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EPR investigation of the influence of side chain protecting groups on peptide–resin solvation of the Asx and Glx model containing peptides

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Abstract—In spite of all progressive efforts aiming to optimize SPPS, serious problems mainly affecting the assembly of aggregating sequences have persisted. Following the study intended to unravel the complex solvation phenomenon of peptide–resin beads, the XING and XAAAA model aggregating segments were labeled with a paramagnetic probe and studied via EPR spectroscopy. Low and high substituted resins were also comparatively used, with the X residue being Asx or Glx containing the main protecting groups used in the SPPS. Notably, the *cyclo*-hexyl group used for Asp and Glu residues in Boc-chemistry induced greater chain immobilization than its *tert*-butyl partner-protecting group of the Fmoc strategy. Otherwise, the most impressive peptide chain immobilization occurred when the large trytil group was used for Asn and Gln protection in Fmoc-chemistry. These surprising results thus seem to stress the possibility of the relevant influence of the amino-acid side chain protecting groups in the overall peptide synthesis yield.

After the seminar proposition of the solid-phase peptide synthesis (SPPS) method in the literature,[1] this innovative approach still suffers from some drawbacks related mainly to the difficulty in achieving complete incorporation of amino acid residues in peptide chain elongation.[2] This usually occurs during the synthesis of particular sequences characterized by presenting a tendency of strong chain association. To overcome this type of problem, which is in most cases sequence-dependent, innumerable studies have appeared in the last few decades aiming at improving the SPPS methodology. These efforts comprised the use of more reactive coupling reagents,[3,4] elevated temperature,[5] alternative solid supports[6,7] and microwave radiation to optimize amino-acid coupling reactions.[8,9] Nevertheless, a significant part of these efforts have failed to give us a better understanding of the physicochemical features of the complex peptide–resins in solvated state. Neither have they helped us discover approaches or strategies that might help overcome specific chain growth difficulties. In this context, many reports have applied the classical procedure of measuring the swelling volume of resins in different solvent systems.[10–12] However, the majority of works have indeed used spectroscopic methods such as IR,[13,14] NMR,[15–17] fluorescence and EPR.[18] In our case, the peptide resin swelling determination was also the first approach tested to better understand the peptide–resin solvation. But in this context, this process is considered a type of solvent effect investigation and the solute is a collection of model peptide–resins. Thus, by handling a dozen peptide–resins and solvent systems differentiated by parameters such as their polarity or acid/base properties, it has been possible to propose some rules that seem to govern peptide–polymer solvation.[19,20] In addition, this investigation also allowed the proposition of a novel and dimensionless solvent polarity scale, which proved to be more practical and sensitive than all those existing to date in the literature.[20] As a continuation of this effort, we started a different strategy where the EPR method was conjugated with the use of the stable free radical TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid)[21] derived from

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its amino group with the *tert*-butyloxicarbonyl (Boc)[22] or 9-fluorenylmethyloxycarbonyl (Fmoc).[23] These groups temporarily protect the TOAC, thus allowing its coupling to peptide sequences in solution or attached to polymers and even to other active group-containing macromolecules or systems. The goal in this case was to determine the mobility of peptide chains in different conditions and make a correlation to the efficiency of coupling reaction in the synthesis.

The latter strategy fortunately has led us to obtain some important information in respect to peptide–resin characteristics, comprising some rules of peptide chain solvation and detection of strong aggregated sequences[24,25] and the effect of an increase in temperature.[26] More recently, a real-time monitoring of the coupling reaction in the resin[27] or calculation strategy of unusual polymer parameters was also demonstrated. Examples of this are the number of sites per bead, the concentration and the distance existing between these sites when in the solvated state.[28] Lastly and of great relevance to the present Letter, it was possible to confirm a direct relationship existing between solvation degree of the bead and chain mobility with the rate of coupling reactions.[25,28,29]

Thus, taking into account all the progress achieved to date in the understanding of the solvation phenomenon during the peptide chain assembly, we now decided to start evaluating the influence of the amino-acid side chain protecting groups in the overall solvation degree of peptide–resins. This effect has not yet been evaluated and we decided to combine for this purpose, the use of the EPR method and the TOAC labeling strategy. To maximize possible problems for appropriate solvation of peptide–resins, two well-known aggregating peptide sequences were selected. For the EPR experiments, the strategy already applied[25] was selected and, the higher the central field peak line-width (W_0) values, the greater the immobilization of the labeled peptide chains.

