Correlation between the Mobility of Spin-Labeled Peptide Chains and Resin Solvation: An Approach To Optimize the Synthesis of Aggregating Sequences[1]

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Resin solvation properties affect the efficiency of the coupling reactions in solid-phase peptide synthesis. Here we report a novel approach to evaluate resin solvation properties, making use of spin label electron paramagnetic resonance (EPR) spectroscopy. The aggregating VVLGAAIV and ING sequences were assembled in benzhydrylamine-resin with different amino group contents (up to 2.6 mmol/g) to examine the extent of chain association within the beads. These model peptidyl-resins were first labeled at their N-terminus with the amino acid spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (Toac). Their solvation properties in different solvents were estimated, either by bead swelling measurement or by assessing the dynamics of their polymeric matrixes through the analysis of Toac EPR spectra, and were correlated with the yield of the acylation reaction. In most cases the coupling rate was found to depend on bead swelling. Comparatively, the EPR approach was more effective. Line shape analysis allowed the detection of more than one peptide chain population, which influenced the reaction. The results demonstrated the unique potential of EPR spectroscopy not only for improving the yield of peptide synthesis, even in challenging conditions, but also for other relevant polymer-supported methodologies in chemistry and biology.

Introduction

Almost four decades after its introduction, Merrifield's Nobel prize awarded solid-phase peptide synthesis (SPPS) technique[2] still presents some drawbacks. Among these, one persistent challenge concerns the synthesis of strongly aggregating sequences assembled in highly substituted resins. Despite the economical advantages of this protocol, it enhances chain association inside the resin matrix, substantially impairing coupling reactions during chain growth, as expected for any solid-supported chemical process. Among several factors, the success of the SPPS is markedly dependent on the degree of solvation of the peptide chain throughout the polymeric matrix.

With the aim of searching for correlations between peptidyl-resin solvation and physicochemical properties of the solvating system, an initial attempt, based on resin swelling measurements in a volumetric frask,[3] led to the proposition of a contour solvation plot relating the resin degree of solvation and the two components of Hildebrand's solubility parameters (δ and δ_h).[4] Alternatively, other investigators used the strategy of microscopic measurement of bead size to estimate resin solvation properties.[5,6] With the same objective, we evaluated[7] the swelling of model peptidyl-resins, varying the polarity as well as the amount of the resin-bound sequence in ca. 30 solvent systems encompassing the entire polarity scale. Using this approach, it was possible to verify that each type of peptidyl-resin displayed a specific solvation profile, facilitating the choice of solvent for optimal synthesis conditions. Moreover, since this solvation study was in fact an investigation of solute−solvent interactions, where each peptidyl-resin was a special model of a heterogeneous and complex type of solute, we were able to propose[7] a novel solvent polarity parameter based on

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(1) Abbreviations for amino acids and the nomenclature of peptide structure follow the recommendations of the IUPAC−IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, *247*, 997). Other abbreviations are as follows: BHAR = benzhydrylamine-resin; Boc = *tert*-butyloxycarbonyl; Bzl = benzyl; 2-BrZ = 2-bromobenzyloxycarbonyl; DCM = dichloromethane; DIEA = diisopropylethylamine; DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; EPR = electron paramagnetic resonance; EtOH = ethanol; FT-IR = Fourier transform-infrared; HFIP = hexafluoro-2-propanol; HOBt = 1-hydroxybenzotriazole; Fmoc = 9-fluorenylmethyloxycarbonyl, HPLC = high-performance liquid chromatography; MeOH = methanol; NMP = *N*-methylpiperidinone; NMR = nuclear magnetic resonance; PSA = preformed symmetrical anhydride; SPPS = solid-phase peptide synthesis; TBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA = triethylamine; TFA = trifluoroacetic acid; TFE = trifluoroethanol; Toac = 2,2,6,6-tetramethypiperidine-*N*-oxyl-4-amino-4-carboxylic acid.

