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Study of the effect of the peptide loading and solvent system in SPPS by HRMAS-NMR

ANA P. VALENTE,[a] FÁBIO C. L. ALMEIDA,[a] CLOVIS R. NAKAIE,[b] SHIRLEY SCHREIER,[c] EDSON CRUSCA JR[d] and EDUARDO M. CILLI[d]*

[a] Department of Medical Biochemistry, Centro Nacional de Ressonância Magnética Nuclear Jiri Jonas, UFRJ, Rio de Janeiro, Brazil
[b] Department of Biophysics, UNIFESP, Rua 3 de Maio 100, CEP 04044-020, São Paulo, SP, Brazil
[c] Department of Biochemistry, Institute of Chemistry, USP, CEP 05599-970, São Paulo, SP, Brazil
[d] UNESP, São Paulo State University, Department of Biochemistry and Chemical Technology, Institute of Chemistry, CEP 14800-900, Araraquara, SP, Brazil

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Abstract: The SPPS methodology has continuously been investigated as a valuable model to monitor the solvation properties of polymeric materials. In this connection, the present work applied HRMAS-NMR spectroscopy to examine the dynamics of an aggregating peptide sequence attached to a resin core with varying peptide loading (up to 80%) and solvent system. Low and high substituted BHAR were used for assembling the VQAAIDYING sequence and some of its minor fragments. The HRMAS-NMR results were in agreement with the swelling of each resin, i.e. there was an improved resolution of resonance peaks in the better solvated conditions. Moreover, the peptide loading and the attached peptide sequence also affected the spectra. Strong peptide chain aggregation was observed mainly in highly peptide loaded resins when solvated in $CDCl_3$. Conversely, due to the better swelling of these highly loaded resins in DMSO, improved NMR spectra were acquired in this polar aprotic solvent, thus enabling the detection of relevant sequence-dependent conformational alterations. The more prominent aggregation was displayed by the VQAAIDYING segment and not by any of its intermediary fragments and these findings were also corroborated by EPR studies of these peptide-resins labelled properly with an amino acid-type spin probe.

Keywords: NMR; high-resolution magic angle spinning; solid-phase synthesis; resin; peptide

INTRODUCTION

Many spectroscopic methods have been used intensively to acquire relevant knowledge about the physicochemical factors that govern the complex solvation process of a polymer structure. Presently, focus has been given to the development of new drugs through the resin-supported combinatorial chemistry approach [1,2]. In this respect, solid phase peptide synthesis (SPPS) [3] has also continuously been selected as a target methodology for spectroscopic investigations, including CD [4], FTIR [5–7] and EPR [8–12]. In addition, NMR spectroscopy has been employed in this area [13–15] with emphasis on the high-resolution magic angle spinning (HRMAS-NMR) [16–19] technique applicable to the broad field of solid phase organic synthesis. The ability of this versatile spectroscopy has been valuable to obtain high quality NMR data on resin bound molecules including peptides [20–25] or even to compare the structural features of different polymeric materials [21,22,26]. Of these studies, only one [27] has dealt to date with the influence of the amount of peptide chain per resin bead.

The present investigation intended to extend the application of the HRMAS-NMR methodology to the physicochemical evaluation of the assembly of a very strongly aggregating peptide sequence attached to a polymeric support, but deliberately in a very high peptide loaded condition. To achieve this goal, batches of benzhydrylamine-resin (BHAR) [28] containing 0.3–3.0 mmol/g amine functions and synthesized in strictly controlled conditions [29] were used for assembling the aggregating VQAAIDYING sequence, corresponding to the (65–74) acyl carrier protein aggregating segment [30]. Variable peptide contents ranging from less than 10% to about 80% were obtained with these peptide-resins and the influence of the solvent system were also examined by comparing the solvating characteristics of the resin beads in $CDCl_3$ and DMSO-d_6 – different polar organic solvents.

Finally, EPR spectroscopy was also applied to help to monitor the dynamics of peptide chains inside the resin beads [8–10,12]. The paramagnetic labelling of peptide-resins was carried out with the paramagnetic amino acid-type spin probe TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) properly derived with the Fmoc-N^{α}-protecting group, which allows its covalent binding to any position in the peptide sequence [31].

