VOLUME 70, NUMBER 12

JOC Articles

JUNE 10, 2005

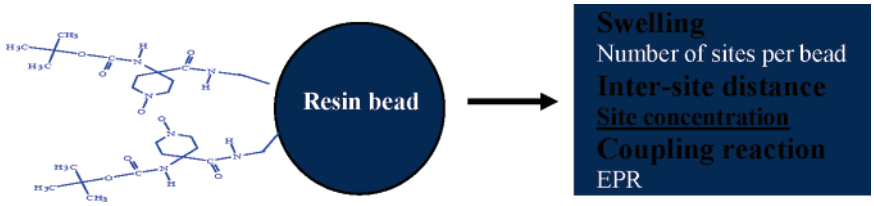
Determination of Site−Site Distance and Site Concentration within Polymer Beads: A Combined Swelling-Electron Paramagnetic Resonance Study

Reinaldo Marchetto,[†] Eduardo M. Cilli,[†] Guita N. Jubilut,[‡] Shirley Schreier,[§] and Clovis R. Nakaie*,[‡]

Department of Biochemistry and Technological Chemistry, Institute of Chemistry, UNESP, Araraquara, São Paulo 14800-900, Brazil, Department of Biophysics, Universidade Federal de São Paulo, Rua 3 de Maio 100, CEP 04044-020 São Paulo, Brazil, and Department of Biochemistry, Institute of Chemistry, Universidade de São Paulo, CP 26077, 05513-970 São Paulo, SP, Brazil

clovis.biof@epm.br

Received September 20, 2004



This work proposes a combined swelling-electron paramagnetic resonance (EPR) approach aiming at determining some unusual polymer solvation parameters relevant for chemical processes occurring inside beads.Batches of benzhydrylamine-resin (BHAR), a copolymer of styrene−1% divinylbenzene containing phenylmethylamine groups were, labeled with the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid (TOAC), and their swelling properties and EPR spectra were examined in DCM and DMF. By taking into account the BHARs labeling degrees, the corresponding swelling values, and some polymer structural characteristics, it was possible to calculate polymer swelling parameters, among them, the volume and the number of sites per bead, site−site distances and site concentration. The latter values ranged from 17 to 170 Å and from 0.4 to 550 mM, respectively. EPR spectroscopy was applied to validate the multistep calculation strategy of these swelling parameters. Spin−spin interaction was detected in the labeled resins at site−site distances less than approximately 60 Å or probe concentrations higher than approximately 1×10^{-2} M, in close agreement with the values obtained for the spin probe free in solution. Complementarily, the yield of coupling reactions in different resins indicated that the greater the inter-site distance or the lower the site concentration, the faster the reaction. The results suggested that the model and the experimental measurements developed for the determination of solvation parameters represent a relevant step forward for the deeper understanding and improvement of polymer-related processes.

Introduction

The innovation of performing chemical processes within an insoluble polymer matrix came into use about four decades ago with the development of the solid-phase peptide synthesis method[1,2] and has been successfully expanded to create efficient synthetic methodologies for other macromolecules.[3] More recently, progressively greater knowledge of solid-phase supported processes has been crucial in successfully launching the unique com-

* To whom correspondence should be addressed. Phone: 55-11-5539 0809. Fax: 55-11-5575 9617.
[†] UNESP.
[‡] Universidade Federal de São Paulo.
[§] Universidade de São Paulo.

(1) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

10.1021/jo0483318 CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/13/2005

binatorial chemistry approach[4] that has allowed the generation of peptide libraries and has had a remarkable impact on the development of new therapeutic agents.[5]

With the scope of using polymers for an ever-widening array of purposes, a large number of different resins has been formulated.[6] In addition, spectroscopic techniques have been applied with the aim of reaching a deeper understanding of polymer-based processes. Among these, investigations based on nuclear magnetic resonance,[7] infrared,[8] fluorescence,[9,10] Raman,[10] and electron paramagnetic resonance (EPR)[11] have been of great value since they provide relevant information about the solvated polymeric network. Properties such as diffusion, adsorption, and distribution of sites within beads were all examined in these reports. We and other groups have described and exploited the use of the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) to investigate the conformation and dynamics of labeled peptides.[12,13] The use of TOAC has been extended to structural investigations of solvated resins and peptide resins.[14]

The interaction between the solvent system and any type of solute is of utmost relevance when examining chemical or physicochemical processes.[15] In the case of polymers or peptide-polymers, the solvent system affects polymer swelling, the average distance between chains (and, as a result, the degree of chain association), controls the rate of motion of components, and regulates reaction kinetics. For this reason, polymer solvation has been investigated with a variety of experimental procedures, among them the microscope measurement of beads.[16]

This latter experimental approach has been of value in the examination of the solvation characteristics of a great number of polymeric materials, adopted as solute models in a large amount of solvents. This approach proved very fruitful since it allowed us to obtain consistent results regarding polymer solvation and to propose a novel amphoteric solvent parameter (AN + DN),[17] which is the sum of Gutmann's solvent electron acceptor (AN) and electron donor (DN) numbers[18] at a ratio of 1:1. The use of the AN and DN concepts was recently extended to predict the potential of a given solvent to dissociate peptide chains not only when bound to a polymer matrix but also when free in solution.[19]

When making use of polymeric matrixes to perform chemical processes it is desirable to understand as much as possible, at a molecular level, the properties of the environment created by these matrixes. In this context, many studies have assessed properties such as dynamics of the polymer backbone, site−site aggregation, and swelling capacity in each solvent system.[7−11,14] To continue these polymer-focused studies, the strategy described in the present work relied primarily on a novel approach to calculate some structural characteristics of

(2) (a) Barany, G.; Merrifield, R. B. *The Peptides*; Academic Press Inc.: New York, 1979; Vol. 2, pp 1−284. (b) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984. (c) Fields, G. B.; Noble, R. L. *Int. J. Peptide Protein Res.* **1990**, *35*, 161. (d) Kates, S. A., Albericio, F. *Solid-Phase Synthesis. A Practical Guide*; Marcel Dekker: New York, Basel, 2000; pp 275−330. (e) Kent, S. B. H. *Annu. Rev. Biochem*. **1988**, *57*, 957.

(3) (a) Fréchet, J. M.; Schuerch, C. *J. Am. Chem. Soc.* **1971**, *93*, 492. (b) Zehavi, U.; Patchornik, A. *J. Am. Chem. Soc*. **1973**, *95*, 5673. (c) Letsinger, R. L.; Finnan, J. L.; Heavner, G. A.; Lunsford, W. B. *J. Am. Chem. Soc*. **1975**, *85*, 3278. (d) Amarnath, V., Broom, A. D. *Chem. Rev.* **1977**, *77*, 183.