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electron acceptor (AN) and electron donor (DN) properties of the solvent.[8]

To further improve the knowledge of the molecular events taking place as a function of solvation inside the resin beads, spectroscopic techniques can provide more direct information regarding the conformational features of peptide chains inside the polymer matrix. For this purpose a great variety of spectroscopic techniques, such as CD,[9] FTIR,[10] and NMR,[11] have been employed in the peptide synthesis field. In this context, electron paramagnetic resonance (EPR) spectroscopy[12] is clearly the method of choice to obtain relevant information concerning the microenvironment and the dynamics of solvated peptide chains attached to the polymer. To our knowledge, the first attempt to monitor peptide solvation during peptide synthesis was made recently in our laboratory.[13] In this study, the paramagnetic amino acid derivative 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (Toac),[14] introduced into the peptide synthesis field either as the *tert*-butyloxycarbonyl (Boc)[15,16] or as the 9-fluorenylmethyloxycarbonyl (Fmoc)[17] N^{α}-protected derivative, was used to label model peptidyl-resins. When compared to more flexible paramagnetic compounds,[18,19] this spin label offers the advantage of binding more rigidly to the system under study as a consequence of its C^{α}-tetrasubstituted cyclic structure containing both the ligand site and the paramagnetic center. This stereochemistry renders this probe more sensitive to conformational properties of the structure to which it is attached. Owing to these features, its use has been largely expanded, encompassing investigations of peptide conformation in singly and doubly labeled sequences,[20] of membrane protein fragments,[21] and of biologically active peptides.[22,23]

In the present work we describe, for the first time in the SPPS field, an evaluation of the relationship between EPR spectral parameters and the rate of coupling reactions of model peptidyl-resins labeled with the Toac spin probe. Following preliminary work,[13] peptides with strong self-association tendency were deliberately chosen since the use of this type of sequence may facilitate the detection in the EPR spectra of relevant conformational features spread throughout the resin matrix. In addition to varying the sequence, the degree of peptide loading was also altered to obtain variable degrees of chain association. The benzhydrylamine-resin (BHAR) introduced for the synthesis of α-carboxamide pepides[24] was selected as the solid support for peptide growth, and differently substituted resin batches were synthesized under strictly controlled conditions.[25] The use of these BHAR batches allowed the synthesis of peptidyl-resin with peptide content varying from 6% to almost 70% (weight/weight).

Results and Discussion

The well-known aggregating sequences VVLGAAIV and ING, corresponding to the 291−298 segment of the murine H-2K protein[26] and to the 72−74 segment of the acyl carrier protein,[27] respectively, were synthesized bound to increasingly substituted BHAR (from 0.2 to 2.6 mmol/g) using the Boc/Bzl chemistry. The calculated peptide contents (by amino acid analysis) of the VVLGAAIV−BHAR were 14% and 68% for BHAR batches of 0.2 and 2.6 mmol/g substitution degrees, respectively. For ING−BHAR the peptide contents were 6%, 16%, and 47% when 0.2, 0.6, and 2.6 mmol/g substituted BHAR was used, respectively. All peptidyl-resins were labeled with Fmoc-Toac at their N-terminal portion as previously described.[13] To avoid spin−spin exchange interactions, which broadens the EPR line shapes,[12] the labeling was kept as low as possible.[13] Moreover, the low labeling protocol allows the physicochemical and steric perturbations to be kept due to the introduction of the spin probe at a minimum, decreasing their influence on the solvation characteristics of the peptidyl-resin under investigation. Before swelling studies were performed or EPR spectra were obtained, a small portion of each peptide sequence was cleaved off the resin with anhydrous HF[2a,b] to check if the material was homogeneous. The purity of all cleaved crude peptides, estimated by analytical HPLC, was ca. 90%. Results from amino acid analyses and mass spectra were also consistent with the expected peptide sequences.

