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ARTICLE

Rabbit IgG-imprinted nanoMIPs by solid phase synthesis: Effect of cross-linker on affinity and selectivity

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Matteo Chiarello,^a Laura Anfossi,^a Simone Cavalera,^a Fabio Di Nardo,^a Thea Serra,^a Fabrizio Sordello^a and Claudio Baggiani^{*a}

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The solid phase synthesis (SPS) of molecularly imprinted nanoparticles (nanoMIPs) represents an innovative method to prepare nanomaterials with tailor-made molecular recognition properties towards peptides and protein. The synthesis of nanoMIPs by SPS usually involves a pre-polymerization formulation where the cross-linker is invariably N,N'-methylene-bis-acrylamide (BIS). To date, the effect on the binding properties of nanoMIPs using other than BIS cross-linkers has never been reported. In this work, in order to investigate the effect of different cross-linkers in protein-imprinted nanoMIPs prepared by SPS, alongside BIS we considered other similar cross-linkers: N,N'-ethylene dimethacrylamide (EDAM), N,O-bis-methacryloylethanolamine (NOBE), ethylene glycol dimethacrylate (EDMA) and glycerol dimethacrylate (GDMA), replacing them for the BIS in pre-polymerization mixtures. Synthesized nanoMIPs were homogeneous, with a polydispersity index of 0.24-0.30 and mean diameter of 129-169 nm in water. The binding properties of the nanoMIPs were measured by equilibrium partition experiments with the template, rabbit IgG (RIgG), and selectivity was evaluated with respect to bovine IgG (BIgG), bovine serum albumin (BSA) and hen egg lysozyme (LZM). The experimental results show that all the cross-linkers, with the exception of EDMA, gave nanoMIPs with high binding affinities for the template (BIS: $16.0 \times 10^6 \text{ mol}^{-1} \text{ L}$, EDAM: $8.8 \times 10^6 \text{ mol}^{-1} \text{ L}$, NOBE: $15.8 \times 10^6 \text{ mol}^{-1} \text{ L}$, GDMA $12.8 \times 10^6 \text{ mol}^{-1} \text{ L}$), medium to high imprinting factors (BIS: 12.3, EDAM: 5.5, NOBE: 7.2, GDMA 11.6) and selectivity towards other proteins good but markedly dependent on the structure of the cross-linker, confirming the importance of the latter in the SPS of imprinted nanoparticles.

1. Introduction

Immunoglobulins G (IgG) are the most abundant proteins with immunological activity, accounting for 75-80% of all immunoglobulins. They strongly bind to the corresponding antigens – usually biomacromolecules foreign to the organism – with specificity, playing a key role in the immune system of the mammals [1]. For this reason, IgG are extremely relevant not only in diagnostics [2,3], therapeutics [4,5] and theragnostics [6] but also in applications where very high selectivity towards a target molecule is mandatory, as (bio)sensoristics [7,8] and affinity chromatography [9,10]. IgG can be conveniently isolated from plasma by the classical Cohn's method based on the fractional precipitation of serum proteins by ethanol [11]. Unfortunately, this method does not assure complete separation of IgG from other serum proteins, and more efficient downstream purification strategies must be used to obtain pure IgG fractions. Several methods based on affinity ligands of natural or artificial origin have been proposed but, at the present, affinity chromatography based on Protein A, a 42 kDa protein with high affinity for the Fc region of IgG, is the

preferred method for preparative and industrial purposes [12-14]. However, this method suffers from high costs and limited stability of Protein A, and harsh elution conditions which can sometimes lead to irreversibly damage IgG. Thus, man-made IgG-binding materials based on the molecular imprinting technology which could overcome these drawbacks are of significant interest. In the last 10 years, several papers describing different approaches to IgG imprinting have been published: cryogels [15,16], films [17,18], hydrogels [19], interpenetrating polymers [20], magnetic particles [21,22], membranes [23,24], microbeads [25,26] and nanoparticles (nanoMIPs) prepared by solid phase synthesis (SPS) [27].

