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Short Communication

Imprinted dispersion polymers: a new class of easily accessible affinity stationary phases

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Abstract

Non-stabilizing dispersion polymerization in combination with molecular imprinting was used to prepare agglomerates of globular micron-sized particles exhibiting molecular recognition properties. These could be prepared either *in situ* in a chromatographic column or separately followed by wet or dry packing of the material. This allowed a rapid chromatographic evaluation of the molecular recognition properties of the materials. Depending on the monomer concentration and the solvency of the dispersion medium the particle dispersity, the degree of particle agglomeration and the average particle size varied. The choice of dispersion medium was mainly dictated by the template solubility and the nature of the interactions between the functionalized monomers (methacrylic acid) and the template used for producing the molecular recognition sites. Addition of water to the dispersion medium allowed imprinting of the poorly soluble template pentamidine (PAM), a drug used for the treatment of AIDS-related disorders. The PAM-imprinted materials prepared *in situ* in the chromatographic column strongly retained the drug in the chromatographic evaluation compared to the retention of PAM on a reference material prepared using benzamidine as template (separation factor $\alpha' = 6.8$). Meanwhile weakly or moderately basic templates from the group nucleotide bases (tri-O-acetyladenosine), herbicides (atrazine) and chiral amino acid derivatives (L-phenylalanine anilide) required low temperature and exclusion of water during imprinting in order to produce the recognition effect.

1. Introduction

In the medical and environmental fields the analysis or isolation of target molecules in complex mixtures is often achieved by the use of biological macromolecules. Immunoassays [1] and affinity chromatography [2] are techniques based on the high selectivity and affinity of antibodies and enzymes towards their antigens and substrates. The biomolecules of these systems often suffer from poor stability and a complicated preparation scheme. Imprinted polymers capable of molecular recognition but without these shortcomings constitute an interesting alternative. These materials are prepared by molecular imprinting [3–10] whereby functional monomers, preorganized around a template molecule, are copolymerized in homogeneous solution with a cross-linking monomer leading to the formation of a highly cross-linked

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network polymer. After washing out the template the materials can be used as affinity stationary phases in the chromatographic mode. Strong, highly selective binding has been observed for enantiomers of basic compounds [4–8], for nucleotide bases [9] and for commercial drugs [7,10].

The imprinted polymers are usually obtained as blocks that need to be ground and sieved before use. This results in irregular particles, poor chromatographic performance and a loss of unsized material. Following a general procedure developed by Svec and Fréchet [11] describing *in situ* prepared continuous rods of macroporous polymer as HPLC separation medium, Matsui *et al.* [12] showed a way to circumvent these difficulties by preparing columns of flow through continuous rods of imprinted polymers. Independently of their work we have developed a dispersion polymerization procedure for *in situ* preparation of imprinted affinity phases in aqueous or polar media [13,14] (dispersion polymerization is defined as a modified precipitation polymerization where the monomer but not the polymer is soluble in the dispersion medium and where well defined polymer particles are formed. Addition of a stabilizer results in the formation of spherical particles of a low dispersity) [15,16]. The resulting materials consist of agglomerates of micron-sized globular particles (Fig. 1) with a microporous (Fig. 1a, b) or mesoporous (Fig. 1c, d) morphology. In analogy with the acrylamide-based materials developed by Hjertén [17] our materials can be prepared *in situ* in a chromatographic column or dispersed separately for column packing by conventional techniques. The resulting columns are stable, they have a low flow resistance and are able to selectively retain the complementary substrate.

2. Experimental

2.1. Chemicals

Phenylalanine (D and L) anilide (PA) were synthesized as described elsewhere [4] whereas tri-O-acetylcytidine (TAC) and the template tri-O-acetyladenosine (TAA) were purchased from Sigma. Pentamidine (PAM) as the isethionate salt was a generous gift from Rhone Poulenc Pharma (Helsingborg, Sweden), the reference benzamidine (BAM) was purchased from Aldrich as the hydrochloride and atrazine (ATR) was purchased from Janssen Chimica. The monomers ethyleneglycoldimethacrylate (EDMA) and methacrylic acid (MAA) (Fig. 2) and the initiator azo-bisisobutyronitrile (AIBN) were all purchased from Aldrich and purified following standard procedures [8]. PAM isethionate was converted to the free base by basifying an aqueous solution (K_2CO_3) of the drug and collecting the hereby formed precipitate. Purification was done by redissolving the precipitate in EtOH, filtration of the EtOH solution and finally evaporation giving PAM as a white solid. BAM–HCl was converted to its free base by extraction into ethyl acetate.