Figure 1 displays the EPR spectra of VVLGAAIV-BHAR (14% peptide content) swollen in various solvent systems. As can be seen, chain mobility decreased in the order 10% HFIP/DCM < NMP < 50% TFE/DCM < DCM < DMF < "magic mixture"[28] < DMSO, as indicated by the progressive line broading of the EPR spectra. In the

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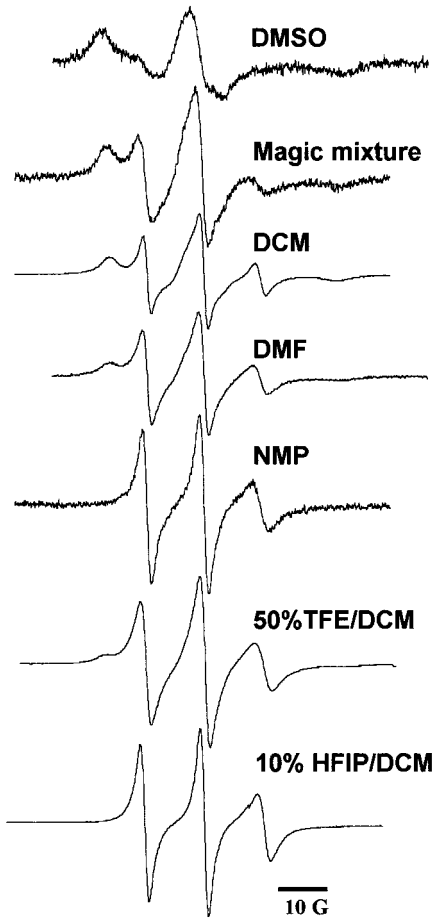

Figure 1. EPR spectra of VVLGAAIV−BHAR (14% peptide content) swollen in different solvents.

poorest solvated conditions (DMSO and the magic mixture), a very broad (powder) spectrum, typical of spin probe immobilization on the EPR time scale, is observed. The spectra of resin solvated in the magic mixture, DMF, DCM, and 50% TFE/DCM display two components, one with broad lines and the other with narrow lines, corresponding to strongly and weakly immobilized spin label populations, respectively. As the solvation increases, both the separation between the outer extreme for the broad line spectra (A_{max}[12], Figure 1) and the line width of the narrow line spectra (as monitored by the low- and high-field peaks in Figure 1) decrease, indicating an increasing degree of motion of both populations. In addition, the broad line component progressively disappears, being essentially undetectable in NMP and in 10% HFIP/DCM. The separations between the outer extremes in the spectra of the strongly immobilized population in Figure 1 are (DMSO) 64.0 G, ("magic mixture") 62.1 G, (DMF) 61.9 G, and (DCM) 60.6 G. The center field peaks in the spectra displaying two components contain the contribution of both populations, strongly and weakly immobilized. Nevertheless, it can be seen that, as the proportion of the weakly immobilized population increases, as well as the overall mobility, the line width of the center peak (W_0) decreases. The values of W_0 are given to the right of the spectra. The spectra in Figure 1 show that, although proposed[28] as an efficient chain-dissociation solvent system for so-called "difficult sequences", the magic mixture composed of DCM/DMF/NMP (1:1:1), 1% Triton, and 4 N ethylene carbonate was not capable of improving the solvation of the hydrophobic sequence used in this study. The results confirm the aggregating tendency of this sequence,[26] a higher chain mobility only being attained when the resin is swollen in NMP and in well-known β-sheet structure-disrupting solvent systems, such as mixtures of polyfluorinated alcohols (HFIP and TFE) and DCM.

Table 1. Correlation among Swelling Degree, Peak Line Width of the Central Field Resonance (W_0), and the Coupling Rate[a] on VVLGAAIV−BHAR (14% and 68% Peptide Content)

solvent	solvent volume within a bead[b] (%)	W_0 (G)	coupling yield[c] (%)
VVLGAAIV−BHAR (14% Peptide Content)			
DCM	67	2.6	51
DMF	63	3.0	45
DMSO	49	8.9	05
VVLGAAIV−BHAR (68% Peptide Content)			
DCM	39	8.8	06
DMF	34	7.6	02
DMSO	60	6.6	25
10% HFIP/DCM	72	4.1	27

[a] Coupling of Boc-Val. [b] [(Swollen volume − dry volume)/swollen volume] × 100. [c] Coupling yield after 15 min with the PSA method. Concentration of acylating components: 2.5×10^{-3} M (1.5 molar excess over the amine component for a 68% peptide content), 1.0×10^{-3} M (equimolar proportion to the amine component for a 14% peptide content).