This latter approach has proved particularly useful for obtaining high affinity protein-imprinted nanoparticles, characterized by selectivity for the template and complete compatibility with aqueous environments [28-36]. Moreover, as the template is covalently grafted onto the solid-phase, the isolation and purification of nanoMIPs is an easy task, and no residual protein remains trapped in the nanoparticles, avoiding product contamination.

The synthesis of protein-imprinted nanoMIPs by SPS is normally performed in water, and the pre-polymerization formulations include a large excess – up to 98% by moles – of functional monomers [37]. The selection of the functional monomer seems to be of lesser importance than in the case of templates made up of small molecules, because it has been shown that functional monomers that differ in their chemical properties are

^a Department of Chemistry, University of Torino, Via Giuria 7, 10125 - Torino, Italy.

E-mail: claudio.baggiani@unito.it

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in any case able to interact with different functions present on the templates, invariably leading to nanoMIPs with good molecular recognition properties [34]. About the cross-linker, it is added in a much more limited amount, and invariably is N,N'-methylene-bis-acrylamide (BIS) [37]. Its prevalent use may be justified by its good solubility in water and compatibility with proteins. It is nevertheless possible to consider using other cross-linkers, of which, however, the effect on the binding properties of nanoMIPs has never been reported in literature to date, with the remarkable exception of the use of N,N'-ethylene dimethacrylamide for the solid phase synthesis of adenosine monophosphate-binding nanoMIPs [38].

In this work, in order to investigate the effect of different cross-linkers in rabbit IgG-imprinted nanoMIPs prepared by SPS, alongside BIS we have considered some other similar cross-linkers whose structural formulas are shown in Chart 1: N,N'-ethylene dimethacrylamide (EDAM), N,O-bis-methacryloylethanolamine (NOBE), ethylene glycol dimethacrylate (EDMA) and glycerol dimethacrylate (GDMA), replacing them for the BIS in pre-polymerization mixtures without changing the molar proportions with functional monomers. The binding properties of the nanoMIPs have been measured by equilibrium partition experiments with the template, rabbit IgG (RIgG) and selectivity has been valued with respect to three other proteins of interest: bovine IgG (BIgG), bovine serum albumin (BSA) and hen egg lysozyme (LZM).

2. Materials and methods

2.1 Chemicals and materials

Glass beads, Spherglass-2429, 70-100 μm average particle size (Potters, UK) were aminated as previously reported [39]. N,O-Bis-methacryloylethanolamine (NOBE) was prepared in according with literature [40].

Acrylic acid (AA), 3-aminopropyltrimethoxysilane (APTMS), ammonium persulphate (APS), bovine IgG (BIgG), bovine serum albumin (BSA), N,N'-diisopropylcarbodiimide (DIC), 4-(N,N-dimethylamino)pyridine (DMAP), ethanolamine, N,N'-ethylenedimethacrylamide (EDAM), ethylene glycol dimethacrylate (EDMA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), glycerol dimethacrylate (GDMA, mixture of 1,2 and 1,3 isomers), N-hydroxysuccinimide (NHS), N-isopropylacrylamide (NIPAm), N,N'-methylene-bis-acrylamide (BIS), morpholinethansulphonic acid (sodium salt, MES), rabbit IgG (RIgG), succinic anhydride, N-

Chart 1: cross-linkers used to prepare RIgG-imprinted nanoparticles

tertbutylacrylamide (TBAm), N,N,N',N'-tetramethylethylenediamine (TEMED) were Sigma-Merck (Milan, Italy). Hen egg lysozyme (LZM) was Boehringer Ingelheim (Milan, Italy). Solvents and all other chemicals were purchased from Sigma-Merck (Milan, Italy). All the solvents were of HPLC grade, whereas all chemicals were of analytical grade. The water used was ultra-purified in Purelab Prima System from Elga (Marlow, UK). Protein stock solutions were prepared by dissolving 25 mg of protein in 25 mL of phosphate buffer (20

mmol L⁻¹, 0.13 mol L⁻¹ NaCl, pH 7.4) and stored in the dark at -20 °C. Coomassie Blu G250 protein assay reagent was from VWR International (Milan, Italy).

