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ORIGINAL ARTICLE

Molecularly imprinted polymers for RGD selective recognition and separation

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Abstract Molecularly imprinted polymers that could recognize the tripeptide Arg-Gly-Asp have been produced with the use of two functional monomers and three different cross-linkers, respectively. Methacrylic acid and acrylamide were used as functional monomers and the role of the ethylene glycol dimethacrylate, trimethylpropane trimethacrylate and *N,N′*-methylene-bisacrylamide as crosslinking monomers, was investigated on their recognition capability. The % net rebinding and the imprinting factor values were obtained, giving for the methacrylic acid–trimethylpropane trimethacrylate polymer the highest values 12.3% and 2.44, respectively. In addition, this polymer presented lower dissociation constant (K_D) value and the higher B_{max}% of theoretical total binding sites than all the other polymers. Rebinding experiments with Lys-Gly-Asp, an analogue of Arg-Gly-Asp, and other different peptides, such as cholecystokinin C-terminal tri- and pentapeptide and gramicidin, further indicated the selectivity of methacrylic acid-trimethylpropane trimethacrylate copolymer for Arg-Gly-Asp giving specific selectivity factor values 1.27, 1.98, 1.31 and 1.67, respectively.

Keywords Arg-Gly-Asp · RGD · Copolymerization · Molecular imprinting · Radical polymerization

Abbreviations

ABCN	Azo-bis-cyclohexane-carbonitrile
RGD	Arg-Gly-Asp tripeptide
KGD	Lys-Gly-Asp tripeptide
CCK-3, CCK-5	Cholecystokinin C-terminal tripeptide and pentapeptide, respectively
EGDMA	Ethylene glycol dimethacrylate
TRIM	Trimethylpropane trimethacrylate
MAA	Methacrylic acid
MIPs	Molecularly imprinted polymers
NIPs	Non imprinted polymers
SEM	Scanning electron microscopy

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Introduction

Molecularly imprinted polymers (MIPs) have gained great scientific attention due to their enormous possibilities of applications (Ye et al. 2000; Mosbach and Haupt 1998). They are being prepared with the idea of inducing selective recognition sites in a polymer matrix using a molecular template in a casting procedure. The template is allowed to form spontaneous interaction/bonds with one or several types of functional elements in prearrangement step, and subsequent co-polymerization of the functional monomer and a cross-linker leads to the formation of a rigid cavity. This cavity accommodates size, shape and chemical functionality complementarities to the template, and thus, after the template removal, the resulting polymer is able to rebind the template with high affinity and specificity. MIPs present remarkable stability in comparison to other man-made materials and antibodies used in analysis. An ideal protein-capture agent should have high specificity and be composed of a stable, robust, non-biological material, as example, the MIPs (Nishino et al. 2006). So, the well-established technique of MIP formation has enormous application potential in the fields of chromatography, immunoassay-type analyses, biosensors, biocatalysis and

bioseparations (Komiyama et al. 2003; Wulff 1995; Mosbach and Ramström 1996).

The first work on imprinting in synthetic polymers was published by Wulff et al. (1973), and refers to the covalent approach case, whereas the most widely applied methodology to produce MIPs is represented by the non-covalent approach (Mosbach 1994) and until recently, only relatively low molecular weight compounds were used as templates. Some works on molecular imprinting field deal with L-phenylalanine anilide (Krlz et al. 1994; Kempe and Mosbach 1995), Boc-L-Phe-OH, Boc-L-Val-OH, Boc-L-Ala-OH, Boc-L-Glu-OH, Z-L-Phe-OH, Z-L-Asp-OH, Z-L-Glu-OH, Z-L-Ala-L-Ala-OMe, Z-L-Ala-Gly-L-Phe-OMe (Kempe and Mosbach 1995; Allender et al. 1997; Sellergren et al. 1988). As far as the free oligo hetero-peptide imprinting is concerned, literature is limited mainly due to difficulties in finding a biologically relevant active peptide, in appropriate amounts with reasonable cost. Other recent reports concern cholecystokinin pentapeptide (Papaioannou et al. 2007), angiotensin II, oxytocin, enkephalin derivatives, Z-aspartame and the peptides Tyr-Pro-Leu-Gly and Gly-Leu-Tyr (Ramström et al. 1994; Kempe and Mosbach 1995; Andersson et al. 1995, 1996; Allender et al. 1997; Ye et al. 1998).