Table 1 shows the correlation among the rate of the coupling reaction and the peptide-loaded resin degree of swelling and chain mobility estimated by the line width of the EPR central field peak in DCM, DMF, and DMSO. The yield of acylation using Boc-Val was measured because valine corresponds to the subsequent residue of the murine H-2K protein fragment under study.[26] It is noteworthy that the higher the swelling of resin beads (percentage of bead volume occupied by the solvent),[7] the narrower the EPR central field peak line width (W_0) and the faster the coupling reaction (DCM > DMF > DMSO). These data demonstrate that the efficiency of the solid-supported coupling reaction strongly depends on chain mobility, which is, in turn, affected by the swelling degree of the peptidyl-resin.

Table 1 also presents the swelling −W_0−coupling rate values measured for highly-peptide-loaded VVLGAAIV−BHAR (68% peptide content). The EPR spectra of this peptidyl-resin are given in ref 13. Chain mobility decreased in the order 10% HFIP < DMSO < NMP ≅ DMF ≅ DCM. When compared to the lowly-peptide-loaded resin, there is a clear inversion of solvation, strong chain immobilization being observed for the highly-peptide-loaded resin in DCM, whereas an increased rate of tumbling is seen in DMSO. These relevant spectral changes can be interpreted in terms of the dominant influence of the apolar polystyrene-1% divinylbenzene BHAR matrix in the lowly-peptide-loaded case, whereas the polar characteristics of the peptide, in particular, the peptide bond, tend to invert this trend as the amount of resin-bound peptide chains increases. The data in Table 1 for the heavily-VVLGAAIV-loaded resin confirm the correlation between the efficiency of the solid-supported coupling reaction and the rate of chain motion as determined by the degree of resin solvation. Much faster coupling was measured in DMSO and 10% HFIP/DCM than in the traditional solvent DCM or DMF. Table 1 shows that the rates of coupling were rather similar in

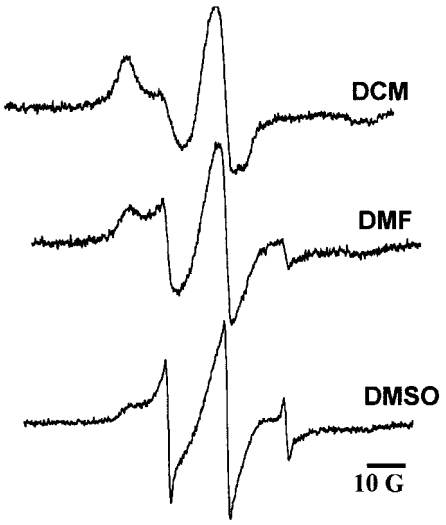

Figure 2. EPR spectra of AAIV−BHAR (53% peptide content) swollen in DCM, DMF, and DMSO.

Table 2. Correlation among Swelling Degree, Peak to Peak Line Width of the Central Field Resonance (W_0), and the Coupling Rate[a] on AAIV−BHAR (53% Peptide Content)

solvent	solvent volume within a bead[b] (%)	W_0 (G)	coupling yield[c] (%)
DCM	38	4.6	11
DMF	48	3.2	27
DMSO	72	1.6	49

[a] Coupling of Boc-Gly. [b] [(Swollen volume − dry volume)/swollen volume] × 100. [c] Coupling yield after 15 min with the PSA method. Acylating components in 2.5×10^{-3} M concentration and with 1.5 molar excess over the amine component of the peptidyl-resin.

DMSO and in 10% HFIP/DCM, although the chain mobility was clearly higher in the latter solvent. This result is likely due to partial consumption of the activator induced by the polyfluorinated alcohol during the acylating reaction.

The C-terminal tetrapeptide fragment of the VVLGAAIV sequence was also assembled in highly substituted BHAR and studied with regard to swelling degree, EPR spectra, and kinetics of coupling. The purpose of this investigation was to verify whether the assembly of a highly aggregating sequence coupled with the use of a highly substituted resin (53% peptide content) can facilitate chain association, usually reported to occur more frequently after coupling the fifth amino acid of the peptide sequence.[29] The EPR spectra of the AAIV-resin (Figure 2) indicated significant chain aggregation as evinced by the strongly immobilized spectrum in DCM. Much before coupling the fifth residue, the strong electron donor DMSO promoted higher solvation of the shorter sequence. The results in Table 2 confirm once more the direct relationship between coupling rate and chain solvation/mobility, previously demonstrated for the lowly- and highly-loaded VVLGAAIV-resins.