(4) (a) Hermkens, P. H. H.; Ottenheim, H. C. J.; Rees, D. *Tetrahedron* **1996**, *52*, 4527 (b) Thompson, L. A.; Ellman, J. A. *Chem. Rev*. **1996**, *95*, 555, (c) Hermkens, P. H. H.; Ottenheim, H. C. J.; Rees, D. *Tetrahedron* **1996**, *52*, 4527.

(5) (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82. (b) Jung, G.; Beck-Sickinger, A. G. *Angew. Chem., Int. Ed. Engl*. **1992**, *31*, 367. (c) Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kasmierski, W. M., Knapp, R. J. *Nature* **1991**, *354*, 82.

(6) (a) Lebl, M. *Biopolymers* **1998**, *47*, 397. (b) Kates, S. A.; McGuiness, B. F.; Blackburn, C.; Griffin, G. W.; Solé, N. A.; Barany, G.; Albericio, F. *Biopolymers* **1998**, *47*, 365. (c) Meldal, M. In *Methods in Enzymology*: *Solid-Phase Peptide Synthesis*; Fields, G., Ed.; Academic Press: New York 1997; pp 83−103.

(7) (a) Ludwick, A. G.; Jelinski, L. W.; Live, D.; Kintamar, A.; Dumais, J. J. *J. Am. Chem. Soc.* **1986**, *108*, 6493. (b) Deber, C. M.; Lutek, M. K.; Heimer, E. P.; Felix, A. M. *Peptide Res.* **1989**, *2*, 184. (c) Warrass, R.; Wieruszeski, M.; Bouitillon, C.; Lippens, G. *J. Am. Chem. Soc* **2000**, *122*, 1789. (d) Furrer, J.; Piotto, M.; Bourdonneau, M.; Limal, D.; Guichard, G.; Elbayed, K.; Briand, J. P.; Bianco, A. *J. Am. Chem. Soc*. **2001**, *123*, 4130. (e) Gambs, C.; Dickerson T. J.; Mahajan, S.; Pasternack L. B.; Janda, K. D. *J. Org. Chem*. **2003**, *68*, 3673. (f) Zhao, T. J.; Beckham, H. W. (g) Zhao, T. J.; Beckham, H. W.; Gibson, H. W. *Macromolecules* **2003** *13*, 4833.

(8) (a) Milton, R. C. L.; Milton, S. C. F.; Adams, P. A. *J. Am. Chem. Soc*. **1990**, *112*, 5039. (b) Hendrix, J. C., Halverson, K. J., Jarret, J. T.; Lansbury, P. T., Jr. *J. Org. Chem.* **1990**, *55*, 4517. (c) Yan, B. *Acc. Chem. Res*. **1998**, *31*, 621.

(9) (a) Li, Y. H.; Chan, L. M.; Tyler, L.; Moody, R. T.; Himel, C. M.; Hercules, D. M. *J. Am. Chem. Soc.* **1975**, *97*, 3118. (b) Shea, K. J.; Sassaki, D. Y.; Stoddarard, G. J. *Macromolecules* **1989**, *22*, 1722. (c) Vaino, A. R.; Janda, K. D. *J. Comb. Chem.* **2000**, *2*, 579.

(10) (a) Kress, J.; Rose, A.; Frey, J. G.; Brocklesby, W. S.; Ladlow, M.; Mellor, G. W.; Bradley, M. *Chem. Eur. J.* **2001**, *7*, 3880. (b) Rademann, J.; Barth, M.; Brock, R.; Egelhaaf, H. J.; Jung, G. *Chem. Eur. J*. **2001**, *7*, 3884. (c) Kress, J., Zanaletti, R.; Rose, A.; Frey, J. G.; Brockesby, W. S.; Ladlow, M.; Bradley, M. *J. Comb. Chem*. **2003**, *5*, 28.

(11) (a) Regen, S. L. *J. Am. Chem. Soc.* **1974**, *96*, 5275. (b) Ward, T. C.; Brooks, J. T. *Macromolecules* **1974**, *7*, 207. (c) Vaino, A. R.; Goodin, D. B.; Janda, K. D. *J. Comb. Chem.* **2000**, *2*, 330.

(12) (a) Nakaie, C. R.; Schreier, S.; Paiva, A. C. M. *Braz. J. Med. Biol. Res.* **1981**, *14*, 173. (b) Marchetto, R.; Schreier, S.; Nakaie, C. R. *J. Am. Chem. Soc.* **1993**, *117*, 11042. (c) Barbosa, S. R.; Cilli, E. M.; Lamy-Freund, M. T.; Castrucci, A. M. L., Nakaie, C. R. *FEBS Lett.* **1999**, *446*, 45. (d) Pertinhez, T. A.; Nakaie, C. R.; Carvalho, R. S. H.; Paiva, A. C. M.; Tabak, M.; Toma, E.; Schreier, S. *Biopolymers* **1977**, *42*, 821. (e) Nakaie, C. R.; Silva, E. G.; Cilli, E. M.; Marchetto, R.; Schreier, S.; Paiva, T. B.; Paiva, A. C. M. *Peptides* **2002**, *23*, 65. (f) Schreier, S.; Barbosa, S. R.; Casallanovo, F., Vieira, R. F. F., Cilli, E. M.; Paiva, A. C. M.; Nakaie, C. R. *Biopolymers* **2004**, *74*, 389.

(13) (a) Smithe, M. L.; Nakaie, C. R.; Marshall, G. R. *J. Am. Chem. Soc.* **1995**, *117*, 10555. (b) Toniolo, C.; Valente, E.; Formaggio, F.; Crisma, M.; Pilloni, G.; Corvaja, C.; Toffoletti, A.; Martinez, G. V.; Hanson, M. P.; Millhauser, G. L.; George, C.; Flippen-Anderson, J. *J. Pept. Sci.* **1995**, *1*, 45. (c) McNulty, J. C.; Silapie, J. L.; Carnevali, M.; Farrar, C. T.; Griffin, R. G.; Formaggio, F.; Crisma, M.; Toniolo, C.; Millhauser, G. L. *Biopolymers* **2000**, *55*, 479. (d) Victor, K. G., Cafiso, D. S. *Biophys. J*. **2001**, *81*, 2241. (e) Pispisa, B., Palleschi, L., Stella, M., Venanzi, M., Toniolo, C. *J. Phys. Chem. B* **1998**, *102*, 7890.