EXPERIMENTAL

Materials

N^{α}-*tert*-butyloxycarbonyl (Boc)-amino acids were purchased from Bachem, Torrance, CA. Benzhydrylamine-resins (BHAR)

*Correspondence to: E. M. Cilli, Department of Biochemistry and Chemical Technology, Rua Prof. Franscisco Degni, S/N-Bairro Quitandinha, Araraquara, SP, Brazil CEP 14800-900;
e-mail: cilli@iq.unesp.br

were synthesized as previously reported [29] 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 the swelling studies were HPLC grade and the chemicals met ACS standards.

Peptide Synthesis

The VQAAIDYING sequence was synthesized manually starting from different BHAR batches following the standard Merrifield Boc/Bzl strategy [32–34]. Cyclohexyl-(OcHex) and 2-Br-carbobenzoxyl- (2-BrZ) side chain protecting groups were used for Asp and Tyr residues, respectively. 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 (2-(1-H-benzotriazole-1-yl)1,1,3,3-tetramethyl-uroniumtetrafluoroborate) in the presence of HOBt (hydroxybenzotriazole) and DIEA (diisopropylethyl-amine) at a 3, 3 and 4 fold excess over the amino component in the resin, respectively, using DMF or 20% DMSO/NMP. After a 2 h coupling time, the qualitative ninhydrin test was performed to estimate the completeness of the reaction and the re-coupling procedure was done when the ninhydrin test was positive. Cleavage reactions were carried out with the low-high HF procedure. The resin was rinsed with ethyl acetate and the peptide extracted in 5% acetic acid aqueous solution and lyophilized. In addition to the expected theoretical yield, the purity of the crude peptides was determined by HPLC. The HPLC conditions were: 0.1 M NaH_2PO_4, pH 7.0 (solvent A) and acetonitrile:H_2O (9 : 1, v/v, as solvent B); a linear gradient from 5% to 50% of B in 45 min, flow rate of 1.5 ml/min, UV detection at 220 nm.

Measurement of Bead Swelling

Before being used in the peptide synthesis and the bead size microscopic measurement, all the amino protonated BHARs batches (Cl^- form) were sized by suspending in DCM, EtOH, and sifting in porous metal sieves to lower the standard deviations of the resin diameters to about 4%. 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), followed by 5×2 min DCM/DMF (1 : 1, v/v) and 5×2 min DCM washing. After this treatment the resins were dried under vacuum until constant weight.

NMR Spectroscopy

All spectra were recorded on a Bruker DRX 400 MHz spectrometer equipped with a 4 mm $^1H/^{13}C/^{15}N$ triple resonance MAS probe head. The experiments were done without lock. Resin containing rotors were rotated at 5000 Hz. The chemical shifts for ^{1}HRMAS NMR are reported relative to water external standard.

EPR Studies

EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 SRC spectrometer at room temperature ($22^\circ \pm 2\,^\circ C$) using flat quartz cells. Labelled peptidyl-resins were pre-swollen overnight in the solvent under study before running the spectra. 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.

RESULTS AND DISCUSSION

BHAR batches with 0.3, 1.6 and 2.6 mmol/g amine group substitution degrees were used to synthesize the VQAAIDYING sequence. A portion of peptide-resin was removed at intermediary positions ING, DYING, AIDYING for further swelling and spectroscopic studies. Peptide contents ranging from 6% to about 80% (weight/weight) were determined by amino acid analysis of the peptide-resins. To ascertain the homogeneity of each sequence synthesized, a small portion of the corresponding peptide-resin was cleaved and the purity of the crude peptides, estimated by analytical HPLC, was ca. 85%. The results from amino acid analysis and mass spectra were also consistent with the expected peptide sequences.

Swelling of Resins

In order to facilitate the interpretation of the resin HRMAS-NMR spectral findings, comparative swelling data of BHAR or peptide-BHAR in DCM and in the polar aprotic solvent DMSO were initially determined and are displayed in Table 1. As a replacement for DCM, more commonly used in peptide synthesis methodology, $CDCl_3$ was considered as a solvent in this study, assuming that both solvents will solvate equally the resin beads. This prediction was based on the similarity of the polarity values of both solvents regardless of the scale chosen. Values of 40.7 and 39.1 or 21.4 and 27.1 for DCM and $CHCl_3$, respectively, were found in the literature when the Dimroth-Reichard ET30 [35] or the (AN + DN) amphoteric polarity [36,37] scales were considered, respectively.