2.2. Polymer preparation

The polymers were prepared using the monomer compositions and solvents indicated in Table 1. As a typical example the preparation of **P5**–PAM (Fig. 2) is described: PAM (0.125 mmol) in the free base form was dissolved in isopropanol (2.8 ml) and EDMA (12 mmol). Addition of MAA (0.5 mmol) caused formation of a precipitate which went back into solution by the addition of water (1.3 ml). Initiator (AIBN, 12 mg) in isopropanol (0.5 ml) was added and the solution purged with nitrogen and heated to 40°C for homogenization. The solution was then transferred under nitrogen to glass tubes (150 mm × 5 mm O.D. × 3 mm I.D.), the tubes sealed and left in an oven at 60°C for 24 h. The remaining polymers were prepared by photoinitiation [8] at 5°C using a Beamboost photolytic reaction chamber except for **P4** where a Hg medium pressure lamp was used.

2.3. Chromatographic evaluation

The tubes containing polymer were cut to a length of 140 mm and equipped with Valco column end fittings containing Vespel ferrules. Alternatively the material could be dispersed in

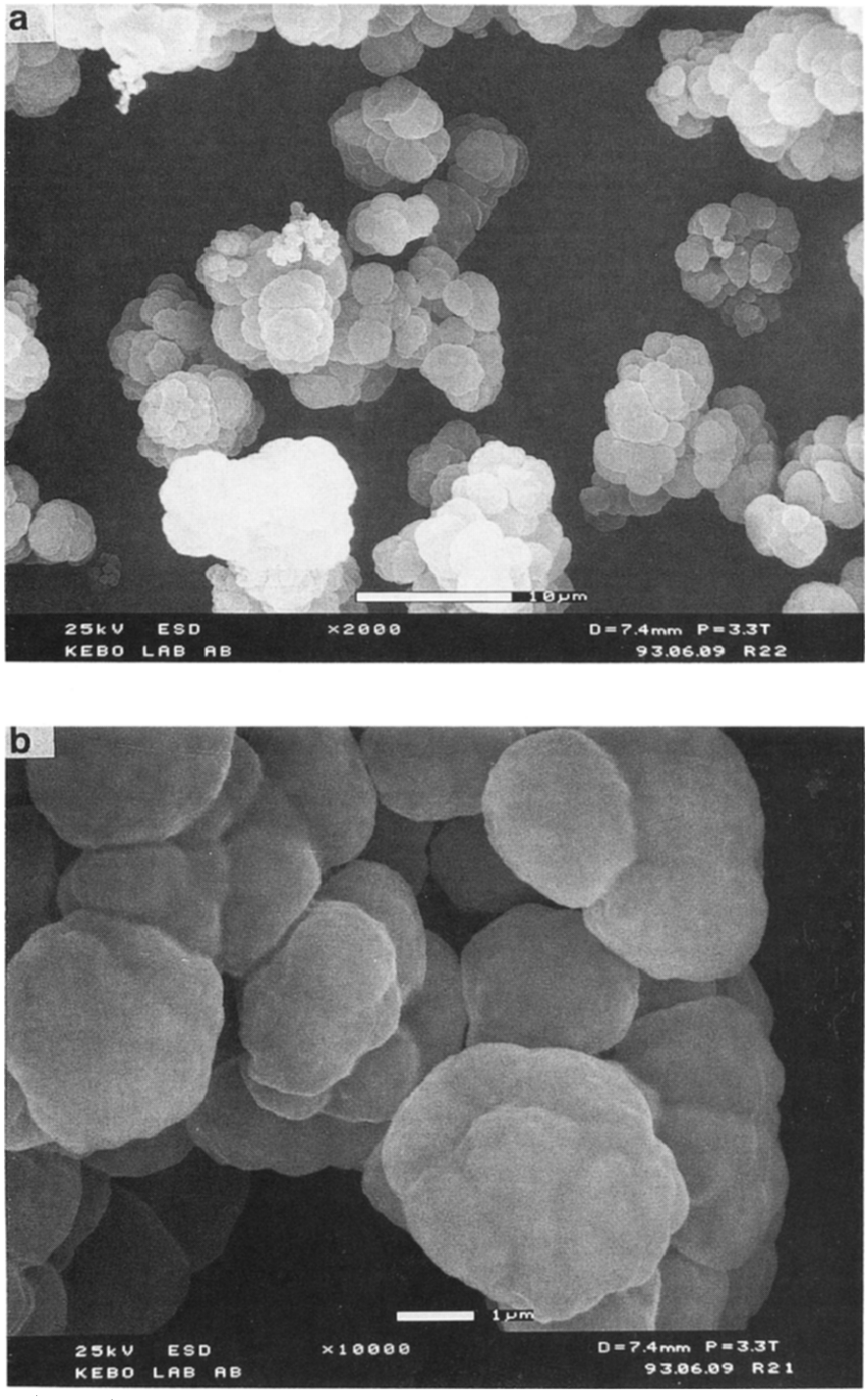
a
10µm
25kV ESD ×2000 D=7.4mm P=3.3T
KEBO LAB AB 93.06.09 R22
b
1µm
25kV ESD ×10000 D=7.4mm P=3.3T
KEBO LAB AB 93.06.09 R21