2.2 Rabbit IgG immobilization on glass beads

In a 100-mL round-bottom flask provided of reflux condenser, 10 g of aminated glass beads (1.1 $\mu\text{mol g}^{-1}$ of amino groups by Kaiser's method [41]), 5 mg (0.05 mmol) of succinic anhydride and about 1 mg of DMAP as catalyst were suspended into 40 mL of anhydrous pyridine. The mixture was heated at 90 °C for six hours, cooled, filtered on a 0.22 μm nylon membrane, and washed with dimethylformamide.

The hemisuccinated beads were transferred in a 100-mL flat-bottom flask containing 40 mL of dimethylformamide 6 mg of NHS (0.050 mmol) and 8 μL of DIC (0.052 mmol). The suspension was incubated at 4 °C for 60 min onto a horizontal roller, filtered on a 0.22 μm nylon membrane, washed with cold dimethylformamide and dried under vacuum suction.

The activated glass beads were transferred in a 100-mL flat-bottom flask and 40 mL of 1 mg mL⁻¹ of rabbit IgG dissolved in bicarbonate buffer (50 mmol L⁻¹, pH 8.5) were added. The suspension was incubated at room temperature overnight onto a horizontal roller, filtered on a 0.22 μm nylon membrane, washed with water, dried under vacuum suction and stored in the dark at 4 °C.

2.3. Synthesis of nanoMIPs

The polymerization mixtures were prepared in according with the literature [34], with minor modifications and adjusting the dilution of monomers to avoid formation of unwanted lumps of polymer. A pre-polymerization mixture (molar ratio BIS : AA : NIPAM : TBAm = 2 : 20 : 30 : 48) was made in 25 mL of ultrapure water by mixing under sonication 0.0065 mmol of cross-linker (BIS: 1 mg, EDAM: 1.1 mg, NOBE: 1.3 mg, EDMA: 1.3 mg, GDMA: 1.5 mg), 4.7 mg of AA (0.065 mmol), 11 mg of NIPAm (0.097 mmol) and 19.8 mg of TBAm (0.156 mmol, dissolved in 0.5 mL of ethanol). Then, 5 mL of mixture was added to 50-mL polypropylene SPE cartridges containing 2.5 g of functionalized glass beads. The cartridges were purged with nitrogen for 5 min, 3 μL of TEMED and 100 μL of 30 mg mL⁻¹ aqueous solution of APS were added and the polymerization was carried out at room temperature for 60 min in a roller-equipped incubator. The supernatant was drained by vacuum aspiration, the dry cartridges were cooled to 4 °C and polymerization by-products and low-affinity nanoMIPs were washed with 10 \times 2 mL of ice-cold water. High affinity nanoMIPs were collected by eluting the cartridges at room temperature with 5 \times 2 mL of 0.1 mol L⁻¹ aqueous HCl. The eluates were immediately neutralized with aqueous ammonium hydroxide 1 mol L⁻¹ and purified by gel-filtration in ultrapure water onto a 26 x 250 mm Sephadex G25 column. The nanoMIPs were isolated by centrifugation at 14000 x g, dried by lyophilisation and stored at 4 °C.

Not-imprinted polymers (nanoNIPs) were prepared in the same experimental conditions in terms of composition of the polymerization mixture and polymerization time, but using glass-beads functionalized with diclofenac as solid phase [42].