In close accordance with these earlier polymer solvation studies [36,37], both BHAR batches displayed enhanced solvation in the less polar DCM as a consequence of the dominant influence of polystyrene backbone. Conversely, when attaching a significant amount of the more polar peptide sequences the very polar DMSO turns out to be the more appropriate solvent for solvating this peptide-resin (see swelling degrees of VQAAIDYING-BHARs in Table 1). It is also possible to verify that this alteration in the swelling

Table 1 Swelling Degree of Resins

Resin	Diam. dry bead (μm)	DCM		DMSO	
		Diam. swollen bead (μm)	Percentage of solvent within bead (%)[a]	Diam. swollen bead (μm)	Percentage of solvent within bead (%)[a]
BHAR 0.3 mmol/g	61	119	87	78	53
ING	56	111	87	82	68
DYING	63	99	74	100	75
AIDYING	67	79	39	98	69
VQAAIDYING	68	89	56	101	70
BHAR 3.0 mmol/g	79	144	84	109	62
ING	82	99	43	142	80
DYING	97	129	57	148	72
AIDYING	109	135	47	156	66
VQAAIDYING	116	145	49	173	70

[a] [(swollen volume − dry volume)/swollen volume] ×100.

behaviour of the resin beads is typically dependent on the degree of peptide loading.

Peptide-free Resins

HRMAS-NMR spectra were initially acquired solely for the 0.3 and 3.0 mmol/g BHAR batches not attaching the peptide sequences. Figure 1 displays the results determined for these two resins swollen in $CDCl_3$ and in DMSO-d^6. 1D and T2 filtered spectra after 20 ms of CPMG [38] are shown in panels A and B (Figure 1), respectively, for both BHAR batches. Due certainly to the lack of swelling of both BHAR batches in DMSO (Table 1), typical signals at 6–7 ppm assigned to polystyrene moieties were more clearly visible. These results confirm, as previously described in the literature [14,24,39], the importance of good solvation to obtain better quality HRMAS-NMR spectra. Worthy of note, was the direct relationship observed between the intensity of a peak appearing at about 7.2 ppm, usually attributed to amine functions, and the amount of this basic group of each resin. This finding suggests that this procedure might be considered as an alternative analytical protocol to estimate the substitution degree of BHAR-type resins by means of NMR experiments.

The T2 filtered spectra after 20 ms of CPMG usually shows only the most flexible components of the spectra [38]. Accordingly, the comparative spectra displayed in panels B (Figure 1) reveal more prominent and sharp peaks in the 6–7 ppm region only when in the improved solvating condition of the polymer matrices (both BHAR in $CDCl_3$, panels B1 and B2). In comparison, a less pronounced peak was observed with the 3.0 mmol/g BHAR batch in DMSO-d^6 (panel B4). On the other hand, owing to the lack of solvation observed for 0.3 mmol/g BHAR in DMSO (53% swelling degree – Table 1), no significant resonance signal at 6–7 ppm was detected in panel B3.

Peptide-Resins

In the following steps, an investigation of the influence of the peptide sequence and loading was initiated. As a model, the intermediary ING sequence assembled in 0.3, 1.6 and 3.0 mmol/g BHAR (6%, 30% and 50% peptide content, respectively) was examined by ^{1}H-NMR. Figure 2 displays the comparative spectra of these peptidyl-resins in $CDCl_3$ and in DMSO-d^6. In agreement with the variation of swelling degrees of these peptide-resins in both solvating conditions (Table 1), a clear spectral change was detected as the amount of polar peptide chains increased in the solid support. The higher the peptide content of the resin, the better was the ^{1}H-NMR peak resolution in the more polar medium (DMSO). As already emphasized, the dominant influence of the apolar polystyrene groups in the low peptide loaded resin (0.3 mmol/g BHAR) was strongly altered when higher amounts of polar peptide chains were attached to the polymer backbone (1.6 and 3.0 mmol/g BHAR). Accordingly, the greater chain mobility indicated by the appearance of well-defined peaks in the 6–8 ppm range (Figure 2) was only observed for the low peptide loaded ING-BHAR (0.3 mmol/g) when in $CDCl_3$, or in DMSO-d^6, for the two more heavily loaded resins (30% and 50% peptide contents). The inadequacy of DCM or $CHCl_3$ solvents to disrupt the strongly associated peptide chains inside the resin beads (maintained by a large amount of inter-chain hydrogen bonding) is well documented and is clearly visible in the immobilized spectra shown in panels B_1 and C_1 of Figure 2.

