP)_{(1-4)}$ (B) and $tBu(DADP)_{(1-4)}$ (C) bound to BHAR (1.4 mmol/g) in DCM (■), DMF (●) and DMSO (▲).

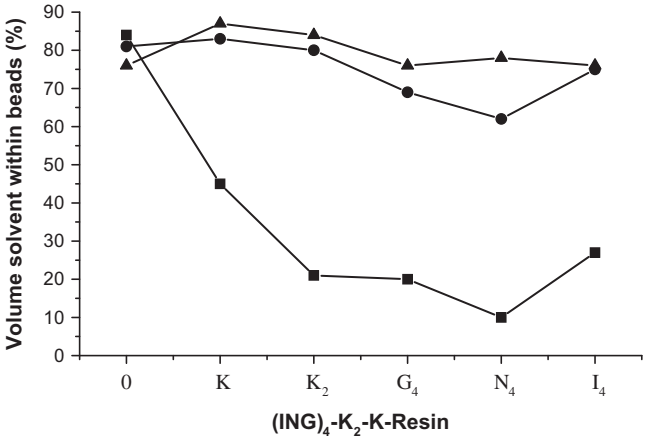

Fig. 2. Swelling degrees of $(ING)_4$-K_2-K-MBHAR (2.6 mmol/g) in DCM (■), DMF (●) and DMSO (▲) during the peptide chain growth.

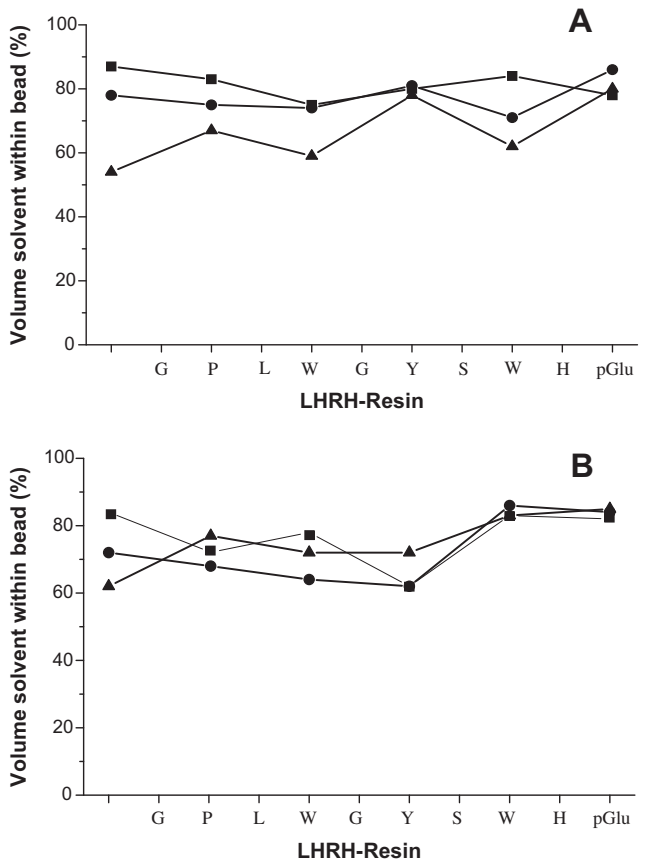

Fig. 3. Swelling degrees of LHRH (salmon sequence) in DCM (■), DMF (●) and DMSO (▲) bound to a 0.3 mmol/g MBHAR (A) and 2.6 mmol/g MBHAR (B).

TEA/DCM mixture (from near 65% to 45%). This swelling value is comparatively higher than that observed when 10% TEA/DMF is used, thus indicating that the change of the classical TEA solution with DCM when in low peptide loading condition is not necessary. Taken together, one may conclude that only for the case of heavily peptide loaded polymers the DCM replacement by the more polar aprotic DMF seemed to be necessarily for co-solvating TEA solution during the neutralization step in the synthesis cycle.