2.5. Determination of nanoMIPs size and charge

Hydrodynamic particle size and zeta potential were measured with a ZetaView® Nanoparticle Tracking Analyzer PMX-120, (Analytik, Cambridge, UK) using a laser source at 488 nm. Solid samples of each of the nanoMIPs were dissolved to working dilution with ultrapure water under sonication, pH was adjusted with HCl 0.1 mol L⁻¹, and about 2 mL of sample immediately injected in the Analyzer. Results are the average of three distinct measurements made at 25.5±0.1 °C.

2.6. Atomic force microscopy of nanoMIPs

Borosilicate glass slides, 10 x 10 mm, were washed with 'piranha' solution (98% sulphuric acid + 30% hydrogen peroxide, 3+1 v/v. Caution! It reacts violently with organic materials) for 10 min, rinsed with ultrapure water, dried under nitrogen and immersed overnight in a 1% v/v solution of APTMS in dry toluene. The aminated slides were washed with ethanol and ultrapure water and covered with an adequate volume of MES buffer (10 mmol L⁻¹, pH 4.7) containing 1 mg mL⁻¹ of NHS-activated nanoMIPs (vide infra), incubated at room temperature overnight, rinsed with ultrapure water and dried under nitrogen.

The Atomic Force Microscopy imaging was performed with a Park System XE-100 microscopes (Park Systems Europe GmbH, Mannheim, Germany) in non-contact mode (scan rate 0.4 Hz) using ACTA-10M cantilevers (Applied Nano Structures, Mountain View, USA).

2.7. Coupling of nanoMIPs to glass beads

In 4-mL vials 1 mg of nanoMIPs were dissolved under sonication in 1 mL of MES buffer, 5 mg of NHS (44 nmol) and 7 mg of EDC (28 nmol) were added and the solutions incubated at 4 °C for 60 min. Then, they were transferred in 3-mL vials containing 1 g of aminated glass beads. The suspensions were incubated at room temperature overnight, filtered on 0.22 µm nylon membranes, washed with ultrapure water, dried under vacuum at room temperature and stored at 4 °C.

2.8. Protein determination

The protein determination was carried out by Bradford assay method. Briefly, 50 µL of protein sample was added to 200 µL of protein assay reagent in polystyrene microplates (12x8 wells, flat bottom, VWR International, Milan, Italy). After shaking for 30s the absorbance was read at 450 and 590 nm. Each experimental point was assessed as the average of four repeated measures. Concentrations were calculated from a calibration graph covering the 0.5-50 µg mL⁻¹ range of protein diluted in same phosphate buffer plotting the ratio A₅₉₀/A₄₅₀ vs. the concentration [43].

2.9. Determination of binding properties

To measure binding isotherms, about 40 mg of glass beads supporting nanoMIPs were exactly weighed in 4 mL flat bottom amber glass vials. Then, 1.0 mL of phosphate buffer (20 mmol L⁻¹

¹, 0.13 mol L⁻¹ NaCl, pH 7.4) containing increasing amounts of proteins ranging from 1 to 50 µg mL⁻¹ was added. The vials were incubated overnight at room temperature under continuous agitation on a horizontal rocking table. Then, the solutions were filtered on 0.22 µm nylon membranes and the free amounts of proteins were measured by Bradford assay. Each experimental point was assessed as the average of three repeated measures. Binding parameters were calculated by using SigmaPlot 12 (Systat Software Inc., Richmond, CA, USA). Non-linear least square fitting was applied to the averaged experimental data. Binding isotherm parameters were calculated by using a Langmuir binding isotherm model:

$$B = \frac{K_{eq} B_{max} F}{1 + K_{eq} F}$$

where B is the protein bound to the polymer, F the protein not bound to the nanoMIPs, K_{eq} the equilibrium binding constant and B_{max} the binding site density.

To assure robust results, weighted (1/y) Pearson VII limit minimization was chosen as the minimization method. To avoid being trapped in local minima, which would give incorrect results, minimizations were carried out several times by using different initial guess values for the binding parameters.