(14) (a) Cilli, E. M.; Marchetto, R.; Schreier, S.; Nakaie, C. R. *Tetrahedron Lett.* **1997**, *38*, 517. (b) Cilli, E. M.; Marchetto, R.; Schreier, S.; Nakaie, C. R. *J. Org. Chem.* **1999**, *64*, 9118. (c) Ribeiro, S. C. F., Schreier, S., Nakaie, C. R., Cilli, E. M. *Tetrahedron Lett.* **2001**, *42*, 3243. (d) Oliveira, E.; Cilli, E. M.; Miranda, A.; Jubilut, G. N.; Albericio, F.; Andreu, A.; Paiva, A. C. M.; Schreier, S.; Tominaga, M.; Nakaie, C. R. *Eur. J. Org. Chem*. **2002**, *21*, 3686.

(15) (a) Katritzky, A. R., Fara, D. C., Hongfang, Y., Tämm, K. *Chem. Rev*. **2004**, *104*, 175, (b) Markus, Y. *Chem. Soc. Rev*. **1993**, *22*, 409. (c) Reichardt, C. *Chem. Rev*. **1994**, *94*, 2319. (c) Pytela, O. *Collect. Czech. Chem. Commun*. **1988**, *53*, 1333. (d) Burton, A. F. M. *Chem. Rev*. **1975**, *75*, 731.

(16) (a) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *102*, 5463. (b) Fields, G. B.; Fields, C. G. *J. Am. Chem. Soc.* **1991**, *113*, 3, 4202. (c) Tam, J. P.; Lu, Y. A. *J. Am. Chem. Soc*. **1995**, *117*, 12058. (d) Pugh, K. C.; York, E. J.; Stewart, J. M. *Int. J. Peptide Protein Res*. **1992**, *40*, 208.

(17) (a) Cilli, E. M.; Oliveira, E.; Marchetto, R.; Nakaie, C. R. *J. Org. Chem.* **1996**, *61*, 8992. (b) Malavolta, L.; Oliveira, E.; Cilli, E. M.; Nakaie C. R. *Tetrahedron* **2002**, *58*, 4383.

(18) (a) Gutmann, V. *Electrochim. Acta* **1976**, *21*, 661. (b) Gutmann, V. *The Donor−Acceptor Approach to Molecular Interactions*; Plenum Press: New York, 1978.

(19) Malavolta, L.; Nakaie, C. R. *Tetrahedron* **2004**, *60*, 9417.

TABLE 1. Swelling Parameters of Differently Labeled Boc-TOAC-BHAR in DCM

	sample									
	col 1	col 2	col 3	col 4	col 5	col 6	col 7	col 8	col 9	col 10
BHAR[a] (mmol/g)	diam dry bead (μm)	diam swollen bead (μm)	vol solvent/ bead (10^5 μm^3)	vol dry sample/g copol[b] (mL)	wt dry sample/g copol (g)	vol dry sample/g sample (mL)	no. of beads/g sample (10^7)	no. of sites/bead (10^{12})	site−site distance (Å)	site concn (mM)
0.003[c]	57	98	4.0	1.8	1.17	1.54	1.59	0.1	169.8	0.4
0.019[c]	57	98	4.0	1.8	1.18	1.53	1.58	0.7	88.9	2.9
0.035[c]	57	98	4.0	1.8	1.18	1.53	1.58	1.3	72.2	5.4
0.050[c]	57	98	4.0	1.8	1.18	1.53	1.58	1.9	63.7	7.9
0.065[d]	57	98	4.0	1.8	1.19	1.51	1.56	2.5	58.1	10.4
0.134[d]	57	98	4.0	1.8	1.22	1.48	1.53	5.2	45.5	21.7
0.646[e]	57	98	4.0	1.8	1.45	1.24	1.28	30.3	25.3	126.3
0.988[f]	58	99	4.1	1.9	1.66	1.14	1.12	52.9	21.2	215.0

[a] Degree of Boc-TOAC-OH labeling. [b] Copolymer of styrene−1% divinylbenzene: d = 0.99 g/mL; average diameter of dry beads = 47 μm. [c] Obtained from 0.050 mmol/g of BHAR. [d] Obtained from 0.14 mmol/g of BHAR. [e] Obtained from 0.80 mmol/g of BHAR. [f] Obtained from 1.40 mmol/g of BHAR.

polymers in solvated conditions. Making use of TOAC labeling, EPR spectroscopy was applied to check the validity of the calculation protocol. The calculation strategy involved the initial microscopic measurement of dry and swollen beads of several spin-labeled batches of the benzhydrylamine resin (BHAR) used to synthesize α-carboxamide peptides.[20] Subsequently, data such as solvent volume inside the bead, number of sites per bead, site−site distance, and site concentration within beads were estimated through a sequential calculation strategy.

EPR spectra of BHAR batches, labeled with *tert*-butyloxycarbonyl-TOAC derivative (Boc-TOAC-OH),[12a] were obtained in order to check the proposed calculation approach. The occurrence of spin−spin interaction was used as a criterion to assess site−site distances and site concentrations inside the beads. The strategy was to verify if this spectral effect is observed at similar probe−probe distances or probe concentrations within the resin beads and when the probe was free in solution.

In addition, experiments were performed to evaluate the relationship between the calculated polymer swelling data, such as site−site distance, site concentration, and the rate of amino acid coupling reactions in model resins or peptide-resins. The results demonstrate that the practical-conceptual approach presented in this work for quantifying resin swelling properties can be applied to other polymeric materials.

Results

Swelling Studies. BHAR batches, with phenylmethylamine group loading ranging from 0.05 to 1.4 mmol/g at the polystyrene−1% divinylbenzene backbone, were synthesized according to a previously described protocol.[20] Boc-TOAC-OH was coupled to BHAR batches under controlled conditions in order to produce resins spin-labeled to different extents.

Approximately 0.5 g of BHAR with substitution degrees of 0.05, 0.14, 0.80, and 1.40 mmol/g were used to couple the Boc-TOAC-OH residue using the conventional Boc-peptide synthesis strategy.[2a,b] If necessary, a 3-fold molar excess was applied to guarantee quantitative spin label incorporation using the diisopropylcarbodiimide/*N*-hydroxybenzotriazole (DIC/HOBt) coupling protocol in DCM. In all BHAR batches, the coupling was complete in about 2 h. When partial incorporation was desired, the coupling step was deliberately carried out with less than equimolar amounts of Boc-TOAC-OH. The exact degree of incorporation was determined by quantitative picric acid method.[21] Using this experimental protocol, eight batches of Boc-TOAC-BHAR with paramagnetic labeling degrees ranging from 0.003 to 0.988 mmol/g were synthesized.