3.1.2.2. Deprotection step. The Fig. 6 displays the comparative swelling data during the α-amine deprotection reaction observed for $(NANP)_{(1-4)}$-BHAR in low and heavily loaded conditions. Regardless the amount of peptide chains attached to the solid support, increased swelling of beads was observed when in 30% TFA/DCM than in 30% TFA/DMF solutions. Unlike what is recommended for the deprotonation reaction, there was no need for replacing DCM for DMF, even when the resin is bearing large amount of peptide chains.

3.2. Comparative synthesis yield in low and highly peptide loaded protocols

In this final topic, most of peptide sequences mentioned in previous items of the present work were examined in terms of

Table 2
(AN + DN) polarity values[a] of solvents used for peptide-resins swelling experiments.

Entry	Solvent	(AN + DN)	Entry	Solvent	(AN + DN)
1	Toluene	3.4	10	Formamide	63.8
2	DCM	21.4	11	50% TFE/DCM	28.5
3	Chloroform	27.1	12	80% TFE/DCM	27.5
4	NMP	40.6	13	20% DMSO/NMP	42.3
5	DMF	42.6	14	50% DCM/DMF	32.0
6	DMSO	49.1	15	50% DCM/DMSO	35.3
7	TFE	53.5	16	50% MeOH/DMSO	60.2
8	EtOH	69.1	17	50% TFE/DMF	48.1
9	MeOH	71.3	18	50% TFE/DMSO	51.3

[a] Refs. [13,18].

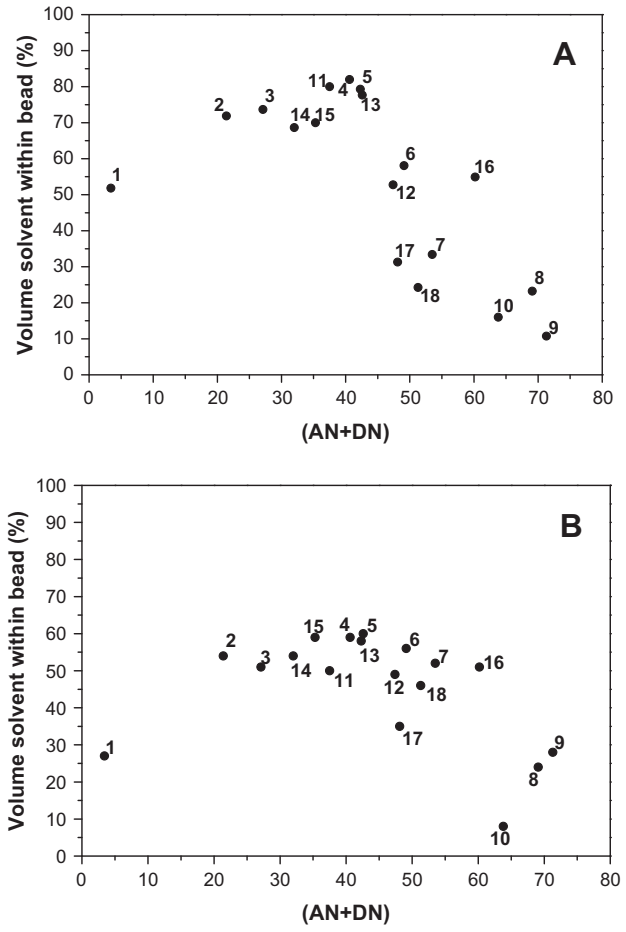

Fig. 4. Swelling degrees of angiotensin II (A) and angiotensin I (B) bound to Boc-Phe- and Boc-Leu-PAMR (1.4 mmol/g) respectively, as a function of solvent polarity (AN + DN) values.

synthesis yield when in low and highly loaded conditions (the PC values ranged from near 30% to 85%). The data shown in the final Table 4 compare the syntheses yields (variation from 65% to 85%), estimated by the analytical HPLC profiles of crude peptide with the use of mass spectrometry assay for confirming the identity of the designed peptide.