The imprinting factor, IF, was calculated as:

$$IF = K_{eq(MIP)} / K_{eq(NIP)}$$

where K_{eq(MIP)} and K_{eq(NIP)} are the equilibrium binding constants measured on nanoMIP and nanoNIP, respectively.

The binding selectivity, α, was calculated as:

$$\alpha = K_{eq(protein)} / K_{eq(RIgG)}$$

where K_{eq(RIgG)} and K_{eq(protein)} are the equilibrium binding constants calculated for RIgG and any other protein, respectively.

3. Results and discussion

In order to investigate the effect of different cross-linkers in IgG-imprinted nanoMIPs, in the pre-polymerization mixtures BIS was replaced with other similar cross-linkers: N,N'-ethylene dimethacrylamide (EDAM), N,O-bis-methacryloyl ethanolamine (NOBE), ethylene glycol dimethacrylate (EDMA) and glycerol dimethacrylate (GDMA), without changing the molar proportions with functional monomers and using the same persulfate/TEMED-induced radical polymerization protocol in water at room temperature. After gel filtration, centrifugation and drying, nanoMIPs were collected as white solids, with yields calculated with respect to the amount of monomers in the polymerization mixtures of 15-18% (1-1.2 mg). When dissolved in water, nanoMIPs gave transparent and colourless solutions,

without any perceivable turbidity. Nanoparticles composition can be influenced by the different reactivity of the monomers, as well as the effective degree of crosslinking, but, because of the limited quantity of nanoparticles obtained, no attempts were made to establish the effective degree of crosslinking. Therefore, as a first approximation, we assume that it does not vary significantly between the different polymers.

3.1 Size and charge of nanoMIPs

Acrylic acid was used as charged functional monomer, thus nanoMIPs can be seen as charged polyelectrolytes at neutral pH. This is confirmed by ζ potential measurements, reported in table 1, where at pH 7 all the nanoMIPs show a net negative potential, with ζ values between -7.4 mV (GDMA) and -23.9 mV (BIS), while at pH 3, in more acidic conditions where carboxyls are fully protonated, ζ turns positive, with values between +1.0 mV (EDMA) and +15.1 (NOBE).

Table 1: hydrodynamic diameter (d_p) \pm 1s.d., relative increasing (swelling capacity) of particle volume between pH 7 and pH 3 (ΔV), polydispersity index (PDI), zeta potential (ζ), and absolute difference of zeta potential between pH 3 and pH 7 ($\Delta\zeta$) measured for nMIPs

	d_p (nm)			PDI		ζ , mV		
	pH 3	pH 7	ΔV	pH 3	pH 7	pH 3	pH 7	$\Delta\zeta$
BIS	171 \pm 83	129 \pm 66	2.34	0.24	0.26	+7.7	-23.9	31.6
EDAM	189 \pm 94	169 \pm 84	1.40	0.25	0.25	+10.4	-8.3	18.7
NOBE	186 \pm 87	148 \pm 72	1.98	0.22	0.24	+15.1	-18.0	33.1
EDMA	158 \pm 79	140 \pm 73	1.44	0.25	0.27	+1.0	-22.2	23.2
GDMA	147 \pm 77	129 \pm 71	1.48	0.27	0.30	+10.6	-7.4	18.0