The ING (65–74) fragment of the acyl carrier protein[30] and polyalanine AAAA[17] sequence, both known as aggregating segment, were synthesized via the conventional Fmoc/*t*-Bu-solid phase method using methylbenzhydrylamine-resin (MBHAR), either in low (0.5 mmol/g) or in highly substituted conditions (2.3 mmol/g). The reason for the use of the latter resin lies in the idea of promoting deliberately stronger chain associations inside the beads. After the assembly of both sequences separately in these two MBHAR batches, guest amino acids (Asx or Glx) were introduced at the N-terminal extremity of their sequence. The Trt (trityl) group was selected to protect the Asn and Gln residues; and the *t*-Bu (*t*-butyl) was selected for Asp and Glu (both used in Fmoc/*t*-Bu chemistry).[31] In the case of Boc chemistry,[32] Asn and Gln were evaluated either in free form or protected with the Xan (xanthenyl) group, whereas the *c*-HxO (*cyclo*-hexyl) or Bzl (benzyl) groups were studied as protecting groups for the Asp and Glu residues. The integrity of the synthesized peptide resins was verified by cleaving a small portion of the sample and the crude peptides were characterized by analytical HPLC, amino acid analysis and mass spectrometry. For the EPR studies, the peptidyl-resins were thus labeled with the Fmoc–TOAC derivative and in order to avoid spin–spin exchange interactions, which may broaden the EPR lines, and to minimize possible physicochemical and steric perturbations, the extent of labeling was kept as low as possible. It was also assumed that the TOAC-labeled peptide chains are dispersed homogeneously throughout the resin matrix and behave similarly to the unlabeled chains in all solvent systems tested. Samples were placed in flat quartz cells and EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer using 298 K as the temperature. 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.

For example, Figure 1 displays the EPR spectra of low peptide-loaded (0.5 mmol/g) DAAAA–MBHAR swollen in DMF, NMP and DMSO solvents. In DMSO, which is a polar and strong nucleophilic solvent, the spectrum displays two components, one with broad lines and the other with narrow lines, corresponding to strongly and weakly immobilized spin label populations, respectively. This second component was found in DMSO in most of the low-substitution peptide–MBHARs. This is in accordance with the dominant influence of the 1% polystyrene–styrene apolar matrix of the solid support over the more polar attached peptide chains. Table 1 summarizes the peptide chain mobility degrees estimated by W_0 values of all these peptide–resins in DMF, NMP and DMSO. In these peptides only the N-terminal residue (Asx or Glx) and the corresponding protecting groups were changed, as necessary.

The evaluation of the W_0 values of this table allows the following conclusions: (i) the ING sequence is more aggregated than the AAAA segment. This conclusion was obtained by the greater average W_0 values of the former sequence, regardless of the substitution degree of the resin or the solvent used; (ii) using these same W_0 solvation data, DMSO seems to be the less appropriate solvent for solvating peptide–resins in a low peptide-content condition (this solvent presented greater W_0

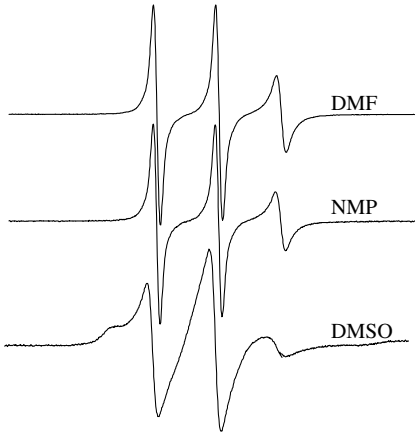

Figure 1. Effect of solvent on EPR spectra of TOAC-labeled low load DAAAA–MBHAR.

