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Polystyrene-type resin used for peptide synthesis: application for anion-exchange and affinity chromatography

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Abstract

This paper deals with an unusual application for a copolymer of styrene–1% divinylbenzene bearing high amount of aminomethyl groups for anion-exchange and affinity chromatography. The so-called aminomethyl resin (AMR), to date only employed for peptide synthesis, swelled appreciably in water and was used successfully to purify negatively charged peptides. By correlating swelling degree of beads with pH of the media, it was possible to estimate that the AMR amino group pK_a is approximately 5.5. In addition, the synthesized acetyl–$(NANP)_3$–AMR succeeded in the affinity interaction with large antibody molecules related to malaria transmission and raised previously against this dodecapeptide sequence.

Keywords: Resin; Peptide; Polymer; Ion-exchange chromatography; Affinity chromatography

1. Introduction

The main characteristic of styrene–divinylbenzene copolymers is their propensity to thoroughly solvate in apolar media. This is due to the dominance of aromatic styrene groups in such copolymers. As a consequence, the solid phase peptide synthesis method [1,2] is usually performed in organic solvents such as dichloromethane (DCM) or chloroform. However, this solvation property of the resin beads can be strongly affected if the structure of the copolymer incorporates great amount of amino groups in the protonated form. Large quantities of positively charged groups induce the resin to begin solvating more intensively in polar organic solvents such as dimethylformamide (DMF) or dimethylsulfoxide (DMSO), as well as in water [3].

Employing this type of solvation study, we have previously demonstrated that a polystyrene-based copolymer (benzhydrylamine resin or BHAR) [4], if attaching large amount of positively charged phenylmethylamine function, can be transformed into a weak anion-exchange resin. Negatively charged gangliosides [5] and carbohydrates [6,7] have been successfully fractionated with highly substituted BHAR batches. This resin is indeed, the first developed exclusively for use in peptide synthesis but applied as solid support for anion-exchange chromatography.

The present report represents a step forward in this type of multi-use approach to resin applications. Our objective was to test a highly substituted polystyrene-type aminomethyl group attaching resin known as aminomethyl resin (AMR) [8,9], as solid support for dual anion exchange and affinity chromatography. For the latter application, a desired peptide sequence was synthesized bound to the AMR structure and this peptide-resin was tested as its capacity to interact

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with antibody molecules raised against this sequence. This latter experiment is actually an inverted strategy in comparison with recent unusual finding that commercially available anion-exchange resins such as DEAE-MacroPrep® (Bio Rad) and DEAE-Sephadex® (Amersham Biosciences) can be successfully used for peptide synthesis and subsequent affinity chromatography [10].

The steps developed in this study included initial AMR synthesis under controlled conditions in order to achieve the highest possible amino group loading. Subsequently, solvation of this resin batch in different solvent systems (organic or water) was studied through measurement of dry and swollen beads under microscopy, according to a previously established protocol [3,11]. This type of physicochemical investigation, which focuses on solute-solvent interaction and uses the resin as the model solute, has indeed been of great value in predicting rules which govern solvation of polymeric materials, as well as in the creation of a more accurate solvent polarity scale [12–14]. This type of approach which is based mainly on the electron acceptor/donor concepts of the solvent molecule also allowed us very recently, to propose rules for classifying strong or weak solvents in terms of dissociation capability of peptide chains when attached to resins or free in solution [15]. Needless to emphasize the relevance of these findings in terms not only for the general polymer field but also for solubilization planning of peptide segments with strong aggregating tendencies found for instance, in many neurodegenerative processes such as the Alzheimer disease.

Since prior knowledge of the value of AMR amino group pK_a is essential for its efficient anion-exchange application, this physicochemical parameter was also determined through a swelling method previously introduced for pK_a determination of the BHAR amino group [16]. In this method, the degree of resin swelling is correlated with the pH of the medium. Since this property is directly related to the number of positively charged amino groups on the resin, a downward curve is observed when deprotonation of these groups occurs during the increase of the solution pH. From the mid-point corresponding to the inflection of this sigmoidal curve, one can estimate the pK_a of the resin amino group.

In term of chromatographic applications, the anion-exchange property of AMR was evaluated by testing its capacity to fractionate two peptide sequences containing −1 and −3 net charges, at a neutral pH. An additional and more relevant objective of this report was to evaluate the feasibility of synthesizing a peptide sequence bound to the AMR structure and testing the affinity degree of this composite peptide-resin against a specific ligand macromolecule. A peptide fragment corresponding to the antigenic and immunodominant epitope of sporozoites of the *Plasmodium falciparum* malaria parasite [17] was synthesized in a highly substituted (4.9 mmol/g) AMR batch, and its ability to retain antibody molecules generated against this sequence was evaluated. Other types of solid supports attaching this same peptide sequence were also comparatively examined.