The hydrodynamic diameter, d_p , measured by laser nanoparticle tracking at pH 7, shows nanoparticles with average diameters just over a hundred nm, ranging from 129 nm (BIS) to 169 nm (EDAM), and with polydispersity index between 0.24 (NOBE) and 0.30 (GDMA), corresponding to moderately polydispersed nanoparticles. In more acidic environment, at pH 3, the formation of aggregates larger than 1 μ m (instrumental limit of the particle tracker set-up), was indirectly observed, because the nanoparticles count fell by two orders of magnitude from 10^5 to 10^3 . About the fraction of nanoparticles remained in solution, the polydispersity index remains essentially constant, but diameters increase markedly, ranging from 147 nm (GDMA) to 189 nm (EDAM). These results show that in the solid phase synthesis the cross-linker structure marginally affect the dimensions of the resulting nanoparticles, which are probably mainly controlled by the formation of dangling long chains of monomers, some or most not cross-linked. Anyway cross-linker in some manner is yet capable of influencing nanoMIP flexibility. In fact, while nanoparticles containing BIS or NOBE are able to double their volume from pH 7 to pH 3, nanoparticles containing EBIS, EDMA or GDMA swell significantly less. It must be noted that nanoparticles swelling ability does not seem to be related to the binding properties (vide infra, section 3.3 for experimental results), as BIS- and GDMA-based nanopolymers show comparable binding constants but very different swelling ability from pH 7 to pH 3. It is also noteworthy that the absolute difference in the ζ values

measured between pH 7 and pH 3 is proportional to the swelling ability of the nanoparticles. This is not unexpected because as the volume changes, the surface charge density changes proportionally (whatever its sign), equally affecting the resulting ζ potential.

3.2 AFM imaging of nanoMIPs

Acrylic acid was used as charged functional monomer, thus The results obtained by laser nanoparticle tracking are confirmed by atomic force microscopy performed onto nanoMIPs covalently grafted onto aminosilanized glass slides (see Electronic Supplementary Information). The imaging – an example of which is reported in Figure 1 – performed on relatively large area of 10 x 10 μ m shows that the glass surface is randomly covered with what seems to be sparse clusters of nanoparticles. NanoMIPs were covalently grafted onto the glass slides at pH 4.7, in conditions within the pH jump covered by the tracking measurements (from pH 7 to pH 3), thus the formation of these structures is likely to be due to grafting of clustered nanoparticles stabilized by electrostatic interactions. The imaging of a cluster at higher resolution (x25) on an area of 2 x 2 μ m (Figure 2) shows an overall shape rather irregular, with an approximate size of 1.2 x 1.2 x 0.3 μ m, apparently composed of several tightly packed globular objects with a slightly wrinkled surface and with individual diameters comparable to those measured by nanoparticles laser tracking, therefore compatible with an aggregate of nanoparticles.

A further evidence of nanoparticles clustering induced by electrostatic interactions comes from imaging of deposited nanoMIPs at higher ionic strength (0.1 mol L⁻¹ NaCl). In this case, clusters are significantly larger for all the nanoparticles examined (Figure 3), often exceeding dimensions of 2 x 2 μ m, even if their height with respect to the underlying glass surface does not seem to grow proportionally.

3.3 Binding properties of nanoMIPs

In the traditional molecular imprinting techniques (bulk, suspension/emulsion, etc.) BIS is very little used and the cross-linker constitutes up to 80% molar of the polymerization mixture, thus exerting a deep effect not only on the morphology of the polymer and its bulk properties, but also on the binding properties [44-46]. On the contrary, in SPS technique BIS is the preferred cross-linker, and in the pre-polymerization mixture it is added in a much more limited amount, practically never more than 3% by moles. Consequently, it is to be expected that the effect on molecular recognition properties by the cross-linker is limited, and that the presence of structurally different cross-linkers is not able to affect these properties.

Table 2: equilibrium binding constants ($\text{mol}^{-1} \text{L} \times 10^{-6}$) \pm 1s.e. measured in phosphate buffer (20 mmol L^{-1} , 0.13 mol L^{-1} NaCl, pH 7.4) for RIgG, BIgG, BSA and LZM on RIgG-imprinted (nMIP) and not imprinted (nNIP) nanoparticles supported onto glass beads