To facilitate the comparative analysis, all the syntheses were deliberately carried out in the same condition, i.e., using a 3.0-fold excess of the classical acylating agents diisoproprylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBT) (1:1) and using 1:1 DCM/DMF mixture as the solvent system for 3 h. When necessary, *N*-(1-H-benzotriazol-1-yl)-(dimethylaminomethylene)-N-methylmethanaminium hexafluorophosphate–*N*-oxide (HBTU)/1-hydroxy-7-azabenzotriazole (HOAt)/diisopropylethylamine (DIEA) in 20% DMSO/N-methylpyrrolidine (NMP) were employed for re-coupling steps. However, as a consequence of the solvation study developed in the previous basic neutralization step (Section 3.1.2.1), the standard 10% TEA/DCM solution was replaced by 10% TEA/DMF mixture, specifically when in the case of highly substituted resins.

As expected, greater difficulties in the coupling reactions were detected in heavily peptide loaded synthesis conditions. However, a single re-coupling procedure allowed in most cases, the progressive elongation of the desired peptide sequence. Of note, a detailed analysis of the syntheses yields presented in the Table 4 revealed that whatever the sequence tested rather equivalent syntheses yields were measured for different peptide-resins. These findings thus pointed to the feasibility in synthesizing peptide even of heavily loaded experimental protocol, despite the potential for the occurrence of severe sterically hindered conditions induced by the greater amount of peptide chains within resin beads.

Noteworthy and emphasizing again the clear influence of the solvation effect affecting the final synthesis results, the Table 4 also demonstrated that the synthesis yield of AII was greater than that observed for AI, using the same synthesis protocol (about 85% vs 70%, respectively). These results are in close accordance with previous findings shown in the contour solvation plots of these two peptide-resins (Fig. 4, Section 3.1.1.2). In this case, greater facility in bead swelling was detected for AII in comparison to AI, when attached to the 1.4 mmol/g BHAR.

4. Discussion

The main objective of the present investigation was to examine, through different approaches, the possibility of synthesizing peptides in heavily loaded conditions. Thus home-made batches of very highly substituted BHAR and MBHAR or PAMR which were made deemed requisite were therefore synthesized following previously reported strategies for both types of resins [36,40], respectively). Next, model peptide sequences were assembled in these

Table 3
Swelling parameters of LHRH–MBHAR (0.3 and 2.6 mmol/g) in DCM, DMF and DMSO.

Solvent	Col. 1 Diam. dry bead (μm)	Col. 2 Diam. swollen bead (μm)	Col. 3 Volume solvent/bead (10^5 μm^3)	Col. 4 Volume dry sample/g copol. (mL)	Col. 5 Weight dry sample/g copol. (g)	Col. 6 Volume dry sample/g sample (mL)	Col. 7 Number of beads/g sample (10^7)	Col.8 Number of sites/bead (10^{12})	Col. 9 Site–site distance Å × 10^5	Col 10 Site conc. (mM)
LHRH–MBHAR (0.3 mmol/g)										
DCM	73	121	7.2	3.9	1.4	2.8	1.37	9.8	9.8	23
DMF	73	139	12.0	3.9	1.4	2.8	1.37	9.8	11.3	14
DMSO	73	124	8.0	3.9	1.4	2.8	1.37	9.8	10.1	21
LHRH–MBHAR (2.6 mmol/g)										
DCM	98	177	24.1	9.5	7.8	1.5	0.30	129	6.1	89
DMF	98	182	26.6	9.5	7.8	1.5	0.30	129	6.3	81
DMSO	98	185	28.2	9.5	7.8	1.5	0.30	129	6.4	76