1.1. Abbreviations

Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of IUPAC-IUB (Commission on Biochemical Nomenclature (J. Biol. Chem. 247 (1971) 997). Other abbreviations are as follows: AMR, aminomethyl resin; BHAR, benzhydrylamine resin; BSA, bovine serum albumin; Bu, ter-butyl; DCM, dichloromethane; C_{18}, octadecyl; DEAE, diethylaminoethyl; DIC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethylsulfoxide; Eac, e-aminocaproic acid; EPR, electron paramagnetic resonance; EtOH, ethanol; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, *N*-[(1 -H-benzotriazol-1 -yl)- (dimethylaminomethylene)]- *N*- methylmethanaminium hexafluorophosphate-*N*-oxide; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MAb, mouse monoclonal antibody; MeOH, methanol; NMP, *N*-methylpyrrolidine; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; TEA, triethylamine; TFA, trifluoroacetic acid.

2. Experimental

2.1. Materials

All amino-acid derivatives were purchased from Bachem (Torrance, CA). Solvents and reagents from Fluka (Buchs, Switzerland) or Sigma-Aldrich (Poole, UK) were of analytical grade and were taken from recently opened containers, without further purification. The Rink amide (4-(2,4-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy)-resin and the copolymer of styrene–1% divinylbenzene were purchased from NovaBiochem Co. (Hornby, Ontario, Canada), Advanced ChemTech (Louisville, KY) and Bio-Rad (Hercules, CA), respectively.

2.2. Methods

2.2.1. Swelling studies

Before use in chromatography and microscopy (for measurement of bead sizes), the amino protonated 4.9 mmol/g AMR batch (Cl^- form) were dried in vacuo using an Abderhalden-type apparatus with MeOH refluxing. After drying, the beads were exhaustively sized by suspension in DCM/EtOH and sifted in pore metal sieves to lower the standard deviations of resin diameters to approximately 4%. Dry and swollen beads (150–200 from each resin) were allowed to equilibrate overnight, then spread onto a microscope slide and measured directly at low magnification in an Olympus, model SZ 11 microscope coupled to ImagePlus software, version 3.0.01.00. Since size distributions in these bead samples are lognormal rather than normal, central values and distributions of the particle diameters were estimated by the more accurate method of calculating geometric mean values and

geometric standard deviations [18]. The average amount (percentage) of solvent absorbed by resin beads was calculated by the equation ((swollen volume − dry volume)/swollen volume) × 100, where bead volumes were calculated from their diameter values.

For determination of the AMR amino group pK_a, the following solutions were used in the bead size measurement: phthalate/HCl at pH 2–2.9; HCl/NaAc at pH 3.3–5.5; NaH_2PO_4/Na_2HPO_4 at pH 5.6–7.9; Tris/HCl at pH 8.0—8.9; $Na_2CO_3/NaHCO_3$ at pH 9.0–10.3; and $Na_2CO_3/NaHCO_3$/NaOH at pH 10.4–12.3. In all cases, the ionic strength of the solution was maintained at 0.05.

2.2.2. Peptide synthesis

The peptides were synthesized manually according to the standard tert-butyloxycarbonyl (Boc) [1] or 9-fluorenylmethyloxycarbonyl (Fmoc) [2] protocols. In the former strategy, after the coupling of the C-terminal amino acid to the resin, the successive α-amino group deprotection and neutralization steps were performed in 30% trifluoroacetic acid (TFA)/DCM for 30 min and in 10% diisopropylethylamine (DIEA)/DCM for 10 min, respectively. Conversely, in the Fmoc strategy, a single 20-min piperidine/DMF treatment was sufficient in order to deprotect and neutralize the amino function of the growing sequence. In most cases, the amino acids were coupled at a three- to five-fold excess using diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) in DCM/DMF and, if necessary, *N*-((1-H-benzotriazol-1-yl)-(dimethylaminomethylene))-*N*-methylmethanaminium hexafluorophosphate-*N*-oxide (HBTU)/HOBt/DIEA in 20% DMSO/*N*-methylpyrrolidine (NMP) for coupling steps. After a 2 h coupling time, a ninhydrin test [19] was performed for monitoring the completeness of the reaction. To check the purity of the synthesized peptide sequence assembled in the resins, a cleavage reaction with small aliquots of peptidyl-resin was performed with either anhydrous HF (Boc-chemistry) or reagent K (Fmoc-chemistry). The crude peptides were precipitated with anhydrous ethyl ether, separated from soluble non-peptide material by centrifugation, extracted into 5% acetic acid in water and lyophilized. Analytical HPLC (Waters), LC/MS (electrospray)-mass spectrometry (Micromass) and amino-acid analysis (Biochrom 20 Plus, from Amersham Biosciences) were used to check the homogeneity of each synthesized resin-bound peptide sequence. Free peptides used in anion-exchange or affinity-chromatography applications were synthesized in the same manner but were purified in preparative HPLC until homogeneity was attained.