		RIgG	BIgG	BSA	LZM
BIS	nMIP	16.0 \pm 1.3	3.5 \pm 0.4	2.2 \pm 0.3	1.7 \pm 0.3
	nNIP	1.3 \pm 0.3	3.4 \pm 0.7	1.0 \pm 0.2	0.6 \pm 0.2
EDAM	nMIP	8.8 \pm 1.9	3.3 \pm 0.8	2.1 \pm 0.5	2.3 \pm 0.6
	nNIP	1.6 \pm 0.3	2.8 \pm 0.9	0.3 \pm 0.2	1.3 \pm 0.2
NOBE	nMIP	15.9 \pm 2.5	6.2 \pm 1.2	0.6 \pm 0.3	0.2 \pm 0.2
	nNIP	2.2 \pm 0.5	1.7 \pm 0.1	0.3 \pm 0.1	0.2 \pm 0.1
EDMA	nMIP	3.4 \pm 0.7	2.4 \pm 0.4	1.4 \pm 0.4	0.3 \pm 0.1
	nNIP	1.2 \pm 0.4	1.6 \pm 0.4	0.9 \pm 0.2	0.1 \pm 0.0
GDMA	nMIP	12.8 \pm 1.5	4.3 \pm 1.0	1.8 \pm 0.3	1.4 \pm 0.3
	nNIP	1.1 \pm 0.3	1.8 \pm 0.5	0.6 \pm 0.2	0.6 \pm 0.0

Surprisingly, the determination of the equilibrium binding constant, K_{eq} , by equilibrium partition experiments shows a distinctly different situation. In fact, as reported in Table 2, all the cross-linker used for the imprinting of RIgG gives nanoMIPs with K_{eq} around $10^7 \text{ mol}^{-1} \text{L}$, with the remarkable exception of EDMA, which gives a significantly lower value of $3.4 \times 10^6 \text{ mol}^{-1} \text{L}$. In comparison, the corresponding nanoNIPs, prepared by SPS with diclofenac as template, show K_{eq} with values significantly lower than the values for the corresponding nanoMIPs and indistinguishable from each other (t-test: $\alpha=0.05$, $n=10$, $t<2.101$).

The differing values of K_{eq} obtained for each nanoMIP have an obvious influence on the imprinting factor, IF, that is an estimate of how much the binding affinity increases for an imprinted polymer respect to a not imprinted of identical composition. In Figure 4 all five nanoMIPs show IF values higher than unity, confirming the success of the SPS technique in the imprinting of RIgG. However, while nanoMIPs containing BIS and GDMA show IF values higher than 10 (BIS: 12.3 ± 3.0 , GDMA 11.6 ± 3.5), corresponding to a very strong imprinting effect, the others show markedly lower IF values (EDAM: 5.5 ± 1.6 , NOBE: 7.2 ± 2.0), demonstrating that the choice of right cross-linker is important to achieve an efficient SPS process, regardless of whether the cross-linker itself is present in the pre-polymerization mixture in very limited quantities compared to the other monomers. It should be noted that the GDMA-based nanoMIPs are to be considered structurally more complex than the other nanoMIPs, as they are composed of an almost equimolar mixture of two different cross-linkers, respectively glycerol 1,2- and 1,3-dimethacrylate. However, the resulting nanoMIP does not appear to behave significantly differently from other nanoMIPs, except of course for those based on EDMA. It is also noteworthy that the polymer with the lowest IF value (2.8 ± 1.1) contains EDMA, which represents the predominant cross-linker used to prepare imprinted polymers with the traditional approaches. This fact is a strong evidence of how the SPS technique differs from the other molecular imprinting approaches, and how it is necessary to paid attention to transfer pre-polymerization mixtures formulations from one approach to another without a careful preliminary evaluation. In addition to the magnitude of the binding constant and imprinting factor, a third essential parameter for evaluating the

molecular recognition properties of nanoMIPs is binding selectivity, α . In this work we evaluated the selectivity of RIgG-imprinted nanoMIPs towards a structurally very similar protein such as bovine IgG (BIgG), a protein structurally different but of similar isoelectric point, bovine serum albumin (BSA), and a protein of different structure and isoelectric point such as hen egg lysozyme (LZM).