On the other hand, it is known that peptides containing the Arg-Gly-Asp (RGD) sequence cause inhibition of platelet aggregation (Basani et al. 2001). This is due to the fact that the specific sequence can be recognized by a family of glycoproteins called integrins, such as IIb/IIIa (GPIIb/IIIa) a glycoprotein receptor found on the surface of the platelets. GPIIb/IIIa is primarily responsible for platelet aggregation, because it binds the plasma protein fibrinogen. Fibrinogen is a dimer of three polypeptide chains (α-, β- and γ-), while each of α- chains contains an RGD sequence, which is recognized from GPIIb/IIIa. Thus, fibrinogen acts as a bridge between platelets and promotes platelet adhesion (Ojima et al. 1995). RGD-containing molecules act antagonistically against fibrinogen, as they compete for the same binding site of GPIIb/IIIa, blocking the platelet receptor and inhibiting by this way the binding of fibrinogen and therefore platelet aggregation.

The involvement of RGD in these biological processes indicated the need for the development of efficient analytical tools for its identification and isolation from biological fluids, as well as for the variety of adhesive proteins containing this moiety.

In this study, MIPs recognizing the RGD sequence were prepared using two common functional monomers: methacrylic acid (MAA), acrylamide and three cross-linkers: ethylene glycol dimethacrylate (EGDMA), trimethylpropane trimethacrylate (TRIM) and *N,N′*-methylene-bisacrylamide, respectively. Six polymers were emerged from this combination and their binding properties are reported.

Experimental

Materials

MAA, acrylamide, EGDMA and TRIM were purchased from Sigma-Aldrich Inc, USA, *N,N′*-methylene-bisacrylamide from Alfa Aesar GmbH & Co, KG (Germany) and the initiator azo-bis-cyclohexanecarbonitrile (ABCN) from Aldrich, USA. Methanol (J. K. Baker, Holland) and acetonitrile (Carlo Erba, Italy), were of HPLC grade.

The RGD peptide was synthesized by the solid phase technique, using the Fmoc/tBu strategy. All the amino acid derivatives, that were used, were of S-configuration and were purchased from CBL (Patras, Greece). 2-Chlorotrityl-chloride resin was used as solid support, while for the coupling reactions, the method of carbodiimides was applied and specifically the reagents *N,N*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) were used. The esterification of the first Fmoc protected amino acid [Fmoc-Asp(tBu)-OH] on the resin was carried out in the presence of *N,N*-diisopropylethylamine (DIPEA), while the removal of the Fmoc group was performed using 20% piperidine solution in DMF at room temperature. The cleavage of the protected peptide from the resin was performed using dichloromethane (DCM):trifluoroethanol (TFE):acetic acid (AcOH) (7:2:1). Subsequently, the removal of the side chain protecting groups of the amino acids used took place in solution, using trifluoroacetic acid (TFA):DCM:scavengers (70:20:10) as deprotection cocktail. The final product was purified by RP-HPLC and identified by ESI-MS. Similar methodology was applied for Lys-Gly-Asp (KGD) synthesis. The cholecystokinin C-terminal tri- and penta-peptide (CCK-3 and CCK-5, respectively) (with unsulphated tyrosine) were synthesized by the solution technique using the appropriate Boc-amino acid derivatives, dicyclohexylcarbodiimide and 1-hydroxybenzotriazole as coupling reagents. Both peptides obtained as hydrochloride or trifluroacetic acid salts, were purified by RP-HPLC using a C18 column Grace Vydac 201TP54, with methanol as eluent and flow rate 1.5 ml/min, prior to their use as templates and for rebinding experiments. Gramicidin was obtained from U.S. Biochemical Corporation.

Polymers preparation

Polymerizations were performed with UV radiation at 322 nm at room temperature for 24 h. RGD was dissolved in methanol at 37×10^{-6} mol and added to the pre-

polymerization mixture. In all cases, the pre-polymerization mixtures were cooled in an ice bath and sparked with nitrogen to remove the dissolved oxygen. Then the tubes containing the polymerization mixture were sealed and placed near the UV source. After polymerization, the template was removed from the polymer by extensive washing with a solution of 5% acetic acid in methanol (v/v), until the template could no longer be detected in the supernatant. The MIPs were then used for rebinding experiments.