Calculation Strategy To Determine Polymer Bead Swelling Parameters. By starting from simple swelling parameters such as diameters of dry and swollen beads as reference points, a sequence of calculations was designed to determine the resin parameters given in Tables 1 and 2. The tables summarize the data calculated for eight batches of Boc-TOAC-BHAR in DCM and in DMF, respectively.

To illustrate how the various resin parameters were sequentially calculated, the detailed procedure will be described below for the 0.134 mmol/g of Boc-TOAC-BHAR batch in DCM (Table 1, row 6).

Example: 0.134 mmol/g of Labeled Boc-TOAC-BHAR Swollen in DCM (Table 1). Columns 1 and 2 list the average diameters of the dry (57 μm) and DCM swollen (98 μm) beads, respectively, measured under the light microscope. The volumes of the dry (0.97×10^5 μm^3) and swollen (4.93×10^5 μm^3) beads were calculated, and the volume of solvent/bead (4×10^5 μm^3, column 3) was obtained by subtracting the dry bead volume from the swollen bead volume.

Column 4: Volume of Dry Sample/Gram of Copolymer (1.8 mL/g of Copolymer). The ratio (diameter dry sample/diameter dry copolymer)^3 represents the relationship between the volume of the dry macroscopic working sample (0.134 mmol/g of Boc-TOAC-BHAR) and that of the dry copolymer used to synthesize the sample. In the example, since the average diameters of beads of dry sample and dry copolymer are 57 and 47 μm, respectively, the ratio between the dry volumes of both resins is $(57/47)^3 = 1.78$. Considering that the number of beads in 1 g of copolymer is the same as in the sample synthesized from this amount of copolymer and taking

(20) (a) Pietta, P. G.; Cavallo, P. F.; Takahashi, K.; Marshall, G. R. *J. Org. Chem*. **1974**, *39*, 44. (b) Marchetto, R.; Etchegaray, A.; Nakaie, C. R. *J. Braz. Chem. Soc.* **1992**, *3*, 30.

(21) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248.

TABLE 2. Swelling Parameters of Differently Labeled Boc-TOAC-BHAR in DMF

	sample									
	col 1	col 2	col 3	col 4	col 5	col 6	col 7	col 8	col 9	col 10
BHAR[a] (mmol/g)	diam dry bead (μm)	diam swollen bead (μm)	vol solvent/ bead (10^5 μm^3)	vol dry sample/g copol[b] (mL)	wt dry sample/g copol (g)	vol dry sample/g sample (mL)	no. of beads/g sample (10^7)	no. of sites/bead (10^{12})	site−site distance (Å)	site concn (mM)
0.003[c]	57	81	1.8	1.8	1.17	1.54	1.59	0.1	140.9	0.9
0.019[c]	57	81	1.8	1.8	1.18	1.53	1.58	0.7	73.7	6.5
0.035[c]	57	81	1.8	1.8	1.18	1.53	1.58	1.3	60.0	12.0
0.050[c]	57	81	1.8	1.8	1.18	1.53	1.58	1.9	52.8	17.6
0.065[d]	57	79	1.6	1.8	1.19	1.51	1.56	2.5	47.0	26.0
0.134[d]	57	79	1.6	1.8	1.22	1.48	1.53	5.2	36.8	54.2
0.646[e]	57	80	1.7	1.8	1.45	1.24	1.28	30.3	20.5	297.1
0.988[f]	58	79	1.6	1.9	1.66	1.14	1.12	52.9	17.0	551.0

[a] Degree of Boc-TOAC-OH labeling. [b] Copolymer of styrene−1% divinylbenzene: d = 0.99 g/mL; average diameter of dry beads = 47 μm. [c] Obtained from 0.050 mmol/g of BHAR. [d] Obtained from 0.14 mmol/g of BHAR. [e] Obtained from 0.80 mmol/g of BHAR. [f] Obtained from 1.40 mmol/g of BHAR.

into account that the volume of 1 g of copolymer is 1.01 mL (d = 0.99 g/mL),[16a] the total volume of dry sample containing 1 g of copolymer is therefore 1.78 × 1.01 mL, or 1.80 mL.

Column 5: Weight of Dry Sample/Gram of Copolymer (1.22 g/g of Copolymer). The 0.134 mmol Boc-TOAC-BHAR sample was synthesized by quantitative incorporation of the Boc-TOAC-OH in the 0.140 mmol/g of BHAR batch. This resin originated from partial phenylmethylamino incorporation into a heavily substituted 1.4 mmol/g benzoyl group-containing copolymer. This copolymer derivative is synthesized in the first step (Friedel−Crafts acylation) necessary to obtain BHAR.[20] Thus, the Boc-TOAC-BHAR sample under consideration still contains (1.4−0.14) mmol = 1.26 mmol/g of remaining benzoyl groups attached to its backbone. Considering the total weight of groups added in all the synthetic steps, one can calculate that the sum of Boc-TOAC-OH and benzoyl groups attached to the initial copolymer corresponds to 0.182 g. Therefore, in 1 g of sample, the mass of copolymer is 1 − 0.182 g = 0.818 g. Thus, for 1 g of starting copolymer, the weight of the 0.134 mmol Boc-TOAC−BHAR is 1.22 g.

Column 6: Volume of Dry Sample/Gram of Sample (1.48 mL/g). This parameter is calculated by dividing the value of (volume of dry sample/gram of copolymer), column 4, by (weight of dry sample/gram of copolymer), column 5. The value obtained (1.48 mL/g) represents the ratio between the volume of the dry sample (1.8 mL) and its total weight (1.22 g) and corresponds to the volume occupied by 1 g of sample in the dry form.

Column 7: Number of Beads/Gram of Sample (1.53×10^7 Beads/g of Sample). This value is calculated by dividing the volume of 1 g of dry sample (1.48 mL, column 6) by the average volume of one dry bead, which is calculated from its diameter (57 μm, column 1). Thus, the volume of one dry bead is 9.7×10^{-8} mL. The ratio 1.48 mL/9.7×10^{-8} mL yields 1.53×10^7 beads in 1 g of sample.

Column 8: Number of Sites/Bead (5.2×10^{12}). The number of sites per bead is calculated by dividing the number of sites/gram of sample by the number of beads/gram of sample (column 7). The former value corresponds to $0.134 \times 6.02 \times 10^{20}$ sites/g. Dividing this number by the number of beads in 1 g of sample (1.53×10^7, column 7) gives 5.2×10^{12} sites/bead.