Fig. 4.

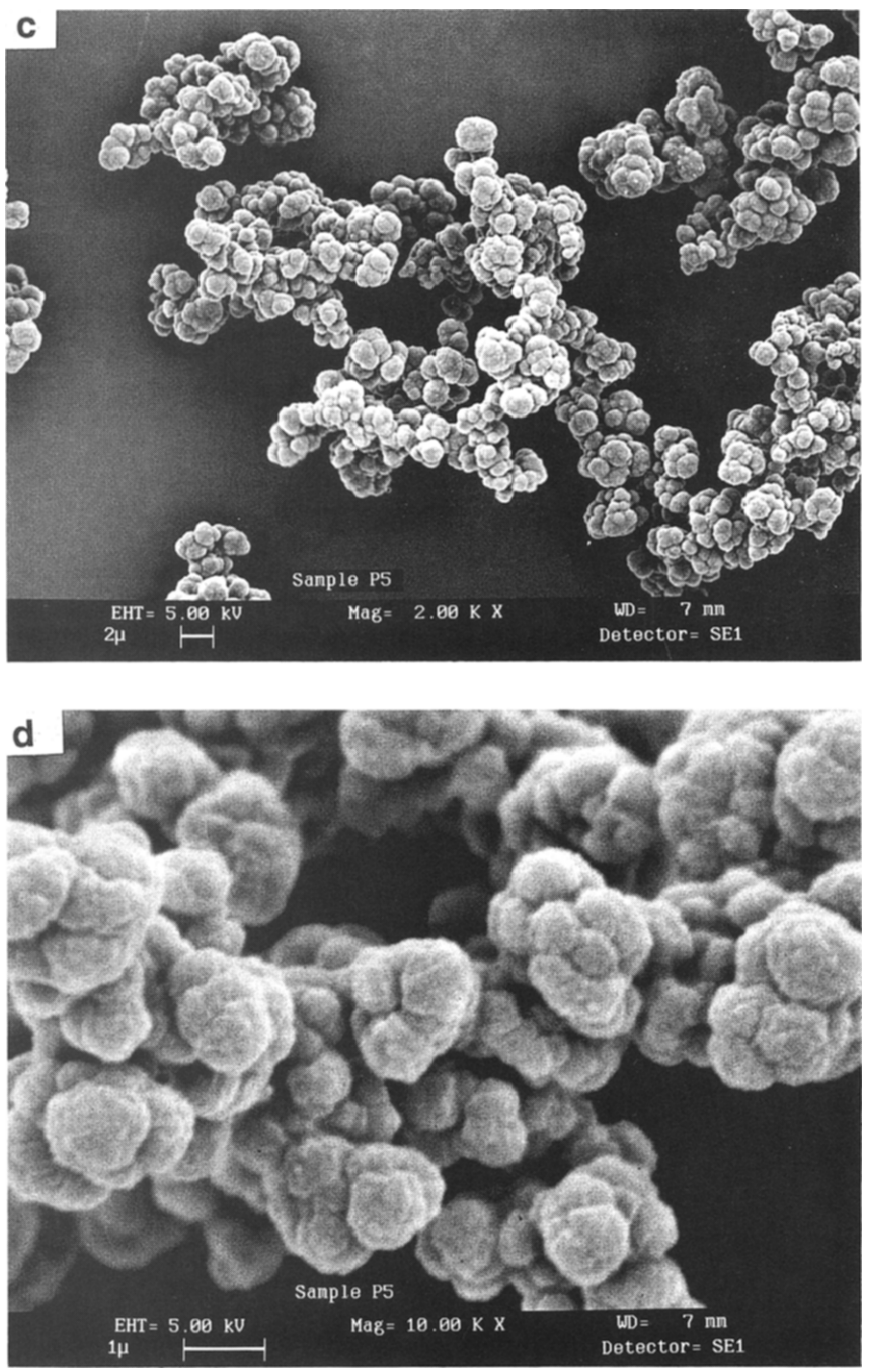

Fig. 1. Scanning electron micrographs of **P5**—PAM (a, b) and **P3**–LPA (c, d). Magnification: (a) 2000 × (Electroscan ESEM); (b) 10 000 × (Electroscan ESEM); (c) 2000 × (Leica Stereoscan 420); (d) 10 000 × (Leica Stereoscan 420).

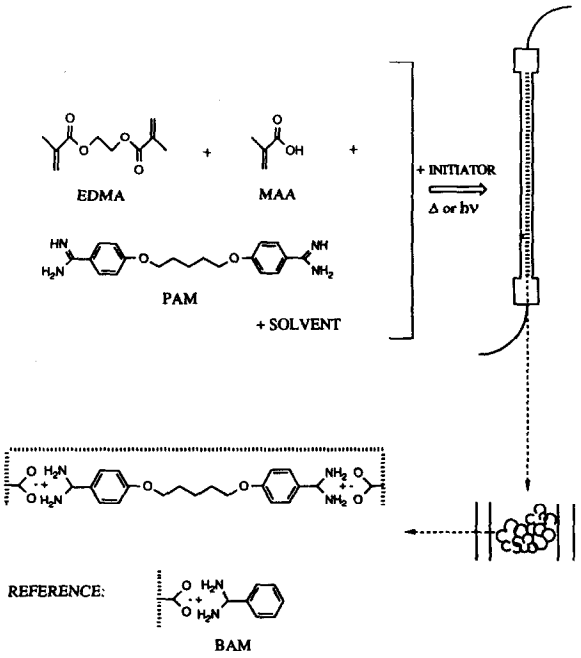

Fig. 2. *In situ* polymer preparation.

ethanol by sonication followed by a traditional column slurry packing. **P1** however was ground and sieved prior to packing. The columns were connected to a simple HPLC equipment and at least 10 ml of EtOH or MeCN–potassium phosphate buffer 0.05 *M*, pH 2 (7:3, v/v) (only for **P5**) was passed at flow-rates giving a back pressure of less than 1500 p.s.i. (1 p.s.i. = 6894.76 Pa). In the initial eluate extracted template was detected by TLC analysis. Due to some compression of the column bed the inlet end fitting was removed and the tube cut to a length of 100 mm. After reconnecting the column MeCN–potassium phosphate buffer 0.05 *M*, pH 5 (7:3, v/v) [in **P4**: MeCN–water (95:5, v/v)] was passed at a flow-rate of 0.3 ml/min until a stable baseline was attained. Substrate or reference (2 nmol in 20 μl eluent) was injected and the elution profile monitored by UV absorption at 270 nm (254 nm for D,L-PA).