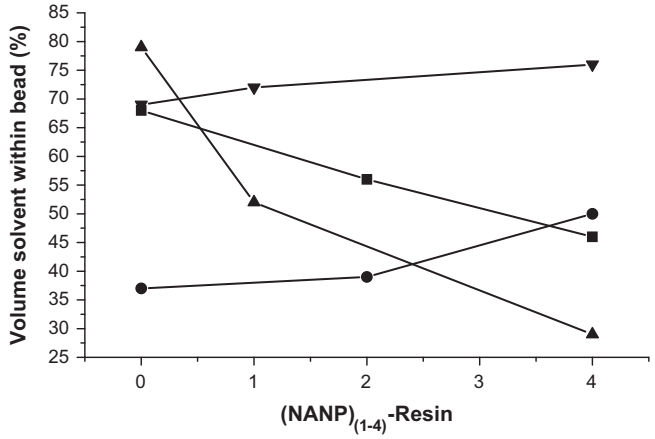

Fig. 5. Swelling degrees of $(NANP)_{(1-4)}$-BHAR (0.2 mmol/g) in 10% TEA/DCM (■) and 10% TEA/DMF (●) and in $(NANP)_{(1-4)}$-BHAR (1.4 mmol/g) in 10% TEA/DCM (▲), and 10% TEA/DMF (▼).

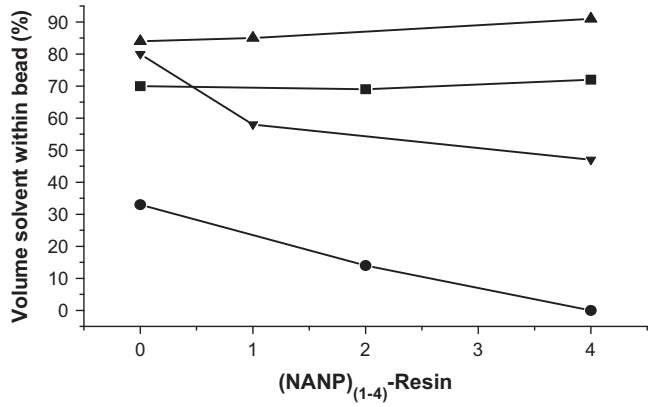

Fig. 6. Swelling degrees of $(NANP)_{(1-4)}$- BHAR (0.2 mmol/g) in 30% TFA/DCM (■) and 30% TFA/DMF (●) and in $(NANP)_{(1-4)}$- BHAR (1.4 mmol/g) in 30% TFA/DCM (▲), and 30% TFA/DMF (▼).

solid supports for comparative solvation and synthesis studies. The achieved PC values in the resins ranged up to near 85%.

The analysis of the swelling data in DCM, DMF and DMSO of $(NANP)_{(1-4)}$-, $Bz(DADP)_{(1-4)}$-, $Bu(DADP)_{(1-4)}$-BHAR (1.4 mmol/g) (Fig. 1), the branched Lys-core containing (ING)4-K2-K-MBHAR (2.6 mmol/g) (Fig. 2) and the salmon LHRH sequence – pGlu-HWSYGLRPG-MBHAR (0.3 and 2.6 mmol/g) (Fig. 3), confirmed the strong influence either of the polarity of the peptide sequence – including the nature of side chain protecting groups – and also of the peptide loading effects affecting the solvation characteristics of each resin.

These results also allowed the detection of a clear inadequacy of the use of DCM as the single solvent system for solvating specific classes of peptide-resins. This assertion was based on the pronounced lack of solvation verified for the $(NANP)_{(1-4)}$-BHAR (1.4 mmol/g), with PC value of 69% and the (ING)4-K2-K-MBHAR (2.6 mmol/g), with PC value of 83%. This particular case of the inappropriateness of the use of DCM for coupling step in the SPPS was already observed with other examples of peptide-resins [4,25,34]. Despite these findings, one must stress that DCM displayed good swelling capacity for other peptide-resins investigated such as those bearing LHRH, AII, AI, $Bz(DADP)_{1-4}$ and $Bu(DADP)_{1-4}$ sequences, thus confirming the critical dependence of the solvation phenomenon to the type of solvent and also of the peptide-resin.

