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Use of spin label EPR spectra to monitor peptide chain aggregation inside resin beads.

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ABSTRACT : An EPR approach to monitor peptide chain aggregation inside resin beads is introduced. Model low and highly peptide-loaded resins containing an aggregating sequence were labeled with a paramagnetic amino acid derivative and studied with regard to their solvation behavior in different solvent systems. For the first time in the peptide synthesis, EPR spectroscopic has allowed the detection of differentiated levels of peptide chain aggregation as a function of solvent and resin loading. © 1997, Elsevier Science Ltd. All rights reserved.

Similarly to other polymer-supported techniques, the success of the solid phase peptide synthesis (SPPS)[1-4] is highly dependent upon the solvation degree of growing peptide chains throughout the resin matrix. Since optimized solvation may indicate greater accessibility of the peptide reactive amino group to coupling reagents, various spectroscopic approaches such as FT-IR [5,6] and NMR [7,8] have been applied to investigate factors that affect the complex solvation properties of peptide-resins. Surprisingly, the use of the EPR method which provides direct information concerning the chain mobility and therefore, the degree of freedom of spin labeled sites in the peptide-resin, has been restricted to one paper published two decades ago.[9] In this communication, alterations in the swelling degree of resin were revealed in the EPR spectra as a consequence of the introduction of a novel linker for peptide synthesis. In order to evaluate the potentiality of EPR spectroscopy in the study of peptide synthesis, we have performed studies aiming at monitoring peptide chain aggregation inside resin beads. The synthesis of so-called aggregating sequences still remains as a serious shortcoming in the SPPS and additional physicochemical information regarding the solvation properties of peptide-resins should be of value for further improvement of the method.

EPR spectra are strongly dependent on the labeling strategy. Ideally, one needs a paramagnetic compound rigidly attached to the peptide backbone to evaluate appropriately the conformational constraints of peptide chains inside resin beads. The stable free radical Toac, (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid), protected either with the tert-butyloxycarbonyl (Boc)[10] or with the 9-fluorenylmethyloxycarbonyl (Fmoc)[11] groups at its amino function for peptide synthesis, meets this criterion. By using the strategy introduced in our laboratory,[11] this paramagnetic compound may be inserted in a peptide sequence as a common amino acid and has been recently used for conformational studies of peptides, either free in solution[12,13] or bound to membranes.[14]

The well-known resin-bound VVLGAAIV aggregating sequence corresponding to the 291-298 fragment of the murine H-2K protein[15] was synthesized manually according to the standard Boc/benzyl strategy[1,2], using low and highly (0.2 mmol/g and 2.6 mmol/g, respectively) substituted batches of previously synthesized[16] benzhydrylamine-resin (BHAR). The scale of each synthesis was 0.2 mmol and all Boc-amino acids were coupled in 20% dimethylsulfoxide (DMSO)/ N-methylpyrrolidinone (NMP) with [benzotriazole-1-yl-oxy-tris-(dimethylamine)-phosphonium hexafluorophosphate] (BOP), N-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIEA) in

a 4:4:5 fold excesses over the amino component of the resin. A qualitative ninhydrin test was performed to estimate the completeness of the reaction. The recoupling procedure was carried out when the ninhydrin test was positive. The crude peptides obtained after anhydrous HF treatment were *ca.* 90 % pure in both syntheses, as estimated by analytical HPLC. The mass spectra and amino acid analyses were consistent with the theoretical peptide sequence. The reason for the use of low and highly substituted resins was to obtain beads displaying variable degrees of peptide chain aggregation resulting from the different amount of the amino group sites in the resins. A very high (68 %, weight/weight) peptide content was obtained with the 2.6 mmol/g BHAR whereas this value decreased to 6% when the low substituted (0.2 mmol/g) BHAR was used.

The Fmoc-Toac labeling of both peptide-resins at their N-terminal portion was kept as low as possible to avoid spin-spin exchange which may interfere with EPR line shapes.[17] Labeling of not higher than 5% of the total amino groups was achieved under controlled coupling conditions monitored by picric acid determination[18]. In addition, low labeling allows to keep the physicochemical perturbation by Toac at a minimum, reducing its influence on the behavior of the peptide sequence under examination, especially its overall solvation properties. It is assumed that the Toac-labeled peptide chains are dispersed homogeneously throughout the resin matrix and behave similarly to the resin-bound unlabeled chains in all solvent systems. Figure 1 depicts the structure of the labeled peptide-resin with the Fmoc-Toac moiety. EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer at room temperature (22 ± 2°C) using flat quartz cells. Labeled peptide-resins were pre-swollen in the solvent under study for 5 h 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.

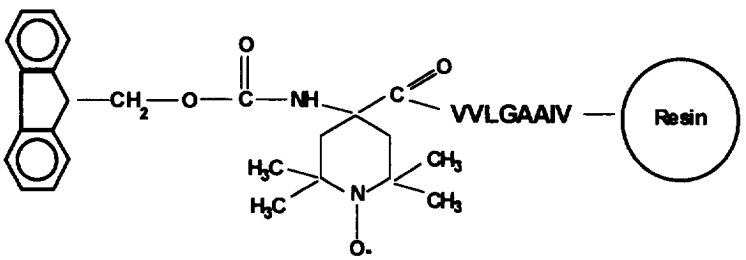

Figure 1: Structure of Fmoc-Toac-VVLGAAIV-BHAR.