3. Results and discussion

The procedure applied for the preparation of a material capable of recognizing PAM, a DNA-binding drug used in the treatment of AIDS-related disorders [18], is outlined in Fig. 2 (see Experimental section and Table 1 for details). Template, monomers (EDMA and MAA) and solvents are simply mixed and homogenized at elevated temperature. After addition of initiator (AIBN) the solution is transferred to a glass column and the polymerization carried out at elevated temperature. The column can then be directly connected to the HPLC equipment and rapidly evaluated. The retention of PAM and the reference BAM on a PAM (**P5**–PAM) and a BAM (**P5**–BAM) column were compared in an organic–aqueous mobile phase. While at pH 2 both compounds eluted essentially with the void volume, *at pH 5 PAM was 7 times more retained on the PAM- than on the BAM- column* (Fig. 3 and Table 2). BAM on the other hand showed a weaker pH dependence and was equally retained on both columns. The PAM column thus exhibits a pronounced selectivity for PAM whereas the BAM column did not appear to recognize BAM. This can be explained considering the number of potential interaction sites that the templates contain towards MAA. While PAM should be present as a bis-methacrylate ion pair (Fig. 2) BAM can only form a 1:1 complex with MAA prior to polymerization. In the complementary polymer PAM will thus be able to bind to the sites by two strong ion-pair interactions, each worth approximately the same in energy as the one ion-pair interaction that is possible between BAM and its complementary site [19]. The difference between the respective binding constants can in such cases amount to several orders of magnitude.

The fact that polymers imprinted with other basic templates, chosen from the group of nucleotide bases (TAA), herbicides (ATR) and chiral compounds (L-PA), also showed molecular recognition properties (Table 2) indicates that the technique may have broad applicability. However, the chiral separation factors (α) of the L-PA imprinted polymers are lower than those previously observed. This is related to the use of low monomer concentrations (compare α of **P1** and **P2**) and strongly hydrogen bonding solvents in the imprinting step [8]. According to our previous investigation [8], of the influence of

Table 1
Polymer preparation and characterization

Polymer[a]	Solvent[b]	EDMA (%)[c]	Monomer (%)[c]	Swelling (ml/ml)[d]	Particle size (μm)[e]	Bulk density (g/ml)[f]	Surface area (m^2/g)[g]	Pore diameter (Å)[g]	Pore volume (ml/g)[g]
P1–L-PA	1	80	40	1.25	<<1 sc	0.19	132	169	0.46
P2–L-PA	1	80	20	1.20	0.5–2	0.21	18	148	0.044
P3–L-PA	2	80	20	1.20	1–2	0.32	36	100	0.050
P3–ATR	2	80	20	1.20	1–2	0.28	43	92	0.078
P3–BL	2	80	20	1.20	1–2	0.28	19	114	0.041
P4–TAA	1	80	20	1.13	0.5–1	0.34	22	317	0.104
P4–BL	1	80	20		0.5–1		18	170	0.060
P5–PAM	3	96	33	1.00	2–4	0.51	210 (150)	22[h]	0.039 (0.066)
P5–BAM	3	96	33	1.05	2–4	0.49	181 (125)	25[h]	0.045 (0.055)
P6–L-PA	1	80	14	1.25	0.5–2	0.13	18	144	0.053
P7–BL	4	80	20	n.d.	< 1 sc	n.d.	n.d.	n.d.	n.d.
P8–BL	1	100	20	n.d.	0.5–1	n.d.	n.d.	n.d.	n.d.

See Experimental section for details on the polymer preparation. n.d. = Not determined.

[a] Polymers that are different only in the template used during polymerization have been indicated with the same number. The templates are indicated after the polymer number (absence of template is indicated as BL = blank).

[b] 1 = Cyclohexanol–dodecanol (4:1, v/v); 2 = cyclohexanol; 3 = isopropanol–water (5:2, v/v); 4 = acetonitrile.

[c] EDMA (%) = Mol percent EDMA present in the monomer mixture with MAA beeing the other monomer. Monomer (%) = volume of monomers/(volume of monomers + volume of solvent).

[d] (Volume swollen polymer)/(volume dry polymer) in MeCN determined as described elsewhere [8].

[e] Approximate range in particle size and degree of agglomeration as judged from scanning electron micrographs (Fig. 1). sc = Strongly coagulated particles where the material had to be crushed before use.

[f] Determined as described elsewhere [8].

[g] Results from nitrogen adsorption using a Micromeritics ASAP 2000 covering pores between 17 and 3000 Å. The samples were degassed at 150°C and an 80-point pressure table was used with a 10-s equilibration time. The surface area was determined from a BET plot, the average pore diameter and the cumulative pore volume using the BJH model on the adsorption isotherm and the micropore surface area and pore volume (values in parentheses) from a *t*-plot using Harkins–Jura average thickness [8].