For each MIP, a corresponding non-imprinted polymer (NIP) was also prepared under the same experimental conditions in the absence of the template, thus giving polymers with the same number of functional groups, but randomly dispersed into the polymer matrix.

Six sets of MIPs and NIPs, respectively, were prepared with the combination of the two functional monomers and the three cross-linkers, respectively, under the same experimental conditions and the same molar ratios of functional monomer, cross-linker and template. The experimental conditions applied are given in Table 1.

Rebinding experiments with RGD

In order to quantitatively evaluate MIPs recognition abilities for the template and to calculate the adsorption isotherms, 20×10^{-3} g of each polymer was incubated with RGD solution in methanol at varying initial concentrations 0.25–2×10^{-3} M, at 30°C for 24 h under slow agitation ($\sim$120 rpm). After the equilibrium was reached, the supernatant was withdrawn and passed through two teflon filter of 0.2 μm diameter, in a series, and the concentration of free peptide remaining in solution was measured by HPLC. The corresponding bound peptide concentration was calculated as the difference between the initial and final concentration in the supernatant.

Table 1 Polymers produced by the combination of different functional and cross-linking monomers

Polymer	Functional monomer	Cross linking monomer
1	MAA	EGDMA
2		TRIM
3		Bisacrylamide
4	Acrylamide	EGDMA
5		TRIM
6		Bisacrylamide

Calculation of % net rebinding and imprinting factor

The percentage of net rebinding was calculated by Eq. 1:

$$\%\ \text{net rebinding} = \frac{(C_{\text{initial}} - C_{\text{freeMIP}}) - (C_{\text{initial}} - C_{\text{freeNIP}})}{100 \times C_{\text{initial}}} \quad (1)$$

where C_{initial} the initial RGD incubation concentration and C_{free} the amount of non-bound template left in solution after the incubation in the supernatant of MIP or NIP, respectively.

The imprinting factor (IF) was calculated according to Eq. 2,

$$\text{IF} = \frac{K_{\text{MIP}}}{K_{\text{NIP}}} = \frac{(\mu\text{molRGD/gMIP})/C_{\text{freeMIP}}}{(\mu\text{molRGD/gNIP})/C_{\text{freeNIP}}} \quad (2)$$

where K the partition coefficient for each polymer, C_{free} the amount of non-bound template left in solution after the incubation and μmol RGD/g polymer is the amount of template rebound per gram of dry polymer, was estimated based on the weight of the polymer and the initial concentration of the template in the rebinding solution (Lanza and Sellergren 2004; Papaioannou et al. 2007).

Selectivity experiments

In order to investigate further the selectivity of polymer **2** (Table 1) for RGD peptide, four other peptides were used. KGD, an analogue of RGD with similar structure and three other completely different, CCK-3, CCK-5 peptides and gramicidin were chosen, in terms of availability, for this series of experiments. Pairs of two peptides at equimolar solution (500×10^{-6} M) were incubated in each case, under the same experimental conditions. If I_1 and I_2 are the IF values of substrate 1 and 2, respectively, then the specific selectivity factor, S (Cheong et al. 1997), is given by their ratio (Eq. 3).

$$S = \frac{I_1}{I_2} \quad (3)$$

The S indicates how many times more the imprinting effect is observed for substrate 1 in relation to substrate 2, on the polymer.

Determination of peptides by HPLC

HPLC analysis was performed with a C18 column, Separon SGX C18 7 μm, 200 × 4.6 mm, isocratically with solvent composition 65:10:25 MeCN:MeOH:H_2O and flow rate 1 ml/min, with a diode array detector at 258 nm for all peptides.

All experiments were performed in triplicate and the numbers reported here are mean values.

SEM images

Scanning electron microscopy (SEM) images were obtained with a JEOL 6300 SEM instrument. The surface of the sample was made conductive by deposition of a gold layer on the sample.

Results and discussion

Figure 1 is a schematic illustration of a non-covalent imprinting process for RGD recognition, using MAA (functional monomer) and TRIM (cross-linker) which was found to express better characteristics in terms of IF and % net rebinding values, than the rest.