Alternatively, the difference in the NMR spectral quality of these peptide-resins can be examined by making use of the quality spectrum. In this case, the number of peaks, line shape and peak intensity of each spectrum were examined by looking at both the peptide and polymer resonances. Emphasizing again

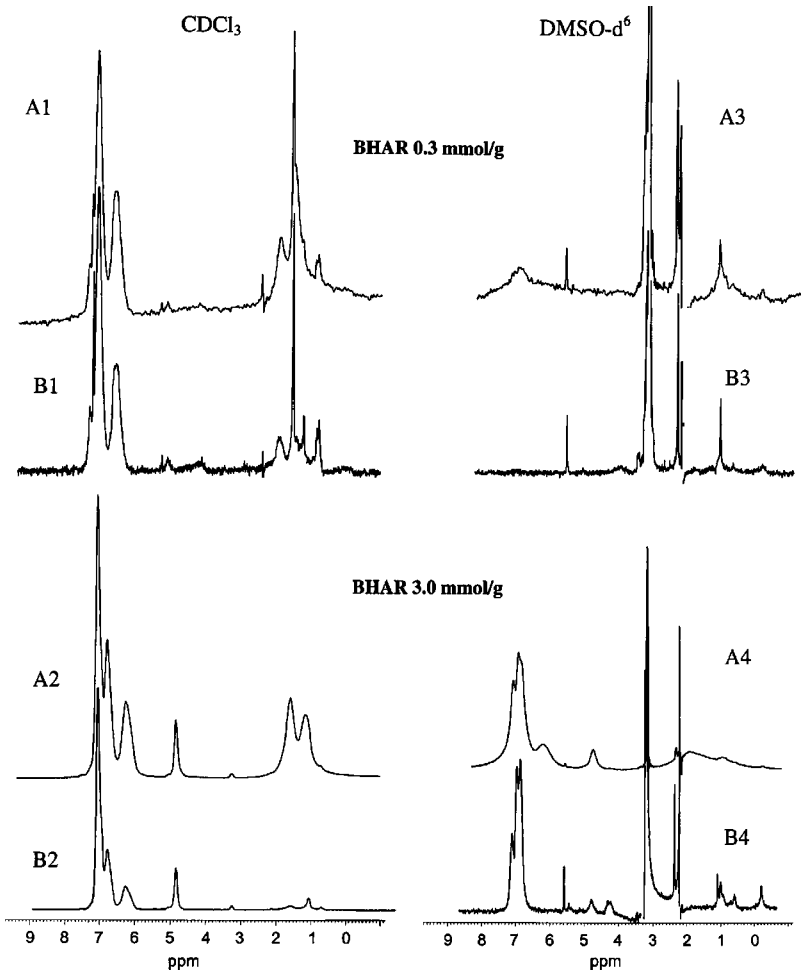

Figure 1 HRMAS NMR ^{1}H spectra (A) and T2 filtered spectra after 20 ms of CPMG (B): A1 and B1, BHAR with 0.3 mmol/g in $CDCl_3$; A2 and B2, BHAR with 3.0 mmol/g in $CDCl_3$; A3 and B3, BHAR with 0.3 mmol/g in DMSO-d^6; A4 and B4, BHAR with 3.0 mmol/g in DMSO-d6. Spectra were recorded at 400 MHz in a 4 mm rotor with ≈5 mg of resin spinning at the magic angle at 5 KHz.

the relevance of the solvation degree of the beads to the NMR evaluation of resins, a better quality of NMR spectra was only observed in $CDCl_3$ and in DMSO-d^6 for lower and heavier peptide loaded resins, respectively.

In addition, a significant decrease in the quality degree was detected when the peptide content of the ING aggregating sequence increased from 30% to 50% (1.6 and 3.0 mmol/g BHAR, respectively), thus suggesting the existence of a limit for the capacity of disruption of peptide chain aggregates, even when using a very strong electron donor solvent such as DMSO. Certainly, this limit will depend upon the peptide sequence and loading, the resin structure and the solvent system.

