Kinetics of Synthesis and Swelling Studies of Highly Substituted Benzhydrylamine-Resins: Implications for Peptide Synthesis and Perspectives for Use as Anion Exchanger Resin

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Estudos cinéticos da síntese de benzidrilamino-resina (BHAR), um polímero rotineiramente empregado em síntese de peptídeos, mostraram que, ao contrário do previsto, a etapa de redução de ceto-grupos pode ser total. Este fato facilita tanto o controle do grau de substituição da resina quanto a obtenção de BHARs mais substituídas do que as descritas até o momento. Medidas dos grãos, por microscopia, indicaram que o inchamento da BHAR é dependente da forma ionizada do seu amino grupo, que pode ter relevância para a síntese de peptídeos em fase sólida. Independentemente do grau de substituição, as resinas desprotonadas incharam melhor em solventes menos polares enquanto que as protonadas apresentaram solvatações crescentes em solventes orgânicos polares, à medida em que se elevou o teor de grupamentos amônio. As BHARs altamente substituídas apresentaram, quando protonadas, inchamentos também em solução aquosa, sugerindo que, além do uso em síntese de peptídeos, elas podem ser também empregadas como novas resinas de troca aniônica.

Kinetic studies carried out during the synthesis of benzhydrylamine-resin (BHAR), a polymer routinely employed in solid phase peptide synthesis, showed that, in disagreement with general belief, the step involving the reductive amination of ketone groups can be nearly complete. This facilitates the control of amine group substitution in the resins and also allows the synthesis of more highly substituted BHARs than previously reported. Important for peptide synthesis, microscopic measurements of bead sizes indicated that the swelling of BHARs is dependent on the ionization of their amine groups. Deprotonated resins, whatever their substitution degree, swell better in less polar solvents, whereas the protonated form showed enhanced solvation in polar organic solvents, as the amount of ammonium groups increased. Highly substituted amine-protonated BHARs also swelled in aqueous solution, suggesting that in addition to their routine use for peptide synthesis, they may also be tested as novel anion exchanger resins.

Key words: benzhydrylamine-resin; peptide synthesis; anion exchange chromatography.

Introduction

An important factor in the solid-phase peptide synthesis methodology¹⁻³ is the degree of substitution of the resins that are employed. The use of heavily substituted resins, containing high degrees of reactive functionalities (>1 mmol/g), presents some advantages, such as the production of larger amounts of peptide per synthesis, and the smaller consumption of solvents. However, the high-loading of the resins also may lead to problems due to the forced proximity between peptide chains inside the resin beads, inducing

chain-chain interactions (aggregation) which interfere with the peptide chain growth. Since these interactions in the highly substituted resins should be better reflected by the solvated bead volume than in the case of low substituted resins, the study of the swelling behavior of the resin beads can be used as a simple but useful tool for an improved understanding of the dynamic processes occurring at the molecular level inside the beads during the peptide chain growth.

In previous reports, we have shown how different factors, such as solvent and peptide sequences^{4,5}, affect the solva-

tion of the resin beads and, consequently, the effectiveness of coupling reactions. We have used benzhydrylamine resin (BHAR) as the model resin for these studies correlating bead swelling with the rate of acylation reaction. One essential prerequisite of these studies is the synthesis of much higher amine-substituted BHARs than usually employed in peptide synthesis. Among different methods proposed in the literature for the synthesis of BHAR, the Friedel-Crafts benzylation of polystyrene-divinylbenzene copolymer followed by Leuckart reductive amination⁶, seemed to be the most interesting for the study of kinetics of its reaction steps due mainly to: (a) the frequently reported^{7,8} difficulties in controlling the resin's amine group content; (b) the reported⁹ incomplete reduction of carbonyl groups during the Leuckart reaction (not exceeding approximately 50%). These factors hamper the synthesis of BHARs with high degrees of substitution, and to better evaluate their role, we have conducted rigorously controlled kinetic studies of BHAR synthesis, which are reported in the first part of this paper.