EPR spectra of the ING−BHAR with peptide contents of 6%, 16%, and 47% swollen in different solvents are shown in Figures 3−5, respectively. Spectral alterations

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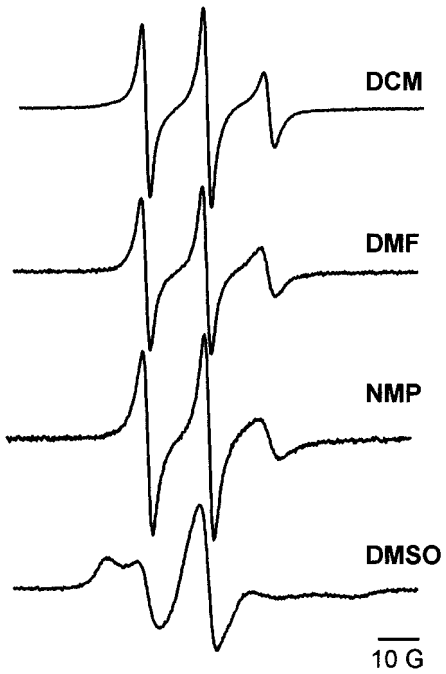

Figure 3. EPR spectra of ING−BHAR (6% peptide content) in DCM, DMF, NMP, and DMSO.

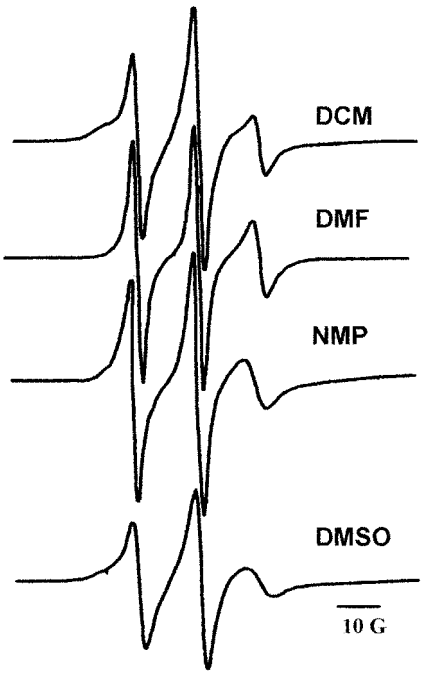

Figure 4. EPR spectra of ING−BHAR (16% peptide content) in DCM, DMF, NMP, and DMSO.

as a function of the amount of resin-bound peptide chains are observed when the polarity of the solvents is taken into account. The proposed (AN + DN) polarity scale[7] yields values of 21.4, 40.6, 42.6, and 49.1 for DCM, DMF, NMP, and DMSO, respectively. In accordance with this scale, the lowest-peptide-loaded (6%) ING−BHAR exhibited improved solvation and, therefore, chain mobility in DCM due to the dominant influence of the apolar polystyrene structure. In contrast, a powder spectrum was observed when the strong electron donor DMSO was used (Figure 3). This solvation tendency was completely inverted in the case of the highest-peptide-loaded ING-

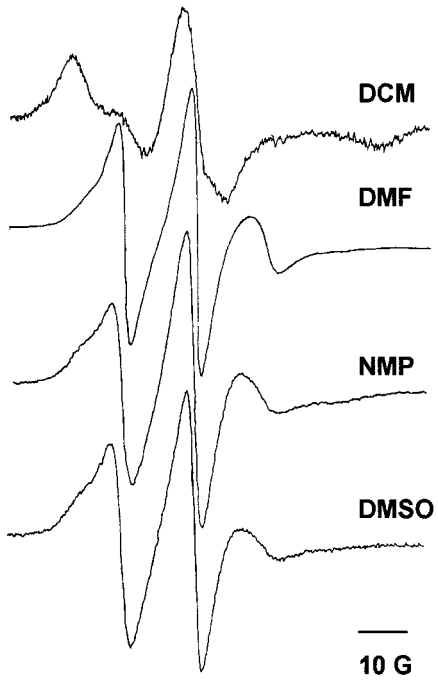

Figure 5. EPR spectra of ING−BHAR (47% peptide content) in DCM, DMF, NMP, and DMSO.