Protonated Peptide Resin

Relevant peptide conformational features may be obtained by altering the α-amine group ionization state, even when bound to a polymeric matrix as occurs during the solid phase peptide synthesis method [32,33]. These changes carry to the introduction of *in situ* neutralization protocols, mainly in Boc chemistry [40,41]. This method gives a significant increase in the efficiency of chain assembly, especially for 'difficult' sequences arising from sequence-dependent peptide chain aggregation, in comparison with standard protocols (neutralization prior to coupling).

To check the influence of the *N*-terminal form (unprotonated or protonated with trifluoroacetate) in the aggregation of the peptide, the ING peptide sequence bound to BHAR of 3.0 mmol/g was examined by ^{1}H-NMR HRMAS.

Figure 3 displays the spectra of 1H-NMR (A) and T2 filtered HRMAS NMR 1D (B) of protonated ING-BHAR of 3.0 mmol/g in DMSO-d^6. When the tripeptide ING was in the protonated form it displayed a better quality spectral degree, mainly in the amide proton region, corresponding to the three amino acids, when compared with the unprotonated form (Figure 2C2). On the other hand, the swelling and EPR mobility did not follow the spectral quality obtained by NMR. In this resin, the swelling was 82% in the unprotonated and 83%

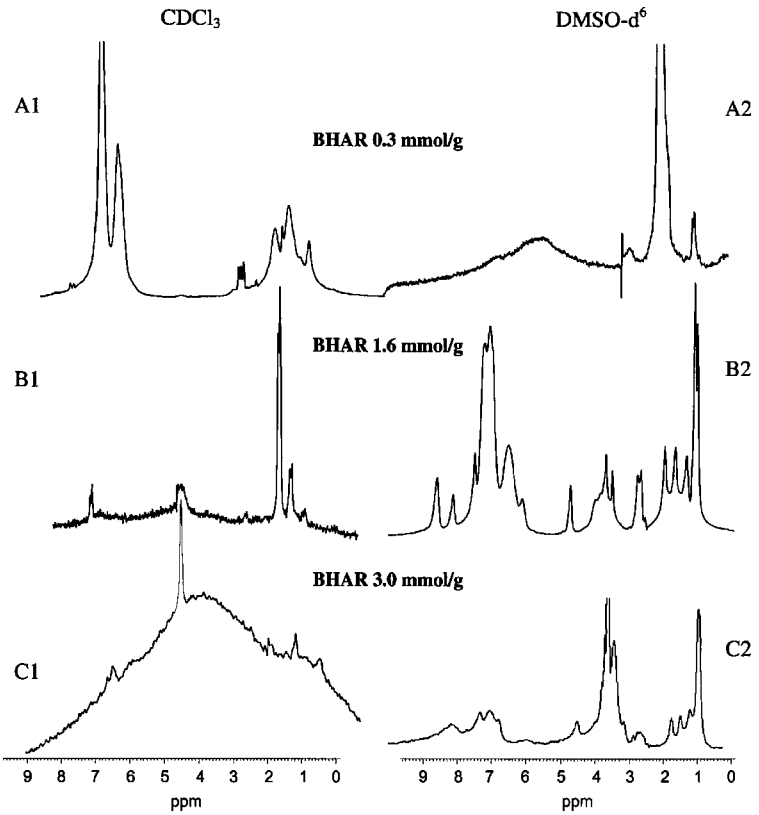

Figure 2 HRMAS NMR ^{1}H spectra of ING-BHAR with 0.3 (A), 1.6 (B) and 3.0 (C) mmol/g in $CDCl_3$ (1) and DMSO-d^6 (2). Spectra were recorded at 400 MHz in a 4 mm rotor with ≈5 mg of resin spinning at the magic angle at 5 KHz.

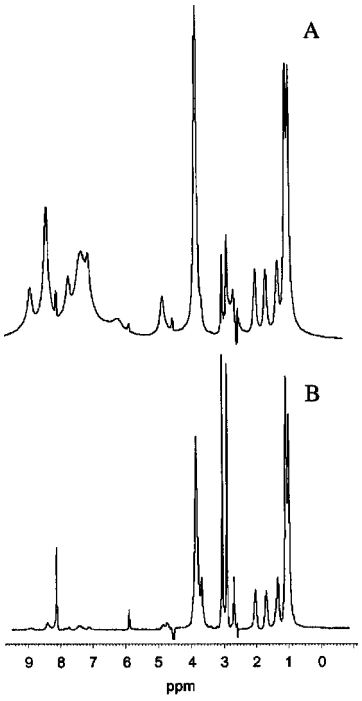

Figure 3 HRMAS NMR ^{1}H spectra (A) and T2 filtered spectra after 20 ms of CPMG (B) of ING-BHAR of 3.0 mmol/g in DMSO-d^6 (amine groups in protonated form). Spectra were recorded at 400 MHz in a 4 mm rotor with ≈5 mg of resin spinning at the magic angle at 5 KHz.