Using a microscopic method for the measurement of bead sizes¹⁰, we have also studied in the second part of this work, the swelling properties of BHARs with different substitution levels. By correlating the swelling ability of beads in different organic solvents with the amount of amine groups (in the protonated and deprotonated forms), we suggest, more specifically for heavily substituted resins, possible alterations in the standard solvent systems employed during solid phase peptide synthesis.

Additionally, the swelling study was extended also to aqueous media, with the expectation that highly substituted BHARS in the protonated form would swell more in water and therefore, besides their use for peptide synthesis, might also be employed as a new class of anion exchanger resins.

Materials and methods

Materials

Dimethylformamide (DMF) was distilled over ninhydrin and stored over 4A⁰ molecular sieves. Unless otherwise stated, all other solvents were dried and distilled prior to use. Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt) and di-(tert-butyloxycarbonyl)-lysine [Boc-Lys (Boc)] were purchased from Bachem Inc. (Torrence, CA). The copoly(styrene-1% divinylbenzene) resin, Bio-Beads SX1, 200400 mesh, was obtained from Bio-Rad Laboratories (Richmond, CA). Hydrolyses of amino acidresins were done with 12 N HCl:propionic acid (1:1, v/v) at 130°C for 40h and amino acid analyses were performed in a Beckman High Performance amino acid analyzer system 6300. Elemental analyses were done in a PerkinElmer 240 instrument and infrared spectra of the resins were recorded as potassium bromide disks (4 mg resin/100 mg KBr) in a Perkin Elmer 137 spectrophotometer.

Resin Synthesis

Benzoyl-Polystyrene-1% Divinylbenzene (Friedel-Crafts reaction): typically, copoly(styrene-1% divinylbenzene) resin (25g) and 150 ml nitrobenzene were added to a 500-ml three-neck round bottom flask, fitted with mechanical stirrer, thermometer and a CaCl₂ drying tube. After bubbling N₂ for 30 min, the mixture was cooled to 0°C in an ice bath. The acylating reagent was prepared by mixing 100 mmol each of aluminum chloride (13.33 g) and of benzoyl

chloride (11.65 ml) in 100 ml nitrobenzene previously deoxygenated with N₂ for 30 min. The acylating reagent was added slowly, so that the temperature of the resin suspension remained about 3°C, and after the removal of the ice bath the mixture was stirred at 35°C (inner temperature). Kinetic studies were done with several 25-g portions of the starting resin, using 4 mmol of acylating reagent per gram of copolymer, in 0.4 M concentration, for various lengths of time (up to 15 h). One independent batch (25 g) of copolymer was submitted for 5h to the same protocol, but with more forcing conditions (7 mmol/g, in a 0.6 M concentration of acylating reagent).

The benzoyl-polymers were filtered and washed exhaustively with nitrobenzene, EtOH, EtOH:H₂O (1:1), MeOH, DCM and MeOH, and then dried in vacuo at 70°C. As expected, the cream-colored resins showed a strong IR band at 1655 cm⁻¹ (C=O). The amount of benzoyl groups in each resin was determined as detailed elsewhere⁹, through the conversion of carbonyl groups to their oxime derivative followed by N elemental analysis.

Phenylaminomethyl-Polystyrene-1% Divinylbenzene (Leuckart reaction): kinetics of the reductive amination step was studied with several portions (5 g each) of a polymer containing 1.44 mmol carbonyl groups per gram. Typically, a mixture of 12 ml (0.3mol) of 88% formic acid, 19 g (0.3 mol) of ammonium formate, 12 ml of formamide and 48 ml of nitrobenzene were added to a 250-ml three-necked flask equipped with a thermometer, an overhead mechanical stirrer, and a Dean Stark trap. Approximately 10 ml of water were removed by distillation as the temperature gradually increased to 140-150°C. The benzoyl-resin was added to the hot mixture and the inner temperature was maintained at 170 ± 1°C. Each portion of resin was submitted to this procedure with reaction times extending up to 50 h. The large proportion of 60 mmol of reagents (at 5 M concentration) per gram of benzoyl-polymer was employed in these kinetic studies. The highest carbonyl-containing resin (2.25 mmol/g) was also reduced in the same manner for 30 h. After the reaction, the resins were collected by filtration, washed and dried as detailed for the benzoyl-polymers.