Table 3. Correlation among Swelling Degree, Solvent Viscosity, Peak Line Width of the Central Field Resonance (W_0), and the Coupling Rate Reaction[a] on ING−BHAR (6%, 16%, and 47% Peptide Contents)

solvent	solvent volume within a bead[b] (%)	W_0 (G)	viscosity (25 °C) (cP)	yield of coupling[c] (%)
	ING−BHAR (6% Peptide Content)			
DCM	77	1.9	0.400	86
DMF	70	2.3	0.796	68
NMP	73	3.0	1.666	45
DMSO	54	4.1	2.000	20
	ING−BHAR (16% Peptide Content)			
DCM	76	2.1	0.400	84
DMF	78	2.0	0.796	83
NMP	77	2.6	1.666	74
DMSO	78	2.9	2.000	46
	ING−BHAR (47% Peptide Content)			
DCM	31	*d*	0.400	01
DMF	81	3.3	0.796	38
NMP	83	3.8	1.666	28
DMSO	81	4.0	2.000	24

[a] Coupling of Boc-2BrZ-Tyr. [b] [(Swollen volume − dry volume)/swollen volume] × 100. [c] Coupling yield after 15 min with the PSA method. Concentration of acylating components: 2.5×10^{-3} M (1.5 molar excess over the amine component for 16% and 47% peptide contents), 1.0×10^{-3} M (equimolar proportion to the amine component for a 6% peptide content). [d] Powder spectra.

resin (Figure 5). Significant chain immobilization was detected for this peptidyl-resin in DCM, whereas the polar DMSO induced the best peptide chain solvation. The 16% peptide content ING-resin presented EPR spectra in the four solvents consistent with its intermediate loading (Figure 4).

Differently from the data for the VVLGAAIV and AAIV resin-bound sequences, those for the ING-resin (Table 3) reveal the influence of the solvent viscosity upon chain motion (as estimated by W_0 values) and, as a consequence, upon the observed coupling rate. This influence was noticed mainly for ING−BHARs of 16% and 47% peptide content, where equivalent swelling degrees in some solvents did not correlate with the values of W_0 and coupling rates. These findings indicate that, although some solvents may swell the resin to similar extent, the coupling is slower in the more viscous ones, emphasizing the importance of diffusional effects in polymer-supported reactions.[30]

It is also worth noting that in some circumstances it was possible to detect the influence of additional factors on the efficiency of the coupling reaction. For instance, the similar coupling rate observed in DCM and DMF for the 16% peptide content ING−BHAR (Table 3) cannot be explained solely by swelling and viscosity effects, as the resin swells equally in both solvents, and the former is less viscous than the latter. The slower coupling in DCM is probably due to the well-known inadequacy of this apolar solvent to dissociate strong peptide chain interactions[31] resulting from a large amount of interchain hydrogen bondings. This hypothesis is strengthened by a second spectral component due to a more immobilized chain population in the spectra in DCM (Figure 4).

In conclusion, the present work demonstrates the possibility of correlating EPR spectral parameters with factors that govern polymer-supported reactions during peptide synthesis. The acylation rate depends on peptide chain mobility, which is, in turn, influenced by a variety of factors such as the resin swelling degree, solvent viscosity, and solvent ability to disrupt aggregated peptide chains. The data showed that, under most of the conditions studied, aggregating sequences gave rise to composite EPR spectra. Under low loading conditions and in solvents (DMF, Figure 3) that promote disaggregation, only a single-component spectrum was observed. Moreover, we have previously shown[32] that Toac-labeled peptide-free BHAR also gives rise to one component EPR spectra, irrespective of the solvent system used.