2.2.3. Analytical HPLC

Analysis was performed in a Waters system consisting of two 510 HPLC pumps, automated gradient controller, Rheodyne manual injector, 486 detector and 746 data module. Unless otherwise stated, peptides were analyzed in a C_{18} Vydac column (4.6 mm × 150 mm, 300 Å pore size, 5 μm particle size) with the solvent systems A (H_2O containing 0.1% TFA) and B (60% MeCN in H_2O containing 0.1% TFA). A linear gradient of 5–95% B over 30 min was applied at a flow rate of 1.5 ml/min and detection at 210 nm.

2.2.4. Preparative HPLC

Purification of peptides was carried out as follows: solvent A (H_2O containing 0.1% TFA); solvent B (60% acetonitrile in H_2O containing 0.1% TFA). A linear gradient was applied, the gradient was dependent upon the retention time, as verified with analytical HPLC of the peptide using the same solvent systems. The flow rate was 10 ml/min, with peak detection at 210 nm.

2.2.5. Anion-exchange chromatography

The 4.9 mmol/g AMR batch (chloride form) was pretreated with 20% ethanol in water and thoroughly washed with the initial buffer before packing the column. The column was previously equilibrated with 0.02 M of ammonium acetate solution, pH 4.5 and a linear gradient from pH 4.5 to approximately pH 2.0 (20%, v/v, AcOH) was applied for elution of negatively charged peptides. A peristaltic pump (LKB), a model GM-1 gradient mixer (Amersham Biosciences, Piscataway, NJ) and a Foxy 200 collector (Isco, Lincoln, NE) were used for chromatography experiments.

2.2.6. Affinity experiments

Microliter plates for ELISA (Corning, NY, USA) were coated with 200 ng/well of purified recombinant protein containing the repeat domain of *P. falciparum* circumsporozoite protein [20], generously provided by Dr. Victor Nussenzweig of the New York University School of Medicine. After overnight incubation at room temperature, the plates were washed three times with PBS containing 0.05% Tween-20 (PBS-Tween). The plates were blocked at 37 °C for 2 h with PBS containing 5% (w/v) nonfat milk and 1% (w/v) of bovine serum albumin (BSA, Sigma, St. Louis, MO).

A purified mouse monoclonal antibody (MAb), specific for the peptide $(NANP)_3$ and corresponding to the immunodominant epitope of the sporozoite of the *P. falciparum* malaria parasite [17], was used in a final concentration of 20 ng/ml to detect the recombinant protein. This MAb was kindly provided by Dr. Ruth S. Nussenzweig, also from New York University School of Medicine, and was produced and characterized as previously described [21,22]. After a 2 h incubation at room temperature, unbound antibodies were washed away with PBS-Tween, and heavy and light chains of peroxidase-conjugated goat anti-mouse IgG, diluted 1:4000, were added to each well. After 1 h of incubation at room temperature, excess labeled antibody was removed through washing, and the reaction was developed with *o*-phenylenediamine. Plates were read at 492 nm on a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). Results are expressed as the average OD_{492} nm of duplicate samples.

The inhibition assay was performed using free Ac-$(NANP)_3$ and Ac-$(NANP)_3$-attaching AMR (4.9 mmol/g), BHAR (1.4 mmol/g) and Rink resin (0.5 mmol/g). As a control, the peptide-free 4.9 mmol/g AMR batch was also included in these comparative assays. These compounds were diluted in PBS (two-fold dilution ranging from 2.0 to 3.9 μg/ml) in a final volume of 100 μl. Control resins without peptide were added in amounts equivalent to those of the peptide-coupled resins. Aliquots of 100 μl of a solution containing 40 ng/ml of MAb, diluted in PBS with 10% non-fat dry milk and 2% BSA, were added to each well. After a 2 h incubation at room temperature with agitation, 60 μl of each sample were transferred to the wells of ELISA plates coated with the recombinant protein. The ELISA was then performed as described above and the percentage of inhibition for each resin or peptide was calculated as follows:

$$\text{Percentage of inhibition} = \frac{\text{Abs}_{492} \text{ with peptide}}{\text{Abs}_{492} \text{ without peptide}} \times 100$$

3. Results and discussion

3.1. Synthesis of highly amino acid-loaded AMR batch

Although an alternative synthesis strategy for AMR has been proposed [23], the present study followed the pioneering procedure described by the Merrifield group [8,9]. In this procedure, incorporation of the aminomethyl-group into the structure of the copolymer of styrene–1% divinylbenzene is carried out in two steps (Fig. 1), and the alternative strategy herein applied to obtain a heavily amino-acid-loaded AMR batch was based mainly on the first step, as detailed below.