Table 1. Effect of side chain protector on the EPR spectra of low and highly loaded TOAC–XAAAA and XING–MBHAR swollen in DMF, NMP and DMSO

X residue/protector	W_0 (G)					
	DMF[a]	NMP[a]	DMSO[a]	DMF[b]	NMP[b]	DMSO[b]
			XAAAA			
Asp (*t*-Bu)	1.74	1.67	2.93	1.80	1.83	1.77
Asp (Bzl)	1.87	1.73	2.95	1.95	2.03	2.24
Asp (*c*-HxO)	2.51	1.99	4.82	1.95	1.94	2.12
Glu (*t*-Bu)	1.79	1.84	3.04	1.85	1.92	2.06
Glu (Bzl)	1.84	1.99	3.20	1.91	2.02	1.92
Glu (*c*-HxO)	1.81	1.88	3.20	1.99	2.04	2.05
Asn (NH_2)	1.79	1.81	2.05	1.90	2.03	1.84
Asn (Xan)	1.77	1.81	1.97	1.95	2.11	1.82
Asn (Trt)	1.91	1.93	2.80	2.10	2.19	2.12
Gln (NH_2)	1.80	1.84	1.99	1.96	1.96	1.82
Gln (Xan)	1.84	1.83	1.96	1.87	1.96	1.99
Gln (Trt)	1.90	1.97	3.25	2.01	2.16	2.10
			XING			
Asp (*t*-Bu)	1.88	1.87	1.89	1.93	2.09	1.92
Asp (Bzl)	1.82	2.17	2.12	2.13	2.60	2.17
Asp (*c*-HxO)	2.30	2.60	2.26	1.99	2.18	2.07
Glu (*t*-Bu)	1.95	1.76	1.86	1.91	2.00	1.94
Glu (Bzl)	1.88	1.95	1.88	2.01	2.26	1.97
Glu (*c*-HxO)	1.91	1.86	1.96	1.98	2.20	2.03
Asn (NH_2)	1.95	2.06	2.02	2.05	2.20	2.10
Asn (Xan)	2.17	2.70	2.08	2.14	2.32	2.01
Asn (Trt)	2.37	1.91	Powder spectra	2.59	2.57	2.67
Gln (NH_2)	2.11	1.79	2.37	2.00	2.30	1.71
Gln (Xan)	1.99	2.01	2.27	2.00	2.34	1.80
Gln (Trt)	2.25	2.21	3.91	2.12	2.35	2.47

[a] 0.5 mmol/g.
[b] 2.3 mmol/g.

values in comparison with DMF or NMP); (iii) in contrast with the aforementioned, no significant difference in the solvation degree was verified among these three solvents when heavily substituted MBHAR is used; (iv) in terms of Boc-chemistry, no significant difference is observed when Asn or Gln residues contain their side chains in free form or attached to the Xan group; (v) otherwise in Fmoc chemistry, the Trt protection induced the more pronounced immobilization of peptide chains listed in Table 1; (vi) when the EPR mobility of Asp and Glu residues attached to the peptides are examined, one can conclude that the *c*-HxO protecting group induces greater chain immobilization than Bzl (in Boc chemistry) and also than *t*-Bu (in Fmoc chemistry).

Collectively, these data strongly point out that the type of single-protecting group significantly affects the overall peptide chain mobility during the peptide synthesis. An increase of about 0.1 G or higher for the W_0 parameter has been previously correlated with a significant decrease in the rate of coupling reaction.[24,25] These findings thus strongly suggest that depending on the side chain protecting groups used for the synthesis, pronounced influence in the overall solvation of the peptide resin can occur with significant consequences to the synthesis. The controversy that still exists in comparing the efficiency of Boc and Fmoc-synthesis strategies[33–35] must be therefore discussed in the light of the solvation influence over the side chain protecting-group mobility of each chemical strategy. This possibility has currently been investigated making different combinations of the presence of these protecting groups in peptide sequence models. This has been done to further the application of the EPR/TOAC strategy aiming at verifying the corresponding influence for the solid-phase peptide synthesis methodology.

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References and notes

1. Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2153.
2. Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957–989.
3. Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398.
4. Sabatino, G.; Mulinacci, B.; Alcaro, M. C.; Chelli, M.; Rovero, P.; Papini, A. M. *Lett. Pept. Sci.* **2002**, *9*, 119–123.
5. Varanda, L. M.; Miranda, M. T. M. *J. Pept. Res.* **1997**, *50*, 102–108.