Figures 2 and 3 give the average percentage of net rebinding and average imprinting factor values of the resultant polymers, respectively, at various RGD concentrations. As it can be seen in these figures, there are variations in the % net rebinding and the IF values, according to the polymer's composition, indicating that the combination of functional monomer and cross-linker is responsible for the final recognition efficacy. Polymer **2** produced by the combination of MAA–TRIM and to less extent polymer **4** by acrylamide–EGDMA, exhibit better rebinding properties than the rest, thus contributing to the above statement that both the functional monomer and the cross-linking monomers are important for the imprinting efficiency. MAA can form strong ionic interactions with basic functional groups on the template while its carboxyl group is also an excellent hydrogen bond donor and acceptor has been widely used. In some other cases it is advantageous to use 'neutral' monomers, such as acrylamide (Yilmaz et al. 2005), which has been used in imprinting of proteins in aqueous systems (Liao et al. 1996) and as hydrogen-bonding functional monomer (Yu and Mosbach 1997). On the other, the role of cross-linker is to fix permanently the functional monomers to stable binding cavities, so it is used in high percentage in the resulting polymer. This has the consequence that the morphology and the chemical environment of MIPs to be greatly affected by the choice of the cross-linker monomer (Yilmaz et al. 2005). EGDMA (Ramström 2005) and TRIM have been used successfully in several cases. In the imprinting of peptides, TRIM has been used to prepare resins with higher sample load capacity and better performance (Kempe 1996). Though, neither EGDMA nor TRIM is water-soluble and for aqueous phase imprinting, other cross-linkers, such as the *N,N′*-methylene-bisacrylamide must be used (Ramström 2005). As it is reported, the amide-based cross-linkers provide a polar microenvironment, more protein-like, which is different from the less polar methacrylates (Glad et al. 1985).

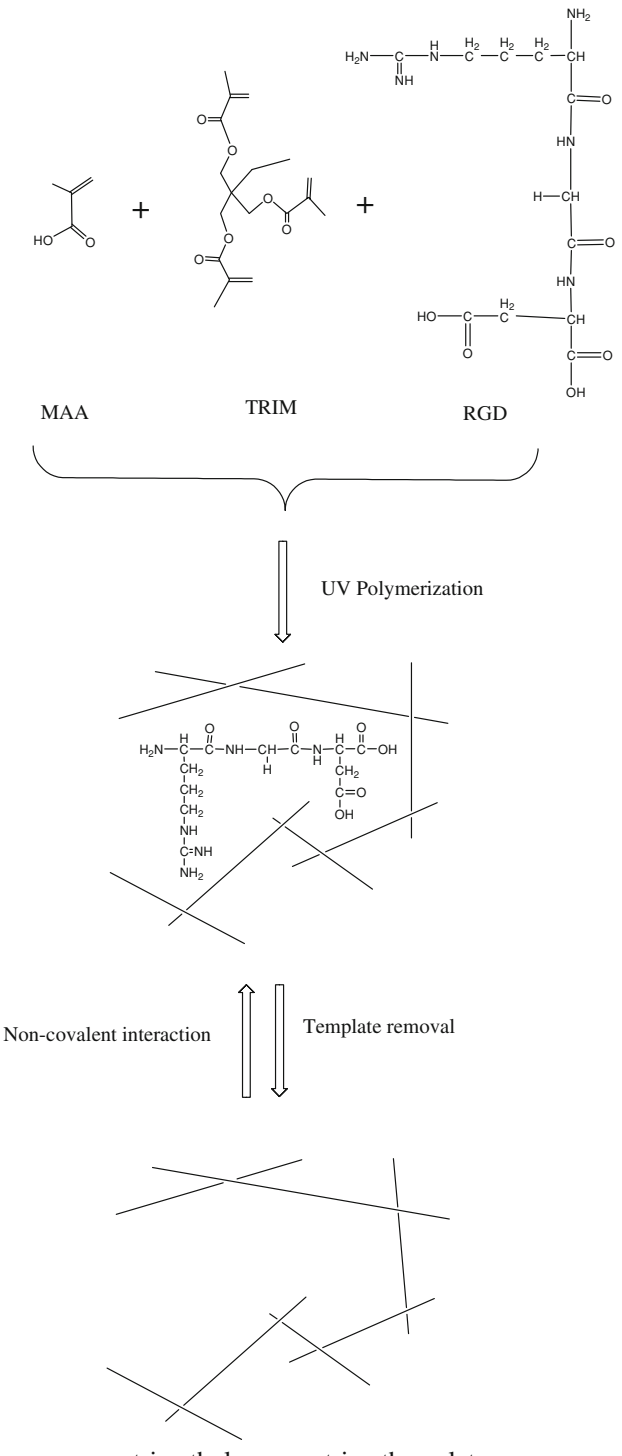

Fig. 1 Molecular imprinting protocol for RGD based on MAA and TRIM polymerization