Figure 2 displays the EPR spectra of highly and low peptide-loaded VVLGAAIV-BHAR swollen in various solvent systems. A clear differentiation in the solvation properties of the two resins is observed which, in turn, depends on the peptide loading and the solvent used. In the case of the highly peptide-loaded sample (Figure 2A), the strongest chain immobilization is seen when the beads were swollen in dichloromethane (DCM) whereas the fastest tumbling of resin-bound chains is observed in 10% hexafluoroisopropanol (HFIP)/DCM, considered a strong β-sheet structure-disrupting solvent.[5] In the very poorly solvated condition (DCM), a broad (powder) spectrum, typical of probe immobilization in the time scale of the experiment is observed. As the beads become more solvated [dimethylformamide (DMF), N-methylpiperidinone (NMP)] a second spectral component appears, displaying a narrower lineshape, characteristic of higher mobility. The contribution of this spectrum increases with increasing solvation, becoming the predominant component in 10% HFIP/DCM. It is noteworthy that the increasing chain mobility revealed by the EPR spectra of highly peptide-loaded resin (Figure 2A) followed exactly the order of decreasing solubility previously measured for this octapeptide sequence free in solution [10 % HFIP/DCM > DMSO > NMP ≅ DMF ≅ DCM].[15] These findings confirm the expected dominating influence of peptide chain properties over the apolar polystyrene resin matrix in the heavily loaded condition (68% peptide-content). Moreover, and relevant for peptide synthesis, the EPR spectra in Figure 2A

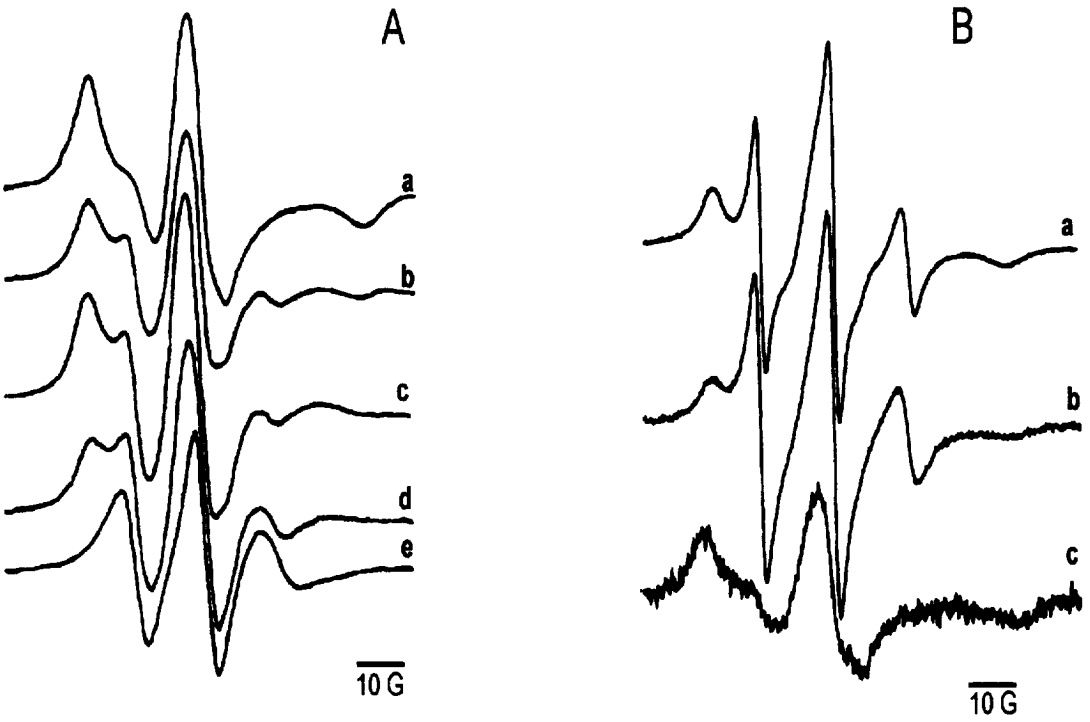

Figure 2 : EPR spectra of VVLGAAIV-BHAR : [A] highly-loaded, in (a) DCM; (b) DMF; (c) NMP ; (d) DMSO ; (e) 10% HFIP/DCM. [B] low-loaded, in (a) DCM; (b) DMF; (c) DMSO.

demonstrate that it is possible to find adequate solvent systems even in very drastic synthesis situations (resin containing a strongly aggregating sequence and in a heavily loaded condition).

In contrast, Figure 2B reveals a distinct solvation behavior of resin beads in the low peptide-loaded condition. The EPR spectra display a narrow component, due to higher peptide chain mobility in DCM and DMF; this component disappears in the more polar aprotic solvent DMSO, where only a powder spectrum is observed. These results are in agreement with the dominant influence of the hydrophobic polystyrene structure over that of the peptide chain in low peptide-loading condition. Interestingly, even in this condition, when the peptide chains are more dispersed inside the polymeric matrix, it is possible to visualize a small amount of the more immobilized chain population in the EPR spectra (Figure 2B), thus demonstrating the high sensitivity of the present approach to detect different interchain association tendencies during peptide synthesis.

The importance of the solvation degree for optimized synthesis conditions has been recognized for polymer-supported reactions. Accordingly, the kinetics of the coupling reaction in the two VVLGAAIV-containing resins used in the present report have been already evaluated[19] and the measured rate of reaction, regardless of the solvent, followed closely the chain mobility levels of resins estimated from the EPR spectra in Figure 2.

In conclusion, the present report allowed for the first time, the establishment of a unique and sensitive procedure making use of EPR spectroscopy to monitor chain-chain association inside a solvated polymeric matrix. And, differently from NMR or FT-IR spectra which reflect the solvation of the whole peptide chain in the bead, the EPR method allows the estimation of the peptide chain

aggregation/accessibility in the N-terminal region, where the extent of steric hindrance is critical for the success of the synthesis.

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