3.1.1. Synthesis of phthalimidomethyl resin

A quantity of 14 g (79 mmol) of *N*-(hydroxymethyl)phthalimide was added to 12 g of copolymer of styrene–1% divinylbenzene in 50% TFA/DCM as the solvent system (180 ml). Subsequently, 50 g (333 mmol/g) of trifluoromethanesulfonic acid were added to the suspension slowly and with constant stirring. The reaction proceeded for approximately 5 h at room temperature. The proportions between reagents of the reaction were therefore approximately 6.6 mmol and 28 mmol per gram of copolymer, respectively. After the reaction, the resin was filtered and washed sequentially with 50% TFA/DCM, DCM, EtOH and DCM in a sintered glass funnel. The resin was dried under vacuum until constant weight (25.12 g) was achieved.

3.1.2. Hydrolysis

A 20 g sample of phthalimidomethyl resin was stirred in 500 ml of 5% hydrazine in ethanol under reflux for 24 h. After this treatment, the resin was quickly filtered (hot) and washed in boiling ethanol and methanol to eliminate the 2,3-dihydro-1,4-phthalazinedione by-product, followed by washings in water, ethanol and DCM. The product was dried under vacuum and the degree of amino group substitution (4.9 mmol/g, chloride form) was determined using the picric acid method [24] and nitrogen elemental analysis. In order to obtain a population of more homogeneously sized beads, the synthesized AMR batch was submitted to a special fractionation treatment, as previously described [3]. These treatments yielded a total of 12 g of the highly substituted AMR batch.

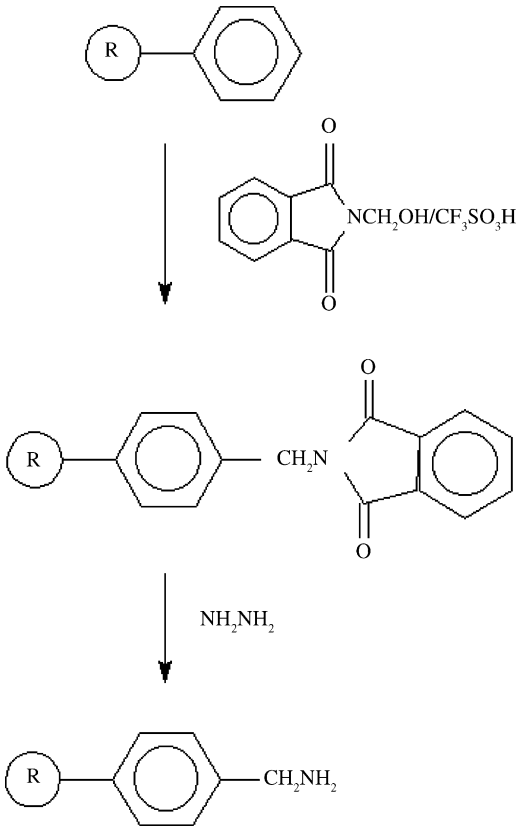

Fig. 1. Scheme of the synthesis of aminomethyl-resin (AMR).

3.2. Swelling studies of the AMR

Table 1 displays data regarding the swelling characteristics of the 4.9 mmol/g AMR batch (Cl^- form) in some organic solvents and in water. Although this resin contains a hydrophobic polystyrene-type matrix, its high positive charge, predictably, resulted in greater swelling in polar solvents, including water. In this solvent, approximately 90% of each swollen AMR bead was occupied by solvent molecules thus suggesting that this capacity might enable this solid support for column chromatography.

3.3. Estimation of the pK_a of the AMR amino group

As previously mentioned, the protocol developed to estimate the pK_a of resin functional groups [16] is based on the measurement of bead swelling in all pH ranges in buffered aqueous solution. By correlating the degree of swelling with the pH of a given medium, a decreasing sigmoid curve is

Table 1
Extent of swelling of 4.9 mmol/g AMR with amine groups in the protonated form in several solvents

Solvent	Diameter of swollen bead[a] (μm)	Volume of solvent within bead[b] (10^5 μm^3)	Solvent within bead[c] (%)
DCM	57.7	0.2	16.9
MeOH	80.4	1.9	69.3
DMF	66.8	0.7	46.7
DMSO	107.9	5.8	87.4
H_2O	111.3	6.4	88.5