The last step of BHAR synthesis, consisting of the hydrolysis of formyl groups to generate free ammonium groups, was performed by refluxing the resin with a mixture of 12 N HCl:EtOH (1:1) for 5 h. After washing and drying, each batch of BHAR (obtained as chloride) was characterized by IR spectroscopy, nitrogen elemental analysis and amino acid analysis and the ammonium group content was determined by the picric acid titration method¹¹. The secondary amine content of the resins was determined by a previously described procedure⁹. Briefly, 200 to 500 mg of BHAR was neutralized and then coupled with a 5 molar excess of Boc-Gly activated by the BOP/HOBt method^{12,13}. After a negative ninhydrin test¹⁴, the coupled resin was washed and submitted to picric acid titration for the detection of secondary amine groups which had not coupled with Boc-Gly.

Lys-BHAR and (Lys)₂-Lys-BHAR: Boc-Lys(Boc) was employed to acylate 3 g of the 2.2-mmol/g BHAR. The double coupling strategy was performed by the BOP/HOBt method using a 3 molar excess of the acylating species for 2 h and changing the solvent from DCM/DMF (1:1) to DMF at the second coupling step. 5.05g of Lys-BAR.2TFA⁻ were ob-

tained after the removal of BOC groups by TFA (30% v/v in DCM for 30 min). Amino acid analysis indicated the presence of 1.28 mmol lysine and 2.55 mmol/g ammonium group were found by the picric acid method.

To obtain the small branched lysine core (Lys)₂-Lys-BHAR.4TFA-, the second level of lysyl residue incorporation was introduced with 2 g of LysBHAR, using Boc-Lys(Boc) for coupling, followed by deprotection with TFA. The synthesis yielded 3.21 g of the product, containing 2.36 mmol lysyl residues (determined by amino acid analysis) and 3.15 mmol ammonium groups (determined by the picric acid method) per gram of resin.

Microscopic measurements of bead size

Before the swelling studies, the amine protonated resins (Cl⁻ or TFA⁻ forms) were sized by suspension in EtOH and fine materials were decanted off. The suspension was allowed to stand until approximately 95% had settled before decanting the supernatant. This was repeated about 10 times. The sizing was continued by suspending the resin in DCM and, after about 95% had floated, the solvent containing fine particles was withdrawn. This was also repeated

about 10 times. Due to the lack of swelling observed in DCM, all resins with high ammonium group content (>1 mmol/g) were submitted also to sequential processes of flotation or decantation using the mixture DCM/EtOH in different proportions. Even after this exhaustive treatment, the standard deviation of dry bead diameters (dried in an Abderhalden apparatus at 70°C), still ranged between 8-10% of the average value. In order to develop the swelling study with as narrowly sized population of beads as possible, the last purification of the resins involved repeated sifting of dry beads through several 44 µm to 88 µm pore metal sieves. This sieving procedure lowered the standard deviation of the resin diameters to about 4%. To compare the swelling properties of amine deprotonated BHAR beads with the respective protonated forms (as chloride), the latter were washed (3 x 5 min), in a sintered glass funnel, with a solution of triethylamine (TEA) in DCM (10% v/v), then washed with DCM and MeOH and dried.

Swelling studies of resin beads were performed as published elsewhere¹⁰, with minor alterations. Briefly, 200 to 250 dry and swollen beads of each resin, allowed to solvate for about 5 h, 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 distributed¹⁵, the central value and the distribution of particle diameters were estimated by the more accurate geometric mean values and geometric standard deviations. The average volume of solvent inside the bead was determined by subtracting the volume of the dry from that of the swollen beads.

For the swelling measurements in aqueous media, the dry resin beads were washed with MeOH (2x) and with 0.05 M NH₄Ac (pH 5.0) buffered aqueous solution (10x), in a sintered glass funnel, and allowed to equilibrate for about 5 h before microscopic measurement of the diameters. The reason for the pre-washing with MeOH was to avoid the partial floatation that occurred mainly in low-substituted resins

Figure 1. Synthetic route to benzhydrylamine-resin.