6. Kates, S. A.; McGuinness, B. F.; Blackburn, C.; Griffin, G. W.; Sole, N. A.; Barany, G.; Albericio, F. *Biopolymers* **1998**, *47*, 365–380.
7. Zinieris, N.; Zikos, C.; Ferderigos, N. *Tetrahedron Lett.* **2006**, *47*, 6861–6864.
8. Yu, H. M.; Chen, S. T.; Wang, K. T. *J. Org. Chem.* **1992**, *57*, 4781–4784.
9. Fara, M. A.; Diaz-Mochon, J. J.; Bradley, M. *Tetrahedron Lett.* **2006**, *47*, 1011–1014.
10. Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *102*, 5463–5470.
11. Tam, J. P.; Lu, Y.-A. *J. Am. Chem. Soc.* **1995**, *117*, 12058–12063.
12. Fields, G. B.; Fields, C. G. *J. Am. Chem. Soc.* **1991**, *113*, 4202–4207.
13. Yan, B.; Sun, Q. *J. Org. Chem.* **1998**, *63*, 55–58.
14. Milton, S. C. F.; Milton, R. C. D. *Int. J. Pept. Protein Res.* **1990**, *36*, 193–196.
15. Valente, A. P.; Almeida, F. C. L.; Nakaie, C. R.; Schreier, S.; Crusca, E.; Cilli, E. M. *J. Pept. Sci.* **2005**, *11*, 556–563.
16. Fitch, W. L.; Detre, G.; Holmes, C. P. *J. Org. Chem.* **1994**, *59*, 7955–7956.
17. Warrass, R.; Wieruszeski, J. M.; Boutillon, C.; Lippens, G. *J. Am. Chem. Soc.* **2000**, *122*, 1789–1795.
18. Vaino, A. R.; Goodin, D. B.; Janda, K. D. *J. Comb. Chem.* **2000**, *2*, 330–336.
19. Cilli, E. M.; Oliveira, E.; Marchetto, R.; Nakaie, C. R. *J. Org. Chem.* **1996**, *61*, 8992–9000.
20. Malavolta, L.; Oliveira, E.; Cilli, E. M.; Nakaie, C. R. *Tetrahedron* **2002**, *58*, 4383–4394.
21. Rassat, A.; Rey, P. *Bull. Soc. Chim. Fr.* **1967**, *3*, 815–817.
22. Nakaie, C. R.; Schreier, S.; Paiva, A. C. M. *Braz. J. Med. Biol. Res.* **1981**, *14*, 173–180.
23. Marchetto, R.; Schreier, S.; Nakaie, C. R. *J. Am. Chem. Soc.* **1993**, *115*, 11042–11043.
24. Cilli, E. M.; Marchetto, R.; Schreier, S.; Nakaie, C. R. *Tetrahedron Lett.* **1997**, *38*, 517–520.
25. Cilli, E. M.; Marchetto, R.; Schreier, S.; Nakaie, C. R. *J. Org. Chem.* **1999**, *64*, 9118–9123.
26. Ribeiro, S. C. F.; Schreier, S.; Nakaie, C. R.; Cilli, E. M. *Tetrahedron Lett.* **2001**, *42*, 3243–3246.
27. Nakaie, C. R.; Malavolta, L.; Schreier, S.; Trovatti, E.; Marchetto, R. *Polymers* **2006**, *47*, 4531–4536.
28. Marchetto, R.; Cilli, E. M.; Jubilut, G. N.; Schreier, S.; Nakaie, C. R. *J. Org. Chem.* **2005**, *70*, 4561–4568.
29. Oliveira, E.; Cilli, E. M.; Miranda, A.; Jubilut, G. N.; Albericio, F.; Andreu, D.; Paiva, A. C. M.; Schreier, S.; Tominaga, M.; Nakaie, C. R. *Eur. J. Org. Chem.* **2002**, 3686–3694.
30. Hancock, W. S.; Prescott, D. J.; Vagelos, P. R.; Marshall, G. R. *J. Org. Chem.* **1973**, *38*, 774–781.
31. Fields, G. B.; Noble, R. L. *Int. J. Peptide Protein Res.* **1990**, *35*, 161–214.
32. Barany, G.; Merrifield, R. B. *Analysis, Synthesis and Biology*; Academic Press: New York, 1980.
33. Beyermann, M.; Bienert, M. *Tetrahedron Lett.* **1992**, *33*, 3745–3748.
34. Rovero, P.; Quartara, L.; Fabbri, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 140–144.
35. Bedford, J.; Hyde, C.; Johnson, T.; Jun, W.; Owen, D.; Quibell, M.; Sdeppard, R. C. *Int. J. Pept. Protein Res.* **1992**, *40*, 300–307.