[h] Pore diameter calculated from the BET plot.

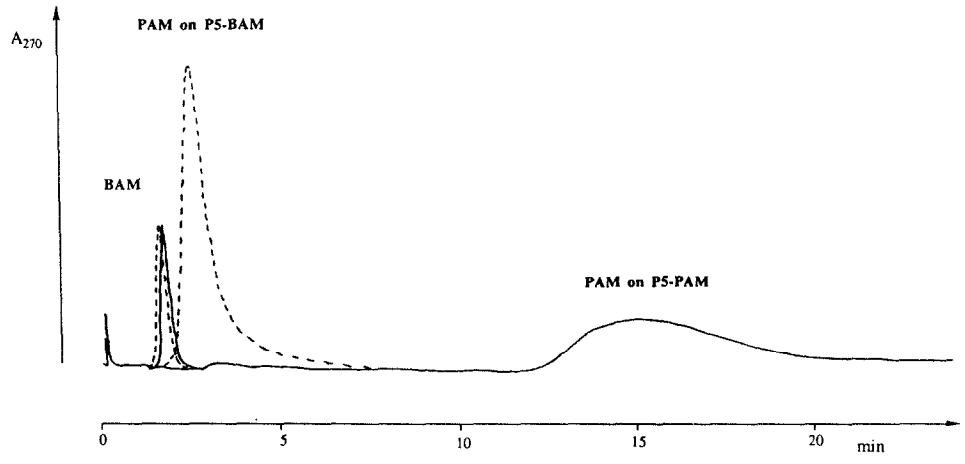

Fig. 3. Elution profiles of PAM and BAM (2 nmol) injected separately on a PAM-imprinted (**P5**–PAM) (solid line) and a BAM-imprinted (**P5**-BAM) (broken line) dispersion polymer prepared *in situ* in a chromatographic column. Mobile phase: MeCN–potassium phosphate buffer, 0.05 *M*, pH 5 (7:3, v/v). Flow-rate: 0.3 ml/min. For other details see Table 2.

Table 2
Chromatographic evaluation of imprinted dispersion polymers

Polymer[a]	Substrate[b]	Reference[b]	Retention[c] k'_{sub}	Separation factor[d] $\alpha(=k'_{sub}/k'_{ref})$
P1–L-PA	LPA	DPA	1.938	2.21
P2–L-PA	LPA	DPA	3.182	1.17
P3–L-PA	LPA	DPA	1.727	1.36
P3–ATR	ATR	DDC	1.243	2.64 (1.3)
P3–BL	ATR	DDC	0.888	2.00
P4–TAA	TAA	TAC	3.000	4.40 (4.2)
P4–BL	TAA	TAC	0.500	1.05
P5–PAM	PAM	BAM	16.0	54 (6.8)
P5–BAM	PAM	BAM	2.294	7.91

[a] The glass columns (150 mm × 5 mm O.D. × 3 mm I.D.) containing polymer were connected to a simple HPLC equipment and at least 10 ml of EtOH or MeCN–potassium phosphate buffer 0.05 *M*, pH 2 (7:3, v/v) (only for **P5**) was passed at 5 ml/min. The flow-rates and back pressures were: **P1**–L-PA: packing: 5 ml/min, < 1000 p.s.i.; **P2**–L-PA: packing: 5 ml/min, < 1000 p.s.i., run in MeCN: 1 ml/min, 250 p.s.i.; **P3**–L-PA: run in MeCN–water–HOAc (94:5:1, v/v/v): 9 ml/min, < 1000 p.s.i.; **P4**–TAA and **P4**–BL: run in MeCN–water (95:5, v/v): 0.1 ml/min, 360 and 630 p.s.i., respectively; **P5**–PAM and **P5**–BAM: packing: 4 ml/min, 643 and 571 p.s.i., respectively. MeCN–potassium phosphate buffer 0.05 *M*, pH 5 (7:3, v/v) [in **P4**: MeCN–water (95:5, v/v)] was then passed at a flow-rate of 0.3 ml/min until a stable baseline was attained.

[b] Substrate (sub) or reference (ref) [TAC = tri-O-acetylcytidine (HCl salt), DDC = dideoxycytidine] (2 nmol in 20 μl eluent) was injected and the elution profile monitored by UV absorption at 270 nm (254 nm for D,L-PA).