Figure 2. Kinetics of benzoyl group incorporation. Conditions: 4 mmol of acylating reagent per gram of copolymer in 0.4 M concentration, at 35°C.

Figure 3. Kinetics of reductive amination of ketone-polymer. Conditions: 60 mmol of reagents per gram of benzoyl-resin in 5M concentration, at 170 °C. The formyl groups were converted to the ammonium groups by acid hydrolysis for 5 h.

when the aqueous medium was added directly to the dry beads.

Results and Discussion

Kinetics of BHAR Synthesis

The synthetic method⁶ detailed in the Figure 1 was chosen for a re-evaluation of the kinetics of its reaction steps. A literature survey^{6,8,9,16-19} showed a large number of variations on experimental details such as the reaction time,

temperature, concentration of reactants, solvents, etc. Since these variations have been restricted to the acylation and reductive amination steps, kinetic studies of both reactions were carried out using roughly an average of all published experimental conditions.

The Friedel-Crafts benzoylation of copolymer was performed at 35°C with 0.4 M concentration of reactants in nitrobenzene, at a proportion of 4mmol per gram of copolymer. The time-course of carbonyl group incorporation in the polystyrene matrix is depicted in Figure 2. A very fast reaction profile can be seen, with the maximum degree of benzoylation (1.44 mmol/g) reached in 1 to 2 h. Taking into account the proportion of acylation reagent employed (4 mmol/g copolymer), the maximum degree of acylation found was about 35%. Nearly the same value was found in an isolated batch of resin when the proportion and concentration of reactants were increased to 7 mmol/g and 0.6 M, respectively. After 5 h of reaction, a higher (2.25 mmol/g) carbonyl containing resin was obtained.

Several small portions of resin containing 1.44 mmol ketone groups/g were then chosen for the study of the Leuckart reaction step. The kinetics of the reaction was followed for up to 50h, using acid hydrolysis to generate free aminecontaining resins. Comparative IR spectra between ketone-resins and BHARs batches showed a clear dependence of the carbonyl band intensity on reaction time. As the time increased the remaining ketone group peak of the BHAR decreased and, after about 30 h, no band was detected at 1,655 cm⁻¹, indicating a complete conversion of the carbonyl to the amine function.

In contrast to the extremely fast reaction observed in the acylation step, the Leuckart reaction was slow, with the maximum of reduction being reached only after about 30h (Figure 3). The maximum degree of substitution obtained (1.4mmol/g) confirmed the nearly complete reduction of the

Table 1. Swelling of amine deprotonated and protonated (Ct) bhar beads in organic solvents.

RESIN	DCM			DMF		DMSO		EtOH	
	Diam. dry beada ³ (μm)	Diam. swollen bead ^a (μm)	Solvent within bead ^b (10 ⁴ μm ³)	Diam. swollen bead ^a (μm)	Solvent within bead ^b (10 ⁴ μm ³)	Diam. swollen bead ^a (μm)	Solvent within bead ^b (10 ⁴ μm ³)	Diam. swollen bead ^a (μm)	Solvent within bead ^b (10 ⁴ μm ³)
Copolymer ^c	49	97	41	65	8	57	3	50	0.2
BHAR (mmol/g)									
0.04	57	98	39	75	13	68	7	61	2
0.24	57	98	40	79	16	69	8	63	3
0.54	57	98	40	80	17	70	9	63	3
1.40	58	99	41	79	16	67	5	67	5
2.20	58	102	46	93	32	79	16	70	8
BHAR ⁺ (mmol/g)									
0.04	59	97	37	76	12	65	4	59	0
0.24	59	90	28	82	18	66	5	64	3
0.54	59	72	9	82	18	90	27	76	12
1.40	61	66	3	86	21	111	59	98	37
2.20	62	62	0	99	38	126	93	119	76

^a geometric means; ^b difference between volumes of swollen and dry beads; ^c copoly (styrene-1% divinylbenzene)

Table 2. Swelling of amine protonated (Cl⁻) resin beads in 0.05 M, pH 5.0, Ammonium Acetate Buffer.