Column 9: Site−Site Distance (45.5 Å). To evaluate this important parameter, we first calculate the average volume per site. This is done by dividing the volume of one swollen bead (4.9×10^{-5} μm^3, calculated from the measured diameter of one swollen bead, 98 μm, column 2) by the number of sites/bead (5.2×10^{12}, column 8). Thus, the average volume per site is 9.4×10^4 Å^3. By assuming a uniformly distributed cubic lattice for the sites within the bead, the site−site distance corresponds to the side of a cube and is given by the cubic root of the volume occupied by one site, i.e., $(9.4 \times 10^4$ Å$^3)^{1/3}$ = 45.5 Å.

Column 10: Site Concentration Inside the Bead (21.7 mM). Finally, making use of the parameters calculated in the previous steps, it is possible to determine the site concentration within the bead. Thus, for the 0.134 mmol/g of substituted Boc-TOAC-BHAR, the site concentration is obtained by dividing the number of sites/bead (5.2×10^{12}, column 8) by the volume of solvent/bead (4×10^5 μm^3, column 3), that is, 1.3×10^7 sites/μm^3, or 1.3×10^{19} sites/mL. Considering that 6.02×10^{20} sites/mL correspond to 1 M concentration, we find that that the site concentration corresponds to an effective Boc-TOAC−OH concentration of 21.7 mM.

By examining the parameters calculated in Tables 1 and 2, it can be seen that the average inter-site distance ranged from a maximum of about 170 Å (for 0.003 mmol/g of Boc-TOAC-BHAR in DCM, Table 1) to a minimum of 17 Å (for 0.988 mmol/g of Boc-TOAC-BHAR in DMF, Table 2). Furthermore, the site concentration within the beads varied from a minimum of approximately 0.4 mM to a maximum of approximately 550 mM. Under very highly loaded conditions (0.988 mmol/g of Boc-TOAC-BHAR in DMF), the concentrations were as high as those typically used during the solution peptide synthesis method.[22]

Some of the parameters calculated in Tables 1 and 2, such as the number of beads per gram of the resin or the number of sites per bead, are relevant for application in polymer studies. Interestingly, values of this latter parameter ranged from approximately 0.3×10^{12} to 50×10^{12} (0.003 mmol/g and 0.988 mmol/g of Boc-TOAC-labeled resins, respectively). A linear correlation between

(22) Gross, E., Meienhofer, J., Eds. *Peptides: Analysis, Synthesis, Biology*; Academic Press: New York, 1989; Vol. 1.

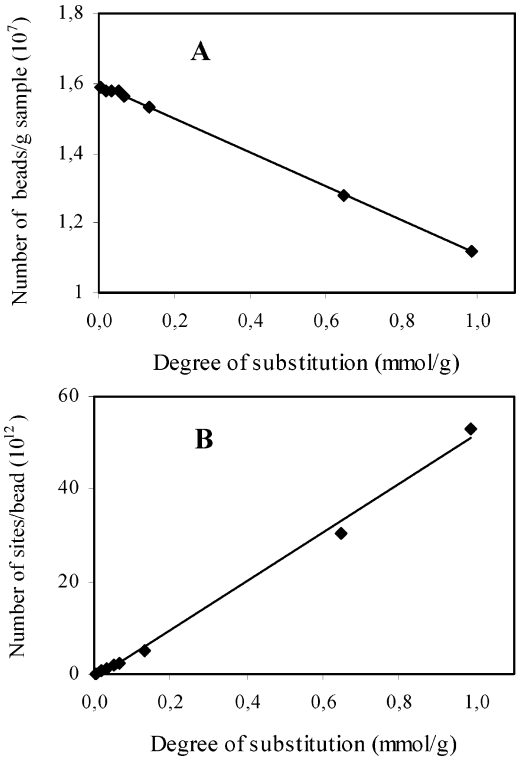


FIGURE 1. Correlation between number of beads per gram of sample (A) and number of sites per bead (B) and the degree of substitution of Boc-TOAC-BHARs.

these two parameters (columns 7 and 8) and the degree of resin labeling is observed in Figure 1. As expected, while the number of beads per gram of resin decreased, the number of sites per bead increased with the increasing degree of substitution.

It should be pointed out that this calculation strategy can be extended to resins containing other chemical groups, including peptides. Besides the dry and swollen bead diameters, it is only necessary to know the density of the starting resin (and therefore the volume occupied by 1 g of resin) and to calculate the overall weight variation due to the incorporation of the desired groups in the composite derivative.

EPR Studies. Figure 2 shows the EPR spectra of eight labeled resins in DCM and DMF (panels A and B, respectively). Spectral line broadening due to spin−spin interaction was observed in both solvents and increased with increasing Boc-TOAC-OH substitution.

The dependence of probe−probe interaction on resin calculated parameters, such as site−site distance and site concentration, was compared with data obtained for Boc-TOAC-OH in solution. Figure 3 shows the EPR spectra of the spin probe in DMF as a function of concentration. Spectral line broadening due to spin−spin interaction can be clearly observed as the probe concentration increases.

Table 3 correlates the Boc-TOAC-OH concentration with the line width of the mid-field line (ΔH) measured for the spectra in Figure 3 and with the average distance between probe molecules free in solution. Similarly to the analysis for the labeled resins (column 9 in Tables 1 and 2), a static model of probe distribution in solution was assumed, namely, the probes are uniformly distributed within a cubic lattice. The average distance between adjacent Boc-TOAC-OH molecules in solution was estimated by calculating the amount of probe molecules at each concentration combined with the average volume occupied by each molecule. Assuming a cubic lattice distribution of probe molecules, the average intermolecular distance between adjacent molecules corresponds to the side of a cube and is given by the cubic root of the volume occupied by the probe. As an example, for 10^{-3} M Boc-TOAC-OH, the average volume occupied by each molecule is 1.7×10^6 Å^3 and the average distance between adjacent molecules is 118.6 Å (Table 3).

Figure 4 displays the dependence of the mid-field spectral line width of both labeled resins and of the spin label in DMF solution on site−site distance (panel A) and site concentration (panel B). Line broadening did not occur for site−site distances larger than approximately 60 Å (probe concentration of ca. 1×10^{-2} M). Similar results were obtained for the probe free in solution and spread throughout resin backbone, strongly suggesting that the sequential calculation designed for quantitative determination of the swelling parameters in Tables 1 and 2 is a valid approach.