Besides the DCM, one must also be aware of the use of other common solvents such is the case of DMF. We have earlier demonstrated that during the assembly of a long and hydrophobic 32-mer transmembrane segment of the angiotensin II receptor, an abrupt shrinking of beads was surprisingly detected near at position 12 of this segment [14,25]. This observation led us to search for more appropriate solvent than DMF in order to successfully achieve the assembly of this type of difficult segment.

In conclusion, this set of solvation data corroborated previous assertion found in the literature which emphasized the need of much care for the choice of the most appropriate solvent system to be used in the coupling step. Besides this aspect, one must be always aware of the effect of the viscosity of the solvent to be used. In cases where similar swelling degree of different solvents is detected for solvation of a determined peptide-resin for instance, in the case of LHRH-MBHAR (Section 3.1.1.1.3], the most viscous one should not be selected (DMSO) due to the diffusion effect that is known to affect seriously the rate of polymer-supported reactions [46].

A more complete swelling investigation, as earlier proposed [13,16] and using almost twenty solvent systems encompassing almost entirely the polarity scale was next applied for comparison of solvation properties of two vasoactive peptides, AII and AI, both attached to a highly substituted 1.4 mmol/g Phe- and Leu-PAMR supports (Section 3.1.1.2). The obtained contour solvation plots of these peptide-resins indicated that they swelled better in solvents with polarity (AN + DN) values around 40. In complement, by examining the maximum swelling values reached by the two peptide-resins in these comparative solvation curves, it was possible to conclude that AII-attached solid support presented greater facility for solvating their beads than did the AI-resin. As these two peptide-resins are characterized by containing rather similar PC values (about 70%), one may thus infer that each sequence acquires specific conformational features with different levels of structural constraints thus influencing their final solvation characteristics.

Next, the complex and unusual solvation investigation involved the estimation of the average chain–chain distance and chain concentration values within the peptide-resin beads [26,32] was carried out comparatively (Table 3, Section 3.1.1.3) with the low and highly peptide loaded LHRH-MBHAR (PC values of near 30% and 80%, respectively). This study indicated that the average chain–chain distance values decreased from near 1×10^{-6} to 0.6×10^{-6} Å and the peptide concentration within beads increased from near 0.2 mM to 0.8 mM in the low and highly loaded resins, respectively.

These findings thus revealed in more microscopic details and closely related to the level of steric hindrance surrounding the growing peptide chains, the significant differences existing with the resin beads when under low and heavily peptide loaded synthesis conditions. In this respect, we have already demonstrated a direct relationship existing between these parameters and the rate of coupling reaction in a model peptide synthesis experimental protocol [32].

Differing significantly from most of previous solvation approaches, the following topic of the present work (Section 3.1.2) examined the solvent effect directly on the standard α-amine group neutralization and deprotection steps, carried out routinely in 10% TEA/DCM (Fig. 5) and 30% TFA/DCM (Fig. 6), respectively. The results revealed initially that, in order to avoid strong shrinking of beads during the α-amine group neutralization step, it was recommended the replacement of the standard TEA/DCM solution for the alternative TEA/DMF mixture but only in the case of heavily peptide loaded synthesis experiments (Fig. 5).

These results are indeed, in agreement with our previous conceptual interpretation of solute–solvent interaction theory based on the electrophilic (AN) and nucleophilic (DN) character of each

component of a solvent mixture [13,16,17]. Improved solvation of the peptide-resins, when in very strong chain associated form as is the case of highly peptide loaded resins, is more easily attained with the use of homogeneous-type solvent systems which is characterized by being composed of components with the same properties in terms of acidity or basicity characters [13,16,17]. The TEA/DMF solution is a typical example of homogeneous solution as TEA and DMF are strong electron donor (basic) solvents, revealed by the Gutmanńs high DN numbers of 33.3 and 26.6, respectively [16,18].