RESIN	Amine group (mmol/g)	Diam. dry bead ^a (μm)	Diam. swollen bead ^a (μm)	Solvent within bead ^b (10 ⁴ μm ³)
Copolymer ^c	—	49	49	0
BHAR	0.24	59	61	1
BHAR	0.54	59	70	7
BHAR	1.40	61	78	13
BHAR	1.70	61	82	17
BHAR	2.20	62	89	24
Lys-BHAR ^d	2.55	63	107	51
Lys ₂ -Lys-BHAR ^d	3.15	65	119	75

^ageometric means; ^bdifference between volumes of swollen and dry beads; ^ccopoly(styrene1%divinylbenzene); ^dtrifluoroacetate form.

Figure 4. Effect of substitution degree on swelling of amine deprotonated BHAR in DCM, DMF, DMSO and EtOH. **Figure 5.** Effect of substitution degree on swelling of amine protonated BHAR (Cl⁻) in DCM, DMF, DMSO and EtOH.

ketone groups, in agreement with the IR spectra. A ketone-polymer batch containing 2.25 mmol/g submitted to the 30h reduction time also showed a nearly complete conversion of ketone to the amine group, yielding a resin with 2.2 mmol/g substitution. It is, to our knowledge, the most highly substituted BHAR reported thus far.

These kinetic findings allow the following conclusions. First, considering that the experimental conditions employed were roughly the average of those found in the literature, it seems that the reason for the often mentioned difficulties in controlling the degree of substitution is due to the absence of more accurate kinetic controls of both the acylation and the reduction steps. For example, small time differences during the initial phase of the faster acylation reaction (Figure 2) will induce a significant variation on the final amine group content of the resin. Only few similar kinetic experiments necessary for the BHAR synthesis have been reported^{19,20}, and they were restricted to the Leuckart reaction. Second, and most important, our results demonstrate that the reduction of ketone groups can be nearly complete, depending on the Leuckart reaction conditions. A previous report⁹ suggested difficulties in obtaining BHARs free of carbonyl groups due to the incomplete conversion during the reductive amination step. In that work, however, the temperature was lower (165°C) and the reaction time was shorter (22 h) than those used in the procedure described in this paper.

Some positive aspects can be foreseen in the complete reduction of the ketone functionality observed at 30 h of the Leuckart reaction. For example, by employing more drastic conditions during the acylation, much more highly substituted BHAR, such as the 2.2 mmol/g batch, can be synthesized. Furthermore, due to the reported difficulties in obtaining a highly reproducible level of amine substitution, alternative methods have been proposed for BHAR synthesis^{7,21,22}. Our results indicate that this shortcoming in the procedure of Pietta *et al.*⁶ can be circumvented by controlling the benzoylation step. Another implication of our findings is related to the well known importance of resin purity, (preferably free of extraneous reactive groups), for the overall efficiency of peptide synthesis²³. With the 30h Leuckart reaction and following our experimental protocol, BHAR containing no carbonyl groups can be obtained, thus avoiding side-reactions in an analogous manner as that described for resins containing aldehydic functions²⁴.

Figure 6. Effect of ammonium group content on swelling of resins in 0.05 M NH_4Ac solution, pH 5.0. Resins: 1-5, BHARs (Cl⁻); 6, LysBHAR (TFA⁻); 7, Lys₂-Lys-BHAR (TFA⁻).

In agreement with an earlier report⁹ on the BHAR synthetic route studied, no undesirable secondary amine formation was detected for any of the resin batches obtained. Also, there was no evidence of damaged or ruptured beads and, with the exception of the resin submitted to a 50-h Leuckart reaction which had a light brown color, all other resins were cream colored.