Correlation between Resin-Swelling Parameters and the Rate of Coupling Reactions. To emphasize the usefulness of the strategy developed to determine resin swelling parameters, the relationship between site−site distance and site concentration and the rate of coupling reactions throughout a polymer network was examined. Table 4 shows the coupling yield of Boc-Pro-OH in DCM to the 1.4 mmol/g of BHAR batch. One can observe that the greater the site−site distance and lower the site concentration, the faster the coupling reaction. The rate of coupling follows the order: DCM > DMF > DMSO. Complementary swelling data of the 1.4 mmol/g of BHAR batch are given in the Supporting Information.

PSA Method in Equimolar Conditions (1 mM Reactants). Table 5 displays another example of the application of this approach, this time extended to peptidyl-resins. Four batches of the aggregating Ile-Asn-Gly sequence[23] bound to BHAR in varying amounts (up to 1.59 mmol/g) were compared under equivalent conditions with respect to the frequency of Boc-(2BrZ)-Tyr-OH coupling. Other swelling parameters of these four peptide resins are also available in the Supporting Information Section. Closely paralleling the results outlined in the previous paragraph, faster coupling reactions occurred systematically with resins that presented larger site−site distance and lower site concentration.

Discussion

The main objective of the present work was to design a novel swelling-EPR approach aimed at multiple goals. First, we intended to develop a sequential calculation that would allow the estimation of polymer swelling parameters for further practical application. Second, we applied EPR spectroscopy to paramagnetically labeled resins in order to determine at what site−site distance or site concentration values significant spin−spin interactions begin to occur. Finally, we verified the correlation between the calculated properties of solvated polymer

(23) Hancock, W. S.; Prescott, D. J.; Vagelos, P. R.; Marshall, G. R. *J. Org. Chem.* **1973**, *38*, 774.

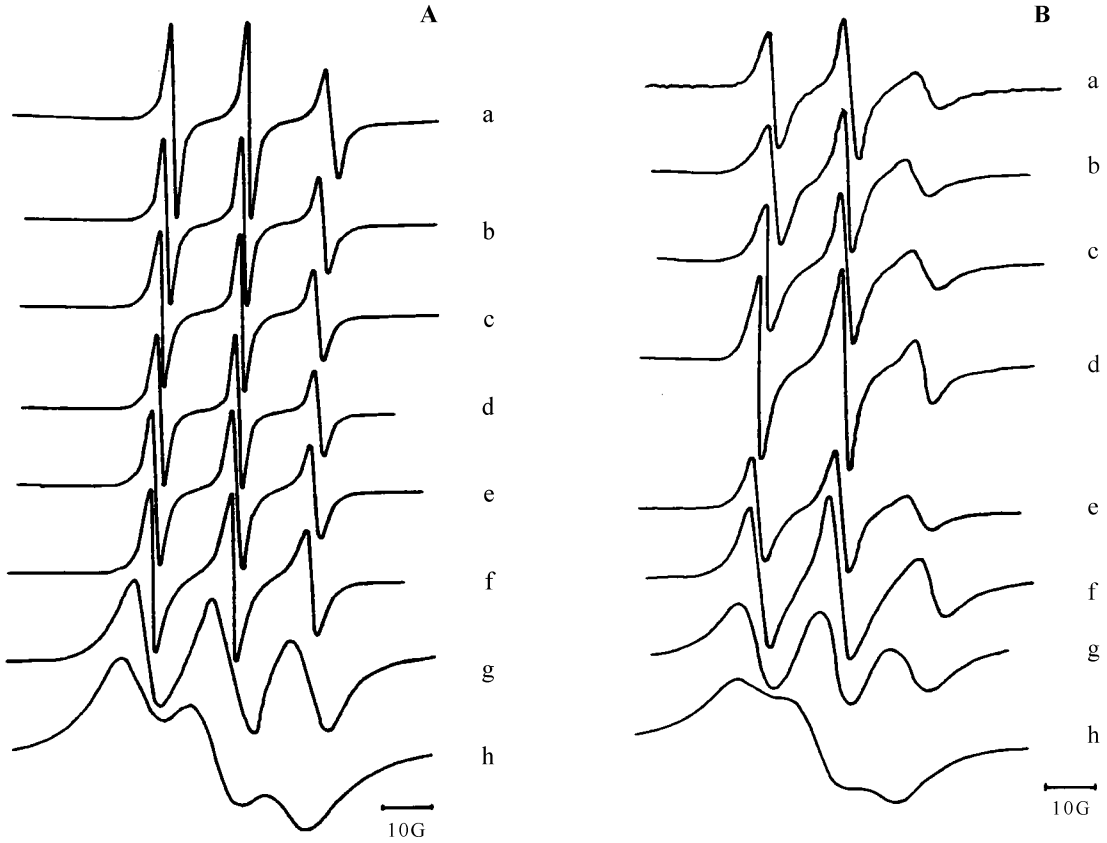


FIGURE 2. Effect of Boc-TOAC-OH loading on the EPR spectra of Boc-TOAC-BHAR in DCM (**A**) and DMF (**B**). Probe loading (mmol/g): a = 0.003; b = 0.019; c = 0.035; d = 0.050; e = 0.065; f = 0.134; g = 0.646; h = 0.988.

and the efficiency of chemical processes taking place in the polymer matrix.

The initial step in this study comprised synthesis and microscopic measurement of the swelling properties of several BHAR batches labeled with the Boc-TOAC-OH spin probe for further EPR studies. By combining swelling and structural data of these resins, a sequential calculation strategy was designed to estimate resin swelling parameters that can be useful in the polymer field (Tables 1 and 2). Among these parameters, the number of sites per bead, average volume occupied by each site, site−site distance, site concentration within beads, should be mentioned.

The combination of EPR spectroscopy with the swelling data of TOAC-labeled resins aimed at testing the validity of the calculation strategy for gauging bead-swelling information. By comparing EPR data for the probe in DMF solution with those for the paramagnetic probe attached to the polymer core at variable degrees in the same solvent, it was possible to estimate the maximum site−site distance (~60 Å) or minimum site concentration ($\sim 1 \times 10^{-2}$ M) for the onset of spin−spin interaction. Similar values were found for the paramagnetic probe attached to the polymer core when the solvent was DCM.