[c] Capacity factor (retention) defined as: $k'_{sub} = (t_{R,sub} - t_0)/t_0$; t_0 was determined from the elution time of MeCN–water (7:3, v/v).

[d] Separation factor defined as: $\alpha = k'_{sub}/k'_{ref}$. The values in parentheses represent a corrected separation factor: $\alpha' = (\alpha$ on templated polymer)/(α on reference polymer).

polymer morphology on the ability of L-PA imprinted polymers to resolve enantiomers, high selectivity is promoted by the use of solvents with a low hydrogen bond capacity, by preparing the polymers at low temperature and by increasing the MAA concentration. Such conditions promote the formation of template assemblies. Nevertheless the column efficiency of **P3**–L-PA was superior to that of the columns packed with crushed polymer particles. Thus the numbers of theoretical plates (N) of D- and L-PA obtained were 2000 and 1640/m, respectively. These numbers are about two times higher than the maximum plate number previously observed [8]. As seen in Fig. 3 however, **P5** exhibited a poorer column efficiency possibly related to the column packing procedure. In this context it should be noted that the flow resistance of the columns was small allowing a maximum flow-rate of 5 ml/min to be passed at a back pressure of less than 1000 p.s.i. (see Table 2 for further details).

The polymer particle size, degree of agglomeration and morphology varied with the solvent and monomer concentration used during polymerization (Table 1). Thus a low total monomer concentration (20%, v/v) and polar solvents favoured the formation of agglomerates (10 μm or less) of globular micron-sized particles (Fig. 1) with an estimated particle size range varying between 0.5 and 1 μm in **P4** and between 2 and 4 μm in **P5**. However, at a total monomer concentration of 40% (v/v) (**P1**–L-PA) the polymer was obtained as a continuous block that could not be dispersed in any solvent. Grinding the block resulted in irregular agglomerates of particles of about 0.1 μm and a poor chromatographic performance of the packed column ($N_{sub} = 110$/m). Likewise the use of MeCN as solvent produced only an ill-defined precipitate impossible to disperse.

In traditional dispersion polymerisations linear polymers are formed and the main role of the solvent is to function as a dispersion medium controlling particle size and dispersity [15,16]. However, in the formation of network polymers

the solvent has an additional role in controlling the morphology of the formed particles or agglomerates. This is reflected in properties such as particle swelling, surface area and porosity. The solvent had a marked influence on the particle morphology in the micro- and mesoporous domains (see Table 1). Polymers prepared using cyclohexanol and dodecanol as solvents (**P2–P4**) can thus be classified as mesoporous with a low surface area and pore volume but with a certain swellability [8]. This differs from the morphology of the polymers prepared using isopropanol–water as solvent (**P5**) which were essentially non-swellable materials with a larger surface area and more than half of the pore volume (total pore volume: 0.1 ml/g) in the microporous domain (<20 Å). An interesting feature is the difference in morphology between templated and reference material. The reason why the bifunctional template PAM gives rise to a material (**P5**–PAM) with a larger surface area (210 m^2/g) than a material (**P5**–BAM) (181 m^2/g) prepared using the monofunctional template BAM is not clear. These observations are in agreement with those made by Dunkin *et al.* [20] in the imprinting of other types of bifunctional templates. It may then be argued that this difference in morphology is the sole origin of the observed selectivity. Thus **P5**–PAM with the higher surface area may contain a larger number of accessible carboxylic acid groups resulting in a stronger retention of PAM. The strongest point against this argument is that the α values were calculated with reference to the retention of BAM. The latter was similar on **P5**–PAM and **P5**–BAM and increased from $k' = 0$ at pH 2 to $k' = 2.9$ at pH 7. Moreover in the enantioselective molecular recognition of L-PA (**P1–P3**–L-PA) the origin of the selectivity is undoubtedly related to substrate binding to imprinted sites [4–8].

In situ preparation of a particulate material with tailor-made affinity for a target substance is thus possible. The use of an aqueous environment during imprinting extends hereby the repertoire of small molecule imprinting to include also templates of low organic phase solubility. The molecular recognition of PAM, a goal in the design of PAM-selective receptors for sensor applications [21], is presently being explored in the development of selective sample enrichment systems for direct drug analysis [22].

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