Swelling of resin beads

Usually, the swelling capacity of resins is studied through measurements of their swollen volume (ml/g or ml/mmol) in a glass-fritted burette. Besides the low accuracy for the detection of small differences in swelling, this simple volumetric method presents some shortcomings, depending on the study to be carried out. Although valid to estimate, for example, the solvation of one resin in different solvents, the measured solvated volume per gram is not always appropriate for comparing the swelling capacities of different resins. This is due to the fact that each resin has its own dry volume, which may contribute differently to the total swollen volume measured in a burette or in a column.

A more accurate and sensitive approach for the evaluation of the swelling properties of resins is the microscopic measurement of bead sizes. This method was earlier employed to analyse the influence of peptide content on the solvation of peptide-resin beads¹⁰ and allows the determination of the average volume of solvent adsorbed by the bead of each resin sample, calculated by subtracting the volume of the dry from that of the swollen bead.

To obtain reliable swelling data, resins were submitted to a rigorous sizing procedure of their beads. Usually, the starting commercial copolymers are polydispersed, showing a wide range of diameters with standard deviations ranging from 10% to 20%, depending on the manufacturer. By using the sizing method described in this paper, we could lower the dispersion of the resin bead population to close to 4%.

Swelling in organic solvents

Table 1 summarizes the swelling, in organic solvents, of BHARs with different amine group contents, in the deprotonated and protonated (as chloride) forms. The average volumes of solvent per bead were measured, and the Table includes, for comparison, the volume of the starting copolystyrene-1% divinylbenzene resin.

The swelling profiles depicted in Figure 4 indicate that, independently of the degree of substitution, DCM is the best solvent for the swelling of amine deprotonated resins. This solvent, therefore, is the most appropriate for the coupling of the first amino acid residue and also for every washing step where deprotonated amine groups are present during the peptide synthesis cycle. More favourable swelling in polar aprotic solvents has only been observed when the peptide content of the amine-deprotonated resin reached about 20% (w/w)^{4,10,25}. This is due to the fact that, as the amount of polar peptide bonds increases, the peptide-resin resembles the more polar polyamide complex. It was recently demonstrated that the swelling of peptide-resins may be also influenced by the peptide sequence, including the overall polarity of their sidechain protecting groups^{5,25}.

Interestingly, very small swelling of beads was observed in EtOH (Table 1). In solid phase peptide synthesis using the coupling reagent dicyclohexylcarbodiimide (DCC), EtOH is usually recommended for the washing cycle after the coupling reaction, because it is a better solvent than DCM for the solubilization of the byproduct $\text{N,N}'$ -dicyclohexylurea (DCU). In spite of this favourable effect, however, the use of EtOH seems to be inappropriate for efficient DCU washing, owing to the near absence of swelling of resin beads in this solvent. Instead, the mixed solvent DMF/DCM is potentially more efficient for washing DCU off, due to the solubilizing effect of this byproduct by DMF¹, and the enhanced bead swelling in DCM.

More profound dependence of the solvation of beads on the degree of substitution was observed with the amine protonated BHARs (Figure 5). The higher the amount of ammonium groups, the better the swelling of resin in all three polar solvents. Intense repulsion among positively charged groups, in addition to the ion-dipole interaction with polar solvent molecules, are responsible for the drastic alteration in the solvation profile. Among these polar solvents, the aprotic DMSO, which is characterized by a strongly negative dipole²⁶, showed the most significant variation, with an increase in swelling by a factor of about 2 as the loading degree reached 2.2 mmol/g. Accordingly, the more apolar DCM showed a complete lack of solvation of heavily positively charged resins. For resins containing more than 0.4 mmol/g substitution, DMF and DMSO were clearly better swelling solvents than DCM.

Relevant specifically to the high-loaded peptide synthesis approach, our findings suggest that DCM must be replaced by more polar solvents during the washing cycle after the acidic cleavage of the Boc protecting group with TFA. This will avoid drastic shrinking of amine protonated beads in DCM, which could induce inefficient washing of reagents and byproducts, and occasional clogging of the sintered glass in the reaction vessel. Even after the attachment of a large amount of peptide chain to the resin matrix, no significant difference on the swelling behavior of amine-protonated peptide-resins was observed²⁷.