in the trifluoroacetate form. For the EPR studies, the mobility estimated by the central peak line width (W_0), which reflects the degree of motion where the probe is located, was the same (3.0 G). This failure is attributed to the greater sensitivity of the NMR technique to find change conformations in the whole peptide, while the EPR shows only the mobility, in this sequence, of the *N*-terminal group.

Monitoring the synthesis of VQAAIDYING in highly loaded conditions

To analyse the viability of peptide syntheses in highly substituted conditions (3.0 mmol/g), the solvation study was extended specifically in DMSO to several minor fragments. Emphasis was given to this challenging protocol, because the synthesis in heavily peptide loaded condition can achieve less consumption of solvents and in time to obtain larger amount of peptide. Even in terms of application for other approaches such as for a peptide library, a greater number of peptide chains per bead would be essential for helping to characterize the amino acid sequences of assembled peptides or for detecting more easily the interaction processes of enzymes with peptide segments.

NMR study. The ^{1}H NMR spectra of ING, DYING, AIDYING and VQAAIDYING bound to highly substituted

3.0 mmol/g BHAR and solvated in DMSO are represented in Figure 4. Due to the occurrence of strong shrinking of the beads in apolar solvents (Table 1), the NMR experiments were not carried out in this type of solvent system (ex. $CDCl_3$). Despite the similarity of swelling of the four peptide-resins in DMSO (Table 1), the ^{1}H-NMR or T2 filtered HRMAS NMR 1D spectra (columns I or II, Figure 4) showed a significant difference between these highly peptide loaded resins. The degree of mobility of the peptide chains inside the resin matrix seems to be clearly sequence dependent and certainly aggravated by the high peptide content inside the resin bead. The stronger immobilization was observed in the complete VQAAIDYING segment (Figure 4D), The greater number of well-resolved background signals in the aromatic and aliphatic regions of the spectra, besides peaks in the 7–9 ppm region, could be attributed to chemical shifts of different hydrogen from the secondary structure under investigation [27]. The smallest sequence (ING) has numerous background signals in the aromatic and aliphatic regions of the spectra and provides good-quality spectra. Of note, a greater motion degree of the peptide-resin backbone was detected more clearly for the intermediary AIDYING sequence, thus stressing again the sensitivity of the NMR spectra to detect the sequence-dependent occurrence of peptide chain associations throughout the polymer matrix.

EPR study. Paralleling the NMR investigation, EPR spectroscopy was also applied specifically to the challenging condition of synthesized aggregating sequence in highly substituted resins. Thus, ING, AIDYING and VQAAIDYING segments assembled in the 3.0 mmol/g BHAR were labelled at their *N*-terminal portion with the Fmoc-TOAC amino acid-type probe [31], as previously described in the literature [8,9]. In order to avoid a spin-spin exchange interaction which broadens the EPR lines [42], the paramagnetic labelling was kept as low as possible [8,9] and quantified by the modified picric acid methodology [43] before