The good agreement between the results obtained with this approach and those obtained making use of the sequential protocol for calculation of swelling parameters (Figure 4) strongly suggest that the proposed protocol is correct. Several geometrical models could be adopted to estimate the average site distribution—spherical, cylindrical, etc. We chose to use a cubic distribution of sites or probes in solution or bound to the resin, based on the classical report by Barany and Merrifield designed to estimate site−site distance within beads.[2a] In this context, it should be recalled that more than 99% of sites are located inside the bead structure and not at its surface.[24]

In previous EPR-solvation studies, we[14] and other groups[11] have monitored the dynamics of resins and peptide-resins with the aim of improving the peptide synthesis methodology. In these studies the mobility of the labeled sites was analyzed by calculating rotational correlation times from measurement of line widths and line heights in the absence of spin−spin interactions, that is, at low label concentrations. Here we took advantage of the occurrence of spin−spin interactions to assess site−site distances and site concentrations inside the polymeric matrixes.

The spectral line broadening obtained with increased labeling of the resins and increased probe concentration in solution provided a clear evidence of the occurrence of increasing spin−spin interaction. The interaction could be due to exchange and/or dipolar mechanisms. The dipolar interaction has been found to be the predominant broadening mechanism in the case of doubly labeled enzymes.[25] In these cases the two paramagnetic centers are located at a fixed distance from each other. Moreover,

(24) Merrifield, B. R.; Littau, V. In *Peptides 1968*; Bricas, E., ed.; North-Holland Publishing Co.: Amsterdam, 1968; p 179.

(25) (a) Taylor, J. S.; Leigh, J. S., Jr.; Cohn, M. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *64*, 219. (b) Mchaourab, H. S.; Oh, K. J.; Fang, C. J.; Hubbell, W. L. *Biochemistry* **1997**, *36*, 307.

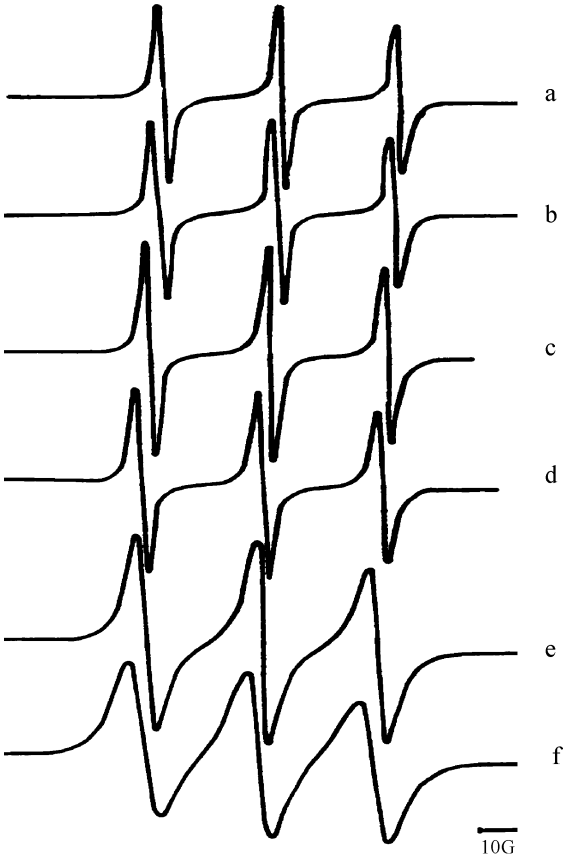


FIGURE 3. EPR spectra of Boc-TOAC-OH in DMF. Concentration (M): a = 0.0001; b = 0.001; c = 0.005; d = 0.01; e = 0.05; f = 0.1.

TABLE 3. Correlation between Concentration of Boc-TOAC-OH in DMF and the EPR Spectra's Central Peak Linewidth and the Site−Site Distance Values

concn (M)	ΔH (G)	site−site distance (Å)
1×10^{-4}	1.40	255.4
1×10^{-3}	1.40	118.6
5×10^{-3}	1.45	69.3
1×10^{-2}	1.70	55.0
5×10^{-2}	2.40	32.2
1×10^{-1}	3.65	25.5

the dipolar interaction has been shown to be detectable up to about 25 Å.[25b] Although the resin beads are formed by a polymeric matrix, the characteristics of these polymers are quite different from those of a protein. In the polystyrene-divinylbenzene branched copolymer, the $-NH_2$ reactive groups are distributed at random, that is, the distances between pairs of attached nitroxides vary. Moreover, these polymers, immersed in solvent, are flexible (as indicated by the spectra at low resin loading, spectra a in Figure 3, panels A and B) and undergo intramolecular motions that lead to variation in the distances separating individual nitroxide groups. In addition, Boc-TOAC-OH bound to one polymer chain can encounter Boc-TOAC moieties bound to other polymer chains in the same bead.

Taking these facts into account, it is seen that the polymeric matrix is a highly dynamic structure and that the distances between Boc-TOAC-OH groups vary in space and time. Thus, the values obtained in the calculations are average values. In this context, it seems plausible to draw an analogy with the situation found in membranes, where their constituent lipid molecules undergo lateral diffusion. Studies at high label concentrations have shown that in model[26] and biological[27] membranes the main contribution to spin−spin interaction is provided by the exchange mechanism. Although in fluid systems the exchange interaction is modulated

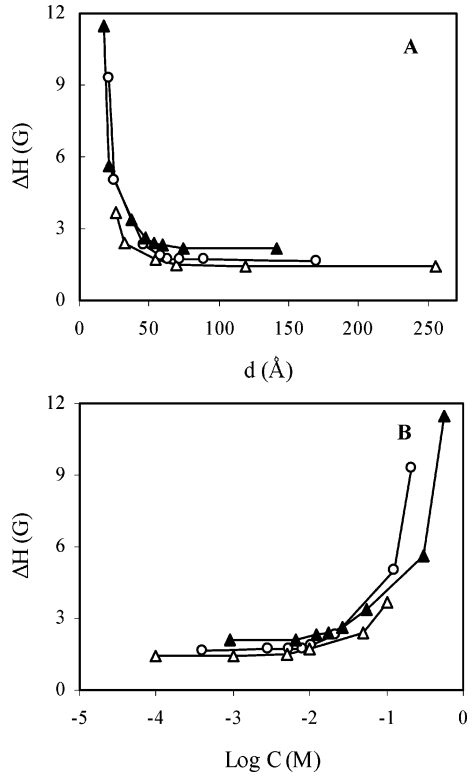


FIGURE 4. Effect of site−site distance (**A**) and site concentration (**B**) on the central peak line width of EPR spectra of Boc-TOAC-OH in DMF solution (Δ) and Boc-TOAC-BHAR in DCM (○) and in DMF (▲).