Swelling in aqueous solution

A 0.05 M ammonium acetate solution, pH 5.0, was chosen as the aqueous medium to investigate the correlation between swelling ability of beads and their content of positively charged ammonium groups. The mono- and tri-branched lysine derivatives of the 2.2 mmol/g BHAR were also synthesized in order to obtain heavier amine loaded resins. Picric acid titration indicated amine group contents of 2.55 mmol/g for the Lys-BHAR.2TFA⁻ and 3.15 mmol/g for the branched (Lys)₂-Lys-BHAR.4TFA⁻. The option for the study of the branched rather than the linear tri-lysine resins (both would contain four amine groups per molecule), resides on the further possibility of synthesizing, in one step, an octa-aminated branched lysyl-core bound to the BHAR. This strategy was earlier introduced for the synthesis of peptides for immunological purposes²⁸, and is much simpler than the conventional stepwise linear elongation with lysil residues for the synthesis of octaaminated compounds.

The swelling values of amine protonated resins in aqueous NH₄Ac solution are summarized in Table 2 and the profile depicted in Figure 6 shows how differently ammonium substituted resins respond to the aqueous medium in comparison to the initial copolymer. As anticipated from the swelling of the hydrophobic polymer in polar organic solvents, they took up virtually no water, whereas the modified resins imbibed proportionally to the amount of positively charged groups. The correlation observed between bead ammonium group loading and swelling was rather linear, with the average volume of solvent inside the bead ranging from $1 \times 10^4 \mu\text{m}^3$ in the lowest substituted BHAR (0.24 mmol/g) to $7.5 \times 10^5 \mu\text{m}^3$ in the most highly substituted (3.15 mmol/g) (Lys)₂-Lys-BHAR. These findings are consistent with the general rule for beaded ionized resins²⁹ by which the swelling, and therefore the porosity, depends on the hydration of the matrix, which, in turn, is controlled by the type and the amount of attached ionic functional groups.

One can better estimate the actual hydration capacity of amino protonated BHARs and derivatives in aqueous media by comparing the swelling values shown in Table 2 with those found for the deprotonated forms in organic solvents (Table 1). For example, the swelling, in aqueous solution, of a highly substituted (2.2 mmol/g) protonated BHAR (about $2 \times 10^5 \mu\text{m}^3$) was rather similar to that of deprotonated BHARs in DMF. It is well recognized that an optimized swelling of the polymeric matrix is important for the efficiency of chemical processes occurring inside beads. With the deprotonated 1.4 mmol/g BHAR^{4,5}, we have demonstrated a clear dependence between the efficiency of coupling reactions and the swelling capacity of beads in different organic solvents. In accordance with the swelling behavior of this resin, the rate of reaction was higher in DCM, followed by those in DMF and in DMSO. Although slower than in DCM, the fast rate of reaction observed in DMF, even in unfavorable kinetic conditions (about 70% of reaction in 30 min), still indicates a favourable diffusional process of reactants through the resin. The similarity of the porosities (reflected by swelling values) between highly amine protonated BHARs in aqueous solution and deprotonated BHARs in DMF solutions suggests that, in principle, highly substituted BHARs or their lysyl derivatives

have sufficient hydration capacity to be tested as optional anion exchanger resins in aqueous solution.

In contrast to most commercial resins which contain secondary, tertiary or quaternary amine groups attached to different polymeric matrices (cellulose, dextran, polystyrene, etc.)^{30,31}, the BHAR-type resins contain the more reactive primary amine function, and may be used advantageously to obtain derivatives for different purposes. The determination of their basicity and of the dependence of swelling on the ionic strength of the medium, as well as the use of highly substituted BHARs for the purification of negatively charged compounds are in progress to better evaluate their potential as anion exchangers.