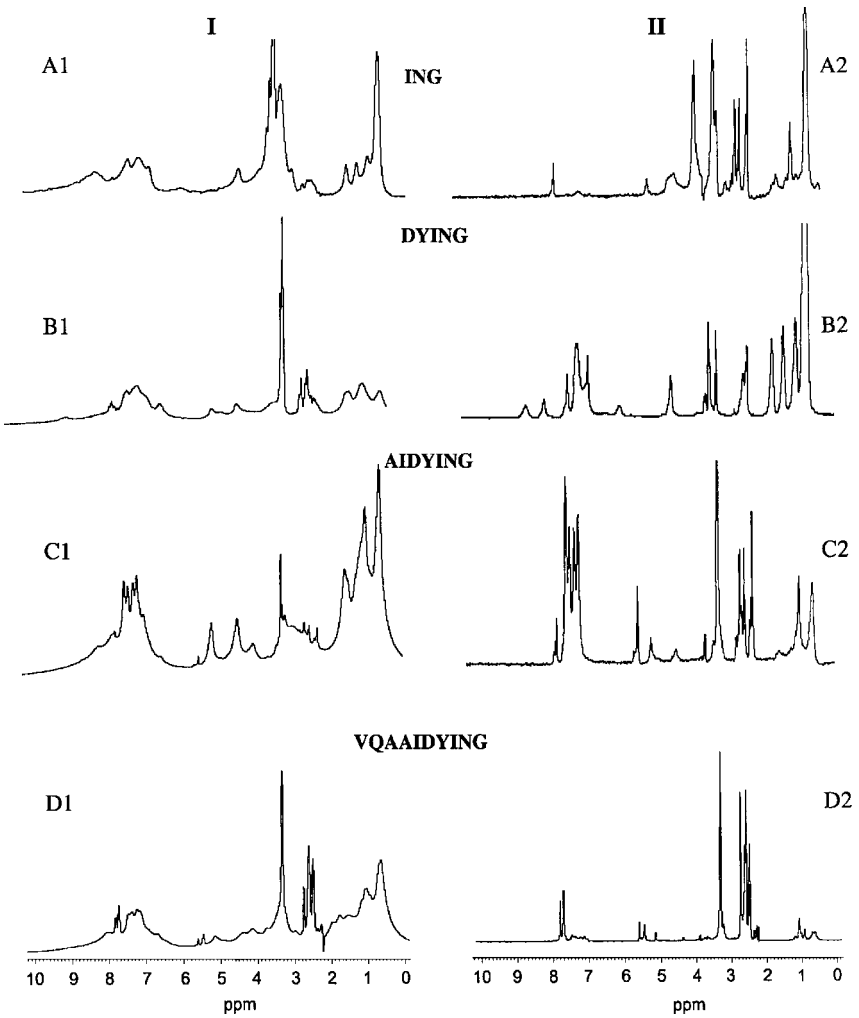

Figure 4 HRMAS NMR ^{1}H spectra (column I) and T2 filtered spectra after 20 ms of CPMG (column II) of ING, DYING, AIDYING and VQAAIDYING fragments bound to 3.0 mmol BHAR and swollen in DMSO-d^6. Spectra were recorded at 400 MHz in a 4 mm rotor with ≈5 mg of resin spinning at the magic angle at 5 KHz.

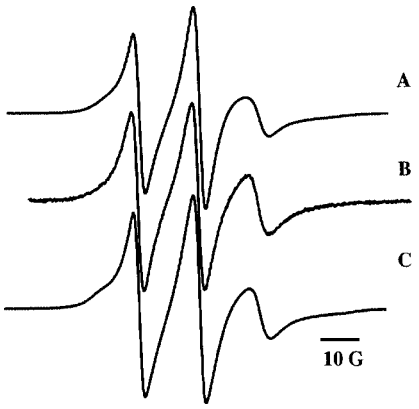

Figure 5 EPR spectra of A (ING), B (AIDYING) and C (VQAAIDYING) bound to 3.0 mmol/g BHAR in DMSO.

and after coupling the Fmoc-TOAC spin label. Moreover, the low labelling protocol allows the physicochemical and steric perturbations due to the introduction of the spin probe to be kept to a minimum, decreasing their influence on the solvation characteristics of the peptidyl-resin under investigation.

Figure 5 displays the EPR spectra of the three peptide-resins. By measuring the mid-field line width (W_o) of the spectra – the greater the W_0 values, the more immobilized is the position where the spin probe is attached – values of 3.0, 3.0 and 3.1 G were measured for ING- AIDYING- and VQAAIDYING-BHAR (3.0 mmol/g) resins, respectively. These results depicted that a more aggregated condition is detected with the octapeptide sequence, in agreement with previous NMR investigation.

CONCLUSION

These findings reinforced the validity of examining the dynamics of solvated polymeric structures using spectroscopic approaches, mainly by the sensitive HRMAS-NMR technique. The combination of this method with EPR seems to be an efficient approach to investigate clusters of microenvironments spread throughout the peptide-polymer backbone, thus helping to overcome the severe synthesis conditions such as those deliberately employed in the present work (synthesis of strong aggregating sequence in very high peptide loading condition).

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