The IF and % net rebinding values reported here for MIPs produced in protic environment (MeOH) are comparable with the values observed in case of MIP produced for Phe-Phe D- and L-dipeptides and Phenylac-Phe in

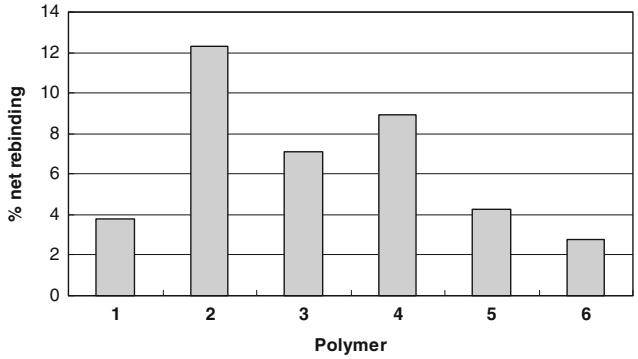

Fig. 2 Percentage of net rebinding of RGD to MIPs

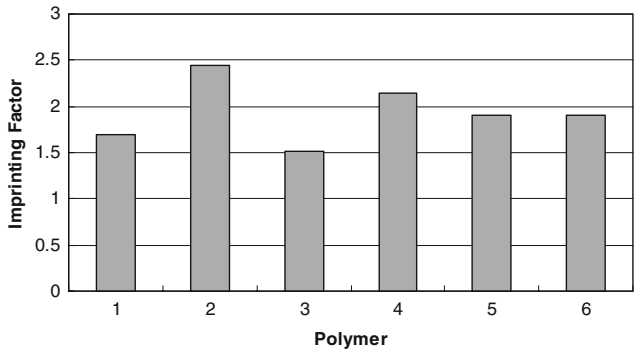

Fig. 3 Imprinting factor (IF) values of MIPs

water with cyclodextrins as functional monomer (Asanuma et al. 2001).

The binding properties of the polymers can also be extracted from the corresponding binding isotherms, which actually give a measure of the concentration-dependent recognition behavior of a system. Generally, the concentration of bound analyte in a solid phase increases with increasing the free concentration. When the polymer is saturated and all sites are occupied, the concentration of bound analyte will level off and remain constant. A binding model specifies a particular relationship between the concentrations of bound and free analyte (template) and therefore, it can be used to model the binding isotherm. The homogeneous binding models are the simplest and easiest to apply and have been used extensively to characterize MIPs, usually in the form of Scatchard plots. The Scatchard equation is a rearranged form of the Langmuir isotherm (Shea et al. 1993). In the present study, we consider that the concentration range is high, in regard to theoretical sites, and thus the system can be modeled by the Langmuir isotherm. The dissociation constant (K_D) and the maximum amount of binding sites (B_{max}) values were calculated from adsorption isotherms using the Langmuir model (Sellergren 2001). The K_D and B_{max} values for polymers **1–6** are given in Table 2. Polymers **5** and **6** with lower dissociation constant values, exhibit stronger guest

Table 2 Dissociation constants for the binding of RGD to MIPs

Polymer	K_D (mM)	B_{max} (μmol/g)	B_{max}% of theoretical total binding sites
1	1.506	14.7	17
2	1.206	13.5	26
3	1.331	14.8	14
4	2.032	34.6	41
5	0.187	4.2	8
6	0.219	2.5	2

binding activity, while their B_{max}% of theoretical total binding sites are lower than the others. These data combined with the % net rebinding and IF data, make polymers **5** and **6** unsuitable for analytical purposes. Polymers **1–4** gave comparable K_D values with polymer **2** exhibiting the lower K_D and higher B_{max}% of theoretical total binding sites. These data together with % net rebinding and IF values, lead to the conclusion that polymer **2** is the most suitable for RGD recognition. Figure 4a gives the