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References

1. R.B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149-2154 (1963).
2. G. Barany and R.B. Merrifield, *The Peptides* (Academic Press, New York, 1980), vol. 2.
3. J.M. Stewart and J.D. Young, *Solid Phase Peptide Synthesis* (Pierce Chem. Co., Rockford, 1984).
4. R. Marchetto, E. Oliveira, A.C.M. Paiva and C.R. Nakaie, *Peptides 1990* (Escom, Leiden, 1991), p. 122.
5. E. Oliveira, R. Marchetto, G.N. Jubilut, A.C.M. Paiva and C.R. Nakaie, *Peptides: Chemistry and Biology* (Escom, Leiden, in press).
6. P.G. Pietta, P.F. Cavallo, K. Takahashi and G.R. Marshall, *J. Org. Chem.* **39**, 44-48 (1974).
7. W.M. Bryan, *J. Org. Chem.* **51**, 3371-3372 (1986).
8. R.C. Orłowski, R. Walter and D. Winkler, *J. Org. Chem.* **41**, 3701-3705 (1976).
9. G.R. Matsueda and J.M. Stewart, *Peptides* **2**, 45-50 (1981).
10. V.K. Sarin, S.B.H. Kent and R.B. Merrifield, *J. Am. Chem. Soc.* **102**, 5463-5470 (1980).
11. B.F. Gisin, *Anal. Chim. Acta.* **58**, 248-249 (1972).
12. B. Castro, J.R. Dormoy, G. Evin and C. Selve, *Tetrah. Lett.* **14**, 1219-1222 (1975).
13. D. Hudson, *J. Org. Chem.* **53**, 617-624 (1988).
14. E. Kaiser, R.L. Colescott, C.D. Bossinger and P.I. Cook, *Anal. Biochem.* **34**, 595-598 (1970).
15. R.R. Irani and C.F. Callis, *Particle size, measurement, interpretation and application* (Wiley, New York, 1963).
16. M. Christensen, O. Shou and V.S. Pedersen, *Acta Chem. Scand. B* **35**: 573-581 (1981).
17. E. Giralt and F. Albericio, *An. Quim.* **77**, 134-137 (1981).
18. V.J. Hruby, F. Muscio, C.M. Groginsky, P.M. Gita, D. Saba and W.Y. Chan, *J. Med. Chem.* **16**, 624-629 (1973).
19. Rivier, J., W. Vale, R. Burgus, N. Ling, M. Amoss, R. Blackwell and R. Guillemin, *J. Med. Chem.* **16**, 545-549 (1973).
20. V.J. Hruby, D.A. Upson and N.S. Agarwal, *J. Org. Chem.* **42**, 3552-3556 (1977).

21. A. Hirao, S. Itsumo, I. Hattori, K. Yamaguchi, S. Nakahama and N. Yamazaki, *J. Chem. Soc. Chem. Commun.* **25-26** (1983).
22. E. Pedroso, J. Josa, J.R. Penalver and E. Giralt, *Peptides 1986* (Walter de Gruyter, Berlin, 1987), p. 159.
23. S.B.H. Kent, *Ann. Rev. Biochem.* **57**, 957-989 (1988).
24. S.B.H. Kent, *Peptides: Structure and Function* (Pierce Chem. Co., Rockford, 1984), p. 99.
25. G.B. Fields and C.G. Fields, *J. Am. Chem. Soc.* **113**, 4202-4207 (1991).
26. D. Martin, A. Weise and H.J. Niclas, *Angew. Chem. Internat. Edit.* **6**, 318-334 (1967).
27. C.R. Nakaie, R. Marchetto, S. Schreier and A.C.M. Paiva, *Peptides: Chemistry, Structure and Biology* (Escon, Leiden, 1990), p. 1022.
28. D.N. Posnett, H. Mc Grath and J.P. Tam, *J. Biol. Chem.* **263**, 1719-1725 (1988).
29. R.L. Albright, P.A. Yarnell and Rohm and Haas Co. *Encyclopedia of Polymer Sciences and Engineering* (John Wiley, New York, 1985), ch. 8, p. 341.
30. P.J. Flory, *Principles of Polymer Chemistry* (Cornell University Press, New York, 1953).
31. J.M. Fréchet, *Tetrahedron* **37**, 663-683 (1981).