The α values in Figure 5 show that all the nanopolymers characterized by high affinity ($K_{\text{eq}} \sim 10^7 \text{ mol}^{-1} \text{L}$) are selective towards the template RIgG, with a limited but substantial recognition ($0.2 \leq \alpha \leq 0.4$) towards BIgG, confirming the results reported in literature for human IgG-imprinted nanoMIPs [27]. This limited recognition can be explained on the basis of the shared presence in the IgG structure of the Fc fragment, which differs little between proteins of different species [1]. As a template RIgG is randomly grafted on the surface of the glass beads, and nanoMIPs produced by SPS will have molecular recognition properties towards different parts of the template structure. Some will have binding sites recognizing the Fc fragment, common to IgG from different species, while others will recognize other portions of the protein, which are typical for IgG of a particular species. Therefore, during the rebinding of BIgG, some nanoMIPs will preferentially bind the Fc fragment, regardless of its origin (rabbit or bovine), while others, more selective, will not be able to bind the BIgG. Thus, the resulting binding will be an average between the full (Fc-binding nanoMIPs) and the weak (non-Fc-binding nanoMIPs) recognition of BIgG.

On the contrary, because of the low affinity resulting in a limited imprinting factor, with EDMA-based nanoMIPs RIgG and BIgG are recognized almost in the same way, confirming a substantial absence of selectivity. It must be noted that the low values of K_{eq} measured for EDMA-based nanoMIPs ($2.4 \pm 0.4 \times 10^6 \text{ mol}^{-1} \text{L}$) and nanoNIPs ($1.6 \pm 0.4 \times 10^6 \text{ mol}^{-1} \text{L}$) are statistically indistinguishable from each other (t-test: $\alpha=0.05$, $n=10$, $t=1.414$). Therefore, in this case the binding to BIgG cannot be attributed for certain to the presence of imprinted binding sites. Concerning BSA and LZM, as these proteins are very different from IgG, for all the nanopolymers the molecular recognition results very limited, lower than that observed for BIgG. However, it should be noted that the binding behaviour presents significant differences, since for BIS-, EDMA- and GDMA-based nanopolymers the recognition follows the order of similarity, i.e. BIgG > BSA > LZM, while in the case for EDAM- and NOBE-based nanopolymers it is different, as the first recognizes the three proteins in the same way, while for the second, BSA and LZM show almost no recognition, confirming that small changes in the nature of the cross-linker – i.e. the replacement of an amide group with an ester group (NOBE vs. EDAM) – exert a significant effect on the binding properties of the nanoMIPs.

Conclusions

The results reported in this work confirm the relevance of the cross-linker structure in the SPS technique. Although present in minimal quantities compared to the other monomers in the

pre-polymerization mixture, they are able to influence the binding affinity and selectivity of protein-imprinted nanopolymers through subtle differences in their structure, i.e. the replacement of an amide group with an ester group (NOBE vs. EDAM), the presence of a hydroxyl group (GDMA vs. EDMA) or the number of atoms in the molecular bridge (BIS vs. EDAM). The experimental results currently available are not sufficient to advance quantitative hypotheses on the relationship between binding properties of nanoMIPs and structural properties of cross-linkers, but it is plausible that a further expansion of the number of cross-linkers tested could provide robust indications on the type of molecular structures optimal to obtain nanoMIPs with high affinity and selectivity for the target molecule.

Author Contributions

Conceptualization, C.B.; methodology, M.C. and C.B.; investigation – laser particle tracking, T.S.; investigation – atomic force microscopy, F.S.; investigation – binding isotherms, M.C. and S.C.; resources, L.A.; data curation, F.D.N. and C.B.; writing – original draft preparation, C.B.; writing – review and editing, M.C.

Conflicts of interest

There are no conflicts to declare.

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