Conversely, the TEA/DCM is classified as heterogeneous-type mixed solvent due to the electrophilic character of DCM (AN of 20.4 and DN of only 1.0) [16,18]. In the case of this type of mixed solvents, each component tends to associates with the other rather than with the solute molecule (peptide-resin). As a consequence, the heterogeneous solvent is not capable of disrupting mainly strong chain–chain associations within beads thus inducing low degree of swelling. This solvent effect is much more prominent when a mixture is composed of very strong electrophilic and strong nucleophilic solvents as is the case, for instance of TFE/DMF or TFE/DMSO, composed of strong electrophilic (TFE, AN = 53.5) and nucleophilic solvents (DMF, DN = 26.6 or DMSO, DN = 29.8).

Following with this rationale, this type of physicochemical interpretation of the solute–solvent interaction based on AN and DN concepts has been also very useful for better understand the solubilization process of strong aggregate peptide sequences when free in solution such as the case of the Aβ-(1–42) amyloid peptide. This well-known insoluble peptide tends to form fibrils in the brain inducing the manifestation of the Alzheimer disease [47]. The dissolution of this aggregated structure was only achieved [48] by using homogeneous solvents such is the case of mixing the strong electrophilic H_20 (AN = 54.8) with TFE or hexafluoroisopropanol (HFIP, AN = 53.5 and 88.0, respectively). As expected and in accord with this rationale, it was not possible to dissolve the amyloid peptide fibrils using the heterogeneous H_2O/DMSO solution [48].

Thus, accordingly to this approach of interpreting solvation of a peptide-resin solute based on the electrophilic/nucleophilic characters of the solvent system, the comparative results displayed in the α-amine deprotection reaction carried out in the classical 30% TFA/DCM solution should be maintained for optimized removal of the Boc group, regardless the peptide loading value of the resin (Fig. 6). Increased swelling of $(NANP)_{(1-4)}$-BHAR (02 and 2.6 mmol/g) in this mixed solution was expected as TFA is classified as one of the most electrophilic solvent (AN = 105) [18] and its mixture will be homogeneous with DCM and not with DMF.

The final topic (Section 3.2) of the present work revealed comparative synthesis data of LHRH-MBHAR, AII-PAMR and AI-PAMR when low and highly substituted resins were used (Table 4). The (ING)4-K2-K-MBHAR (2.6 mmol/g) was also included in this study as an extreme model of difficult sequence to be assembled as previously demonstrated [49] due to its very high PC values of 83% and branched structure. Specific details of theses synthesis were all revealed in the Section 3.2.

No significant differences in the synthesis yields were observed with LHRH-MBHAR, AII-PAMR and AI-PAMR when synthesized in low or in highly loaded protocols. Moreover, in accordance with the previously discussed greater facility in swelling their beads (Fig. 4, Section 3.1.1.2), the synthesis yield of the AII-PAMR was higher (∼85%) than that observed for AI-PAMR (∼70%). Lastly, the synthesis yield of the (ING)4-K2-K-MBHAR obtained in heavily loaded condition reached 64%, thus suggesting that the peptide synthesis through the so-called "prohibitive" heavily loading chemical strategy is in many cases, feasible.

One explanation for these similar results in terms of the final synthesis yield, even in the case of the more difficult highly loaded synthesis condition might be mainly due to the unique possibility in using advantageously much greater concentration of acylation reagents without increasing the molar excess of reactants, thereby inducing faster rate of coupling reaction. This different experimental condition is allowed simply by the fact that, due to their high degree of substitution, much smaller amount of resins is needed for the heavily peptide loading protocol, thus allowing the use of lesser amount of solvent during the coupling step. In this respect, the third column in the Table 4 displays comparatively the calculated concentration values of coupling reactants used when in low and highly loaded synthesis conditions for each of examined peptides. Increase of three to seven folds in these values could be observed when highly substituted resins are used. In addition, one can not also neglect a possible positive influence for the synthesis success related to the application of the innovative use of TEA/DMF instead of TEA/DCM solutions for neutralization step but only in the case of highly peptide loaded synthesis.