TABLE 4. Correlation between Yield[a] of Boc-Pro-OH Coupling to BHAR (1.40 mmol/g) and Site Concentration and Site−Site Distance Values

solvent	site concn (M)	site−site distance (Å)	coupling (%)
DCM	0.21	21.7	90
DMF	0.55	17.0	67
DMSO	1.76	14.2	25

[a] Yield of Boc-Pro-OH coupling after 30 min, at 25 °C with PSA method in equimolar conditions (1 mM of reactants).

TABLE 5. Correlation between Boc-(2BrZ)-Tyr-OH Coupling[a] Yield to ING-BHAR in DCM and Site Concentration and Site−Site Distance Values

ING-BHAR[a] (mmol/g)	site concn (mM)	site−site distance (Å)	coupling yield[b] (%)
0.19	39	38	86
0.54	89	33	63
1.16	625	17	21
1.59	1963	14	1

[a] Degree of ING substitution. [b] Yield of Boc-(2BrZ)-Tyr-OH coupling after 15 min, at 25°C with PSA method in equimolar condition (10 mM concentration of reactants).

by the collision frequency between molecules, calculations were performed using a static model to estimate critical distances for the onset of spin−spin interaction.[26b−d] These calculations yielded results in good agreement with the site−site distances found in the present study.

The data regarding the swelling parameters shown in Tables 1 and 2, when compared to those generated in previous swelling approaches to peptide resins,[16a] represent a step forward in the gauging of differentiated physicochemical factors that govern polymer solvation. In this context, the various swelling parameters evaluated in this study could be of great help in deepening the understanding of the resin solvation phenomenon at the microenvironment level. Accordingly, a clear relationship between site−site distance and site concentration values and the rate of the acylation reaction is demonstrated for the first time in the present report (Tables 4 and 5).

Modern science has progressively broadened the scope of the use of polymeric materials for a variety of purposes. Resin applications range from simple use as a solid support for liquid chromatography to complex methods for synthesizing macromolecules such as peptides[2] and oligonucleotides,[3a,b] as well as in combinatorial chemistry[4] conducted for the development of new drugs. The present report describes an alternative method for the determination of polymer swelling properties in combination with the investigation of model resins by means of EPR spectroscopy. We believe that this dual approach can be of great applicability in many areas involving the use of polymeric matrixes.

Experimental Section

Materials. Reagents and solvents were of analytical grade, were collected from recently opened containers and were not further purified. Boc-TOAC-OH was synthesized according to previous reports[12a]

Methods. Peptide Synthesis. The Ile-Asn-Gly sequence was synthesized manually by standard Boc chemistry[2a,b] on about 0.5 g of 0.22, 0.62, 1.62, and 2.62 mmol/g of BHAR. Coupling was performed using a 2.5 excess of Boc-amino acid/DIC/HOBt (1:1:1) in DCM/DMF for approximately 2 h. All couplings were monitored by qualitative ninhydrin test, and when positive, acetylation was performed with 50% acetic anhydride in DCM for 15 min. A small portion of the peptide-resin was cleaved in anhydrous HF and the crude peptide was characterized with regard to identity and homogeneity using mass spectrometry, amino acid analysis, and analytical HPLC.

Measurement of Peptide-Resin Swelling. Before swelling measurements of Boc-TOAC-OH-labeled resins, all batches of synthesized BHAR were sized by suspension in ethanol and fine material was decanted. The suspension was allowed to stand until approximately 90−95% had settled before decanting the supernatant. This procedure was repeated five times and was followed by suspending the beads in DCM. Solvent containing fine particles was withdrawn; this latter procedure was also repeated five times. To develop the swelling study with as narrowly sized population of beads as possible, the last resin purification step involved repeated sifting of dry beads through several 44−88 μm pore metal sieves. This sieving procedure lowered the standard deviation of the resin diameter to about 4%.

Swelling studies of the small-diameter bead populations were performed as published elsewhere[16a,17] after the resins were dried in a vacuum using an Abderhalden-type apparatus. Subsequently, about 200 dry and swollen (allowed to solvate overnight) beads from each resin were spread over a microscope slide and measured directly with a microscope coupled with Image-Pro Plus software. The values of bead diameter distribution were estimated by the geometric means and geometric standard deviations, as published elsewhere.[28]

EPR Studies. EPR measurements were carried out at 9.5 GHz on a Bruker ER 200D-SRC spectrometer at room temperature (22 ± 2 °C) using flat quartz cells from Wilmad Glass Co. (Buena, NJ). 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. Details of the procedure for TOAC-labeling of resins have been reported.[14] Labeled peptide resins were pre-swollen overnight in the solvent under study.

Yield of the Coupling Reaction. In a reaction vessel thermostated at 25 °C, 50−100 μmol of BHAR or ING-BHAR was equilibrated with the desired solvent. Preformed symmetrical anhydride (PSA) of the Boc-Pro-OH and Boc-2-Br-carbobenzoxyl (2BrZ)-Tyr-OH residues, respectively, were produced by mixing with DCC in equimolar conditions (for 1 h, at 0 °C). The white precipitate was removed by filtration and the solution was evaporated for further dissolution with the desired solvent for comparative coupling experiments. The PSA method was deliberately chosen for these experiments as it is less susceptible to the effect of solvent polarity.[2a,b] The rate of rotation of the reaction flask was 20 rpm. The acylating reagents were dissolved in the solvent under investigation and added in equimolar condition (at 10^{-2} M concentration of reactants) to the reaction vessel containing peptide resin preswollen in the same solvent. The coupling yield was monitored by the picric acid method,[21] and each experiment was performed in duplicate.

Acknowledgment. This work was funded by grants from Fapesp, CNPq, and Capes. R.M., E.M.C., S.S., and C.R.N. are recipients of CNPq research fellowships.

Supporting Information Available: Tables with swelling parameters for the 1.4 mmol/g of BHAR and ING-BHAR (four batches) are available. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0483318

(26) (a) Devaux, P., McConnell, H. M. *J. Am. Chem. Soc*. **1972**; *94*, 4475. (b) Sackmann, E.; Träuble, H. *J. Am. Chem. Soc*. **1972**, *94*, 4482. (c) Träuble, H. J.; Sackmann, E. *J. Am. Chem. Soc*. **1972**, *94*, 4492. (d) Sackmann, E.; Träuble, H. *J. Am. Chem. Soc*. **1972**, *94*, 4499.

(27) (a) Scandella, C. J.; Devaux, P.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2056. (b) Sackmann, E.; Trauble, H.; Galla, H. J.; Overath, P. *Biochemistry* **1973**, *12*, 5360.

(28) Irani, R. R.; Callis, C. F. *Particle Size: Measurement, Interpretation and Application*; Wiley: New York, 1963.