The present data reveal the unique sensitivity of spin label EPR spectra to monitor polymer-supported processes and point to the great potential of this approach in the expanding area of the use of polymeric materials for a variety of chemical and biological purposes. Finally, in terms of peptide synthesis methodology, the results evince that EPR spectroscopy can be a valuable tool in the selection of the best solvation conditions to overcome extreme difficulties such as the synthesis of strongly aggregating sequences in highly-peptide-loaded solid supports.

Experimental Section

Materials. N^{α}-*tert*-Butyloxycarbonyl (Boc)-Asn, -Ile, -Gly, -Val, -Leu, and -Ala were purchased from Bachem, Torrance, CA. Benzhydrylamine-resins (BHARs) were synthesized as previously reported[25] to obtain highly substituted resin batches. Solvents and reagents were purchased from Aldrich or Sigma Chemical Co. DMF was distilled (over P_2O_5 and ninhydrin under reduced pressure) before use. All solvents used for swelling studies were HPLC grade, and all chemicals met ACS standards.

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Peptide Synthesis. The peptides were synthesized manually accordingly to the standard Merrifield Boc/Bzl strategy.[2a−c] Briefly, the α-amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and in 10% DIEA/DCM (10 min). The synthesis scale was 0.4 mmol, and all Boc-amino acids were coupled with TBTU[33] in the presence of HOBt and DIEA (at 3-, 3-, and 4-fold excess over the amino component in the resin, respectively), using DMF or 20% DMSO/NMP for ING or VVLGAAIV syntheses. After 2 h, the qualitative ninhydrin test was performed to estimate the completeness of the coupling reaction; the recoupling procedure was done when the ninhydrin test was positive. Cleavage reactions were carried out with the low−high HF procedure.[34] The resin was rinsed with ethyl acetate and the peptide extracted in 10% acetic acid aqueous solution and lyophilized. In addition to the expected theoretical yield, the purity of the crude peptides was determined by high-performance liquid chromatography (HPLC). The HPLC conditions were 0.1 M NaH_2PO_4 (pH 7.0, solvent A) and acetonitrile/H_2O (9:1, v/v, solvent B), linear gradient from 5% to 50% of B in 45 min, flow rate of 1.5 mL/min, and UV detection at 220 nm.

Measurement of Bead Swelling. Before use in peptide synthesis and microscopic measurement of bead size, all the amino-protonated BHAR batches (Cl^- form) were sized by being suspended in DCM and EtOH and being sifted in porous metal sieves to lower the standard deviations of resin diameter to about 4%. Swelling studies of these narrowly sized bead populations were performed and published elsewhere,[5] with minor modification in some calculated swelling parameters.[7] Briefly, 150−200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly at low magnification. Since the sizes in a sample of beads are not normally but log-normally distributed, the central value and the distribution of the particle diameters were estimated by the more accurate geometric mean values and geometric standard deviations. All resins were measured with the amino groups in the unprotonated form obtained by 3 × 5 min TEA/DCM/DMF (1:4.5:4.5, v/v/v) washings followed by 5 × 2 min DCM/DMF (1:1, v/v) and 5 × 2 min DCM washings. Resins were dried under vacuum using an Abderhalden-type apparatus and refluxed in MeOH.

EPR Studies. EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 SRC spectrometer at room temperature (22 ± 2 °C) using flat quartz cells. Labeled peptidyl-resins were preswollen overnight in the solvent under study before the spectra were run. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects.

Determination of Coupling Yields. A thermostatic reaction vessel (at 25 °C) containing 50−100 μmol of the amino component of the peptidyl-BHAR was submitted to the coupling reaction with the proper Boc-amino acid derivative using the preformed symmetrical anhydride method (PSA),[2a−c] generated in DCM at 0 °C (60 min). After this time, the reaction was stopped by filtration to remove the dicyclohexylurea precipitate, and the DCM filtrate was evaporated. The symmetrical anhydride generated was dissolved in the solvent under investigation and added to a reaction vessel containing peptidyl-resin preswollen in the same solvent. The coupling yield was monitored by the picric acid method;[35] each experiment was performed in duplicate.

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