The present work thus intended to examine with more details, the feasibility of applying the highly loading peptide synthesis approach. There are indeed in the literature, some reports that have already addressed this issue but most of them, dealing with resins bearing smaller amount of peptide chains than used in the present work or related to the Fmoc-chemistry [50,51] and even to the Boc-strategy, but using resins with different types of copolymer backbone [52]. However, the great majority of these studies involved synthesis of comparatively smaller amount of model peptide sequences and/or with the use of lower substituted resins.

In this context, the present report differed significantly from these previous studies as much greater PC values of peptide content were achieved, reaching in some cases, almost 85% (weight/weight) in the resin and with near twenty types of peptide sequences tested in different approaches coupled to different resins. In this work, the Boc-chemistry was deliberated selected due to our previous experience in producing different types of solid supports with much higher substitution degrees which, in turn, would facilitate not only the industrial application for the SPPS but also for the contemporary combinatorial chemistry applicable not only for peptide synthesis but also for new drug developments [53].

One important issue still not raised in the present work is related to the feasibility of using the alternative Fmoc-synthesis strategy [2], specifically for the case of highly peptide loading chemistry. Similar approach herein explored with the Boc-chemistry is currently in progress in our laboratory but initial findings have already indicated greater synthetic difficulties in comparison with those herein reported. Possibly, the already observed higher propensity to peptide chain aggregation induced by the more hydrophobic Fmoc-moiety and some of the side chain protecting groups used in this chemistry [5,54] can be determinant for inducing more severe steric hindrance of the peptide chains mainly in very heavily loaded condition.

Table 4
Comparative peptide syntheses yields in low and highly substituted resins.

Peptidyl-resin	PC[a] (%)	Concentration (M)[b]	HPLC purity (%)[c]
LHRH–MBHAR (0.3 mmol/g)	31	0.10	78
LHRH–MBHAR (2.6 mmol/g)	79	0.70	80
AII-PAMR (0.5 mmol/g)	43	0.10	84
AII-PAMR (1.4 mmol/g)	68	0.34	85
AI-PAMR (0.5 mmol/g)	47	0.10	65
AI-PAMR (1.4 mmol/g)	71	0.34	72
$(ING)_4$-K_2-K-MBHAR (2.6 mmol/g)	80	0,70	64

[a] Peptide content (in %) at end of the synthesis, including the weight of side chain protecting groups.
[b] Concentration of acylating reagents during the coupling reaction.
[c] Experimental conditions – see Section 2.2.

In terms of potentials for the large scale SPPS with direct consequence for the industrial application of the SPPS method, a recent work [55] stressed adequately the main shortcomings still remaining for optimization of the application of this unique synthesis strategy. However, no mention was given to the highly loaded synthesis procedure within those limitations existing in SPPS. The set of results deriving from different approaches applied herein, allowed to conclude that in many cases, the peptide synthesis in this "difficult" heavily loaded conditions which has been avoided since the remarkable inception of SPPS strategy near five decades ago [56] is feasible and that the valuable economical advantages proceeding from different approach must be considered.

5. Conclusions

This work showed for the first time, a systematic evaluation of peptide synthesis of a great variety of model peptide sequences comparatively in low and highly loaded conditions. Several experimental approaches with focus on the physicochemical aspects of the solvation features of peptide-resin beads were developed and the final topic correlated the collected information with those related with the final synthesis yield in this severe sterically hindered chemical condition. One may conclude that, despite the expected aggravation of the peptide-peptide interaction rendering difficulties to the chain assembly within resin beads, the possibility of carrying out coupling in very highly concentration reactants, due to the use of highly substituted resins, seemed to help overcome partially this reported shortcoming, thus allowing the peptide synthesis in this unique and advantageous experimental procedure.

Acknowledgments

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