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A novel method of molecular imprinting applied to the template cholesterol

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ABSTRACT

A novel method is successfully tested for non-covalent imprinting. Conditions are used which practically exclude the formation of prepolymerization complexes. The template is cholesterol, and no so-called functional monomer is used. The polymers contain only an acrylic diester crosslinker. The porogen isopropanol prevents even hydrogen bonding between the template and the monomer in the prepolymerization solution. Despite of these apparently very disadvantageous conditions, appreciable imprinting factors for cholesterol and imprinted selectivity against some other steroids are observed, similar to other cholesterol MIPs with proven analytical usefulness.

1. Introduction

Molecular imprinting is a widely used technique to obtain selective adsorbents, like chromatographic