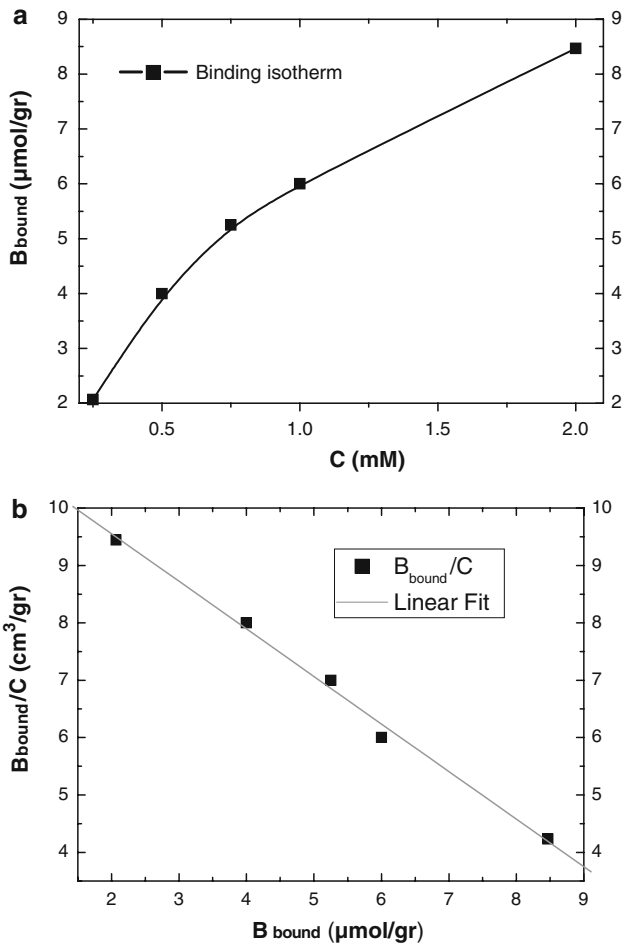

Fig. 4 **a** Binding isotherm and **b** the corresponding Scatchard plot for the binding of RGD to polymer **2**

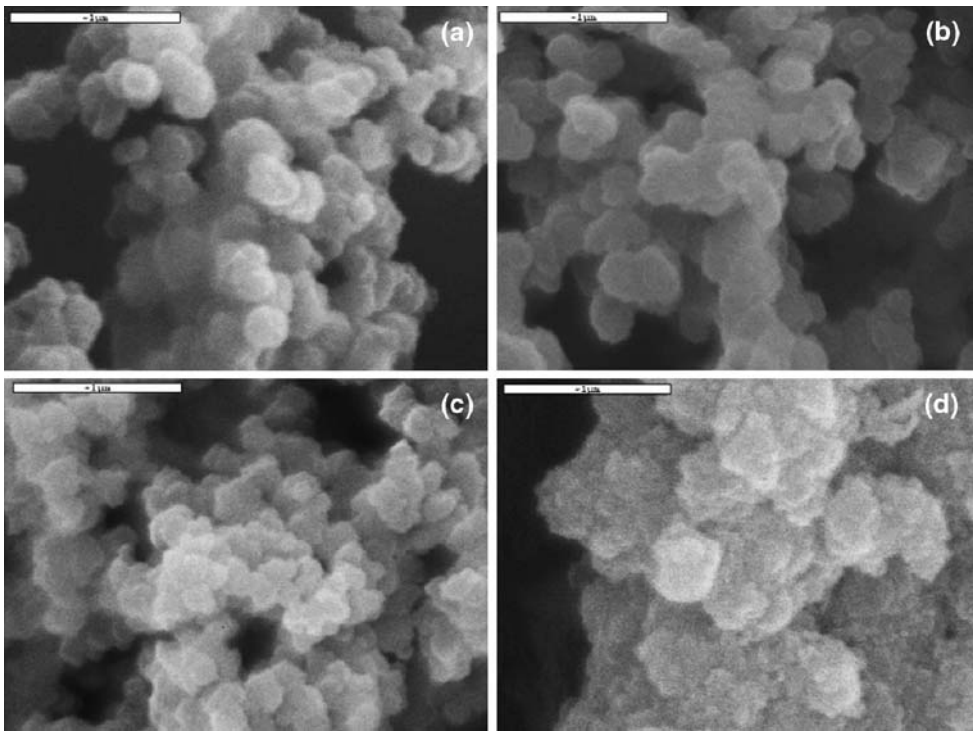

Fig. 5 SEM images of MIPs: **2**, **5** (**a**, **b**) and **1, 3** (**c**, **d**). The number assigned to each MIP is shown in *brackets*. The scale of all pictures is 1 μm

Table 3 Average aggregate size and the minimum and maximum size of the resultant polymers

Polymer	Average size of aggregate per μm^2 (nm)	Minimum (nm)	Maximum (nm)
1	119	54	248
2	175	96	299
3	121	49	331
5	188	88	248

adsorption isotherm and Fig. 4b the Scatchard plot for this polymer.