[a] Geometric mean values.
[b] Swollen volume – dry volume.
[c] Volume of solvent within bead/swollen volume) × 100 using the value for measured diameter of dry beads: 54.2 μm.

obtained as the pH increases, and the mid-point of inflection of this curve corresponds to the pK_a of the amino group of the resin. As can be seen in Fig. 2, the pK_a of the AMR amino group is approximately 5.5, indicating that any anion-exchange experiments with this cationic resin should be carried out at pH 4.5 or lower in order to guarantee complete protonation of its amino function. Otherwise this low pK_a for the AMR amino function seems to be due to the low dielectric constant of the polystyrene backbone contributing to the reduction of the basicity of this group. Similar result has been reported for the amino group of tetracaine when located internally to an apolar environment found in the interior of a lipid bilayer [25].

3.3.1. Peptide synthesis

The following peptides necessary for anion-exchange or affinity experiments were synthesized:

(a) P_1 = Asp-Arg-Val-Tyr-Ile-His-Pro-$CONH_2$
This peptide was synthesized through Boc-chemistry using a 0.2 mmol scale and starting from a 0.65 mmol/g methylbenzhydrylamine-resin (MBHAR) [26] in order to obtain the C^{α}-carboxamide peptide sequence. The final cleavage was performed with anhydrous HF, and yielded 180 mg of crude peptide. After preparative HPLC purification, 114 mg of pure compound for further anion-exchange chromatography were obtained. Characterization: single peak in analytical HPLC; ESI/MS, *m/z*: 898.0 (theoretical), obtained (899.1); AAA = Asp (0.98), Arg (1.03), Val (0.99), Tyr (0.97), He (1.02), His (1.00), Pro (1.01).

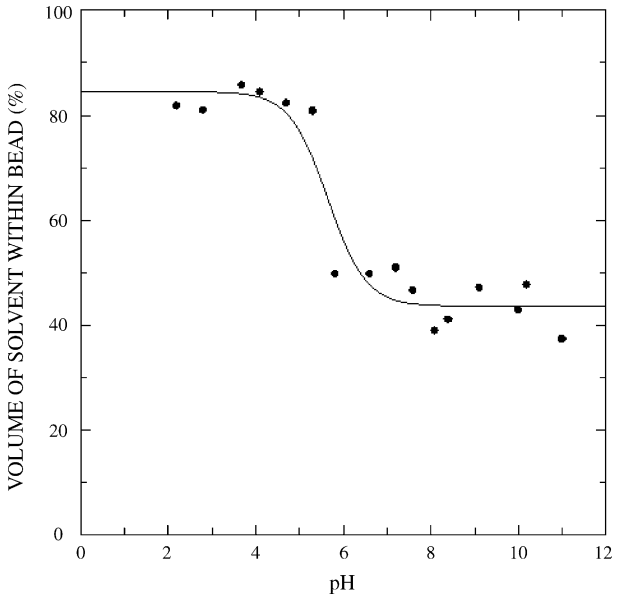

Fig. 2. Degree of swelling of the 4.9 mmol/g AMR as a function of the pH of the medium.

(b) P_2 = Asp-Glu-Val-Tyr-Ile-His-Pro-Phe-COO^-
This peptide was also synthesized with the Boc strategy using a 0.2 mmol scale starting from a 0.6 mmol/g 4-(oxymethyl)-phenylacetamidomethyl-resin (PAMR) used for obtaining free-α-carboxyl sequences. After final HF cleavage, 165 mg of crude sample were obtained and preparative HPLC purification yielded 105 mg of pure compound. Characterization: single peak in analytical HPLC; ISI/MS, *m/z*: 1019.1 (theoretical); obtained (1019.7); AAA = Asp (1.03), Glu (1.01), Val (0.97), Tyr (0.98), He (1.00), His (1.00), Pro (0.99), Phe (1.02).

(c) P_3 = Asp-Glu-Val-Tyr-Ile-Glu-Pro-Phe-COO^-
This peptide was synthesized in the same manner as the previous sequence. The final cleavage yielded 182 mg of crude peptide and, after HPLC purification, 108 mg of homogeneous peptide was obtained with the following characterization: single peak in analytical HPLC, ISI/MS; *m/z*: 1011.1 (theoretical), obtained (1012.0); AAA = Asp (1.00), Glu (2.04), Val (0.98), Tyr (0.99), He (1.01), His (0.99), Pro (1.01), Phe (0.98).

(d) Fmoc-NANP-COOH
This peptide was synthesized using the (4-hydroxymethylphenoxyacetic acid, HMPA) linker [2] attached to a 0.65 mmol/g BHAR to release the peptide from the resin with free carboxyl function after reagent K treatment. The scale of synthesis was 1 mmol using the Fmoc chemistry, and the 20% piperidine/DMF treatment to remove Fmoc group was not applied at the end of synthesis before final cleavage with reagent K. A total of 610 mg of crude peptide were obtained and, after HPLC purification, 485 mg of homogeneous compound necessary for further assembling of the $(NANP)_3$ sequence were obtained. Characterization:

single peak in analytical HPLC; ESI/MS, *m/z*: 637.0 (theoretical); obtained (637.4); AAA = Asn (1.93), Ala (0.97), Pro (1.04).

(e) Ac-$(NANP)_3$-COOH
This peptide was also synthesized with the Fmoc-strategy starting from the HMPA-BHAR (0.65 mmol/g) using a 0.2 mmol scale with three successive couplings of the previously prepared Fmoc-NANP-COOH segment. After cleavage with reagent K, 62 mg of crude peptide were obtained and yielded 35 mg after HPLC purification. Characterization: single peak in analytical HPLC; ESI/MS, *m/z*: 1249 (theoretical); 1248.8 (obtained); AAA = Asn (1.90), Ala (0.95); Pro (1.06).

3.4. Anion-exchange chromatography with the 4.9 mmol/g AMR

The potential of the highly substituted AMR as anion-exchange support was tested by fractionating the peptides P_1 (Asp-Arg-Val-Tyr-Ile-His-Pro-$CONH_2$), P_2 (Asp-Glu-Val-Tyr-Ile-His-Pro-Phe-COO^-) and P3 (Asp-Glu-Val-Tyr-Ile-Glu-Pro-Phe-COO^-), with net charges of approximately +2, −1 and −3 at neutral pH.

Due to its positive charge at neutral pH, the P1 peptide was used as a control for the chromatography conducted in this study. Approximately 10 mg of each peptide were applied to a column containing 0.65 g (3.2 mmol of ammonium groups per column) of the 4.9 mmol/g AMR, equilibrated in a 0.02 M, pH 4.5 ammonium acetate buffer. After injection of the three-peptide mixture, a pH gradient was applied starting from the equilibrium solvent system up to AcOH 10% solution, pH 2.2 (130 ml each). After the gradient, the resin was rinsed sequentially with 5% AcOH (50 ml) and 20% AcOH (250 ml). The collected volume per tube was 5 ml, the flow rate was 15 ml/h, and peak detection was at 275 nm.

Fig. 3 shows the elution profile obtained with this experimental protocol. As expected, the positively charged peptide P_1 was not retained in the column, and elution occurred in the void volume of the column. Otherwise, the negatively charged (−1 or −3) peptides were retained in the cationic resin and, predictably, eluted separately, with higher

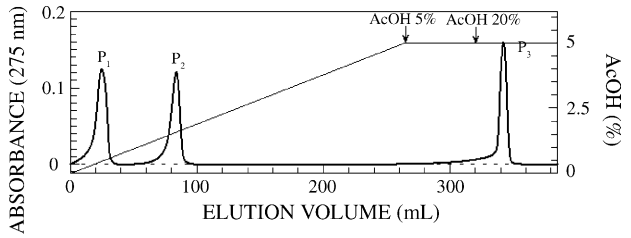

Fig. 3. Anion exchange chromatography of DRVYIHP-amide (P_1), DEVYIHPF (P_2) and DEVYIEPF (P_3) in 4.9 mmol/g AMR (3.2 mmol/g/column, bed volume 2.7 cm^3). Linear pH gradient from 0.02 M ammonium acetate solution, pH 4.5–10% AcOH, pH 2.2 (130 ml each). Aqueous solutions containing 5 and 20% AcOH were applied to the column at 260 and 320 ml, respectively.

retention of the −3 compound. These results indicate that the AMR support, to date used only for peptide synthesis, can be applied as a cationic resin for chromatography, as was previously reported for 2.4 mmol/g BHAR, another polystyrene-type resin that attaches phenylaminomethyl groups instead. This latter solid support presented similar efficiency in the fractionation of negatively charged gangliosides [5] or carbohydrates [6,7] if compared with commercial anion exchanger resins such as the dextran-based DEAE-Sephadex A25 (Amersham Biosciences) or the quaternary ammonium-bearing polystirene–2% divinylbenzene-based AG1-X1 (Bio Rad Co.).

Despite its comparatively low exclusion limit if compared, for instance, to those containing highly hydrophilic dextran- or agarose (Sepharose)-type anion exchanger matrices, the presence of more reactive primary amino groups in the AMR structure can be advantageous since this basic function could be easily derived in order to obtain novel resin derivatives. Depending on the process, these polymers could be used as alternative hybrid supports, either for column chromatography, synthesis of specific macromolecules or in combinatorial synthesis strategy [27,28].

3.5. Affinity assays with peptide-AMR

Initial experiments of peptide cleavage from the AMR support with reagent K indicated that the peptide-resin linkage is very stable. Less than 10% of peptide chains were removed after 5 h under this treatment, suggesting that the AMR support might be used for the synthesis of a desired peptide sequence and further assay as an affinity resin.

To test this possibility, the repetitive Ac-$(NANP)_3$ sequence, which, as previously mentioned, is the immunodominant epitope found in the circumsporozoite protein of *P. falciparum* [17], was synthesized in the 4.9 mmol/g AMR. Before assembling the peptide sequence, the ε-aminocaproic acid (Eac) was coupled to the resin as a spacer in order to improve, by increasing the distance between the peptide and resin core, the possibility of affinity binding of the ligand macromolecule to the solid support. In order to better assess the efficiency of the peptide-AMR support in terms of affinity capacity, the 1.4 mmol/g BHAR and the 0.5 mmol/g Rink-resin were also used to synthesize the Ac-$(NANP)_3$-Eac segment. Thus, after incorporation of the Eac spacer, these resins were submitted to three successive couplings of the previously synthesized and purified Fmoc-NANP-COO^- segment in order to obtain the desired dodecapeptide $(NANP)_3$-ligand for affinity assay. The Fmoc-strategy as detailed in the Section 2.2, was applied to these syntheses. The last step comprised Fmoc removal of the N-terminal portion of the assembled peptide, followed by acetylation reaction, in order to rule out the possibility of anion-exchange interaction with the ammonium groups of the peptidyl-resin during the affinity experiment. The corresponding degree of Ac-$(NANP)_3$-loading of each AMR, BHAR and Rink resin was determined through amino acid analysis.

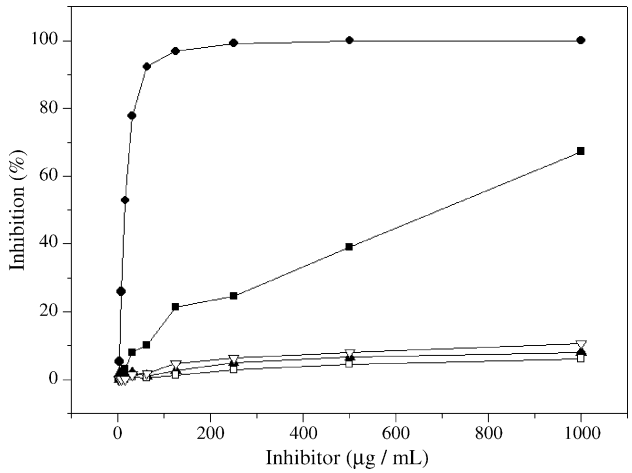

Fig. 4. Inhibition of binding of the purified monoclonal antibody to a recombinant protein containing the epitope $(NANP)_3$ segment with Ac-$(NANP)_3$ (●), Ac-$(NANP)_3$-Eac-AMR (■), Ac-$(NANP)_3$-Eac-Rink resin (▲), Ac-$(NANP)_3$-Eac-BHAR (1.4 mmol/g) (▽) and 4.9 mmol/g AMR (□).

As previously explained, the affinity assay consisted of measurement of the capacity of each peptidyl-resin to retain antibody molecules generated by the $(NANP)_3$ sequence. As a necessary control, the peptide-free AMR and the Ac-$(NANP)_3$ sequence were also included in these comparative experiments. The determination of the affinity values of the acetyl-$(NANP)_3$ segment, free or bound to each of the three resins was based on the (percentage) inhibition of the binding of a purified recombinant protein containing the repetitive domain $(NANP)_n$ of the circumsporozoite protein of *P. falciparum* to a purified mouse monoclonal antibody, generated for this peptide segment.

Fig. 4 displays the inhibition curves obtained by incubating the free peptide Ac-$(NANP)_3$-COO^-, the Ac-$(NANP)_3$-Eac fragment bound to AMR, the Rink resin and the 1.4 mmol/g BHAR and also the peptide-free 4.9 mmol/g AMR. As in the previous study [10], in order to compare the inhibitory capacity of the Ac-$(NANP)_3$-inhibitor when free in solution with that of those attached to resin structures, the amount of each peptide-resin required for these comparative inhibition experiments was calculated according to the previously determined peptide content of each sample. As expected, the highest inhibition occurred with the free peptide, followed by the peptidyl-AMR support. Neither other peptidyl-resins nor the peptide-free AMR batch alone inhibited interaction between the antibody macromolecules and the recombinant protein containing the $(NANP)_n$ epitope. Quantitatively, the degree of inhibition can be estimated by the amount of peptide inhibitor, free or bound to the resin, necessary for inducing 50% of maximum inhibition (IC_{50}). In the present case, the values calculated from Fig. 4 were 12.5 and 700 μg/ml of Ac-$(NANP)_3$-COO^- when free in solution or bound to the AMR structure, respectively. These findings indicate that the linkage to the AMR backbone reduces, by approximately 50 times, peptide affinity for the antibody molecules.

In our previous report [10], we evaluated with this same approach, the peptide-bearing commercial anion-exchange DEAE-Sephadex (dextran-based) and DEAE-MacroPrep (acrylamide-based) resins and obtained values of 108 and 222 μg/ml, respectively. Comparing these values with those of the present work, one can conclude that, probably owing to the well-known smaller exclusion limit of polystyrene-type supports, the AMR affinity efficiency is lower.

In the other hand, the lack of inhibition observed when using other types of polystyrene resins such as the peptide-Rink or peptide-BHAR resins might be likely due to their comparatively low swelling capacities in aqueous solution. In this respect, we have to consider that, when attaching large amount of positively charged ammonium groups, the swelling data obtained for these resins in water would be considerably different than those for resins bearing coupled peptide chains for affinity purposes. This difference is clearly dependent upon the quantity and nature of the resin-bound peptide segment. In the present case in particular, the Ac-$(NANP)_3$ sequence is one of most well-known hydrophilic segments that is evaluated by its amino-acid composition, with a hydrophobicity value of approximately-1, as previously reported [29]. One may therefore, infer that even a very hydrophobic polymer, if bearing a large quantity of this type of peptide sequence, will certainly display enhanced solvation in polar media (including water), as has been demonstrated in previous studies [13,15]. Therefore, based upon the calculated degree of peptide loading in the highly polar Ac-$(NANP)_3$-sequence assembled in the 4.9 mmol/g AMR, 1.4 mmol/g BHAR and 0.5 mmol/g Rink resins (87, 66 and 41%, respectively), one can predict that the solvation capacity of resin beads in the aqueous buffered system used during the affinity chromatography will follow this decreasing order. Owing to its enhanced swelling values, the steric hindrance effect for the peptide-antibody interaction throughout the resin matrix would thus be less pronounced in the AMR support since it has higher peptide content and therefore, greater swelling. Nevertheless, the possibility that interaction between the antibody molecules and the peptide chains occurs predominantly on the surface of AMR support beads can not be ruled out.

Despite this possibly lower efficiency of AMR for affinity purposes when compared with other types of more hydrophilic commercial resins that usually contain much higher exclusion limit values provided for instance, by agarose-type matrix, the findings obtained herein are innovative and valuable. In the present study, we demonstrated that a hydrophobic polystyrene-type resin can function reasonably as an affinity support. In contrast to this type of AMR support weakness, especially for affinity purposes, one must consider that its degree of ligand loading is several orders of magnitude greater (at the mmol/g level) than that typically found in commercial resins. Moreover, its structure is much more physically and chemically stable under a variety of conditions.

In addition, the classical procedure applied to commercial affinity resins usually comprises the covalent coupling

of previously purified ligand molecules to specific sites on the solid support. In many cases, multiple or nonspecific binding to these attaching groups, as in the case of peptides with several reactive sites in their sequence, may occur. Due to differences in the means of binding the ligand to the resin, a decrease in the efficiency of affinity purification is expected. To circumvent this limitation, there is indeed the option of performing a prior synthesis of the desired protected peptide segment, using an appropriate linker between the assembled peptide sequence and the resin core. However, in this case, it is necessary to include additional steps in order to cleave the protected peptide from the resin and subsequently and purify it prior to its coupling with the determined affinity resin. As emphasized for AMR in this study, one must synthesize only the desired sequence within its structure and apply that directly to the affinity experiments.

Furthermore, this aminated resin can be also tested to combine simultaneous anion-exchange and affinity interactions. It is chemically possible to assemble peptide chains in only a fraction of the large number of amino groups present in the AMR backbone, leaving the remaining basic sites for anion-exchange interaction. Undoubtedly, the weakness of this approach is the lack of specificity for affinity purposes caused by the appearance of the anion exchange effect. Nevertheless, this unique chromatographic protocol would, in some circumstances, enable a less-expensive, single-step purification protocol to be used for some types of negatively charged macromolecules.

In summary, the results of our assessment of the highly amino-acid-loaded AMR represent a continuation of earlier efforts to explore the prospects for a multi-use approach to polymer application studies. This has been demonstrated in the case of the anion-exchange capacity of BHAR [5–7] and in the unexpected peptide synthesis and affinity capacities of some commercial anion-exchange resins [10]. Additional physicochemical and chromatographic experiments are currently being undertaken in order to further evaluate the potential of the AMR support.

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