SEM images of MIPs are shown in Fig. 5 and the average aggregate size, their maximum and minimum size, found from the SEM image measurements using the digital image analysis program SemAfore demo Ver 4.0 (JOEL), are summarized in Table 3. The MIPs produced with MAA or acrylamide and TRIM have almost the same size, about 180 nm (Fig. 5a, b). Polymers produced with MAA and EGDMA or *N,N′*-methylene-bisacrylamide, respectively, gave smaller particles with an average size 120 nm (Fig. 5c, d). The same particles size is observed in case of EGDMA or *N,N′*-methylene-bisacrylamide and acrylamide as functional monomer (data not shown). Though, the particles size distribution is quite wide ranging from 50 to 300 nm. The NIPs also presented the same morphology (data no shown). Despite the actually slightly bigger size of MIP produced with TRIM and MAA (Fig. 5c) than the rest polymers, as it is shown above, it exhibits better recognition characteristics.

The results from selectivity rebinding experiments with polymer **2** are summarized in Fig. 6. As it was expected, the net rebinding values of RGD and KGD peptides (Fig. 6a), are the same about ~8–10%, while for the structurally different peptides (CCK-3, CCK-5 and gramicidin) are smaller about ~2–4%. Interesting, only in case of RGD, the relative IF value (Fig. 6b) is higher than two units, meaning preference of polymer **2** for this compound. Accordingly, the *S* values in case of MIP **2** for KGD, CCK-3, CCK-5 and gramicidin are 1.27, 1.98, 1.31 and 1.67, respectively. The ability of polymer **2** to recognize specifically the RGD sequence is of great importance and indicates that this polymer can be used for RGD isolation and analysis in biological fluids.

Conclusion

The effectiveness of MIPs against RGD, produced by the combination of two functional monomers with three cross-linkers was evaluated. Polymer **2** produced by co-polymerization of MAA and TRIM was found to exert better characteristics as it is supported from IF values, the % net rebinding, as well as with Scatchard plot analysis. Furthermore, the selectivity experiments with this polymer indicated its ability to recognize specifically the RGD moiety from other different peptides, giving satisfactory

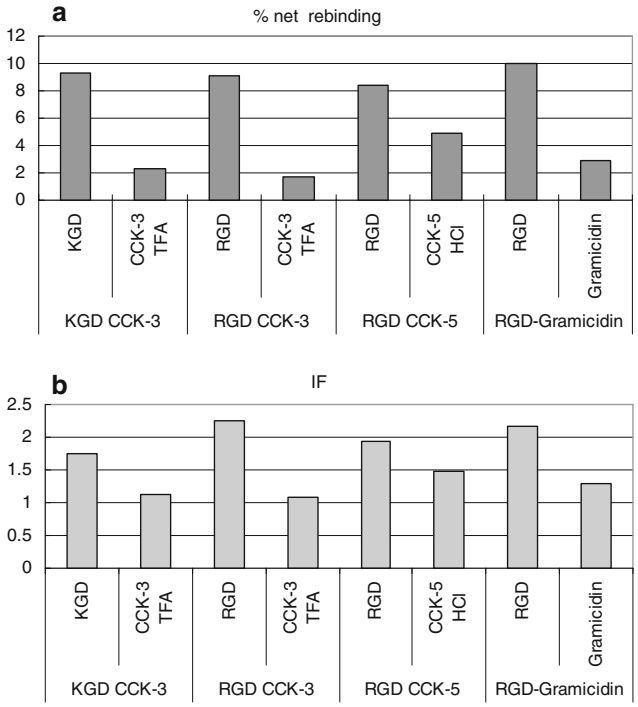

Fig. 6 Selectivity experiments with polymer **2**. **a** % net rebinding and **b** IF values

specific selectivity factor values. The data support the use of molecular imprinting technology for molecular recognition and selective separation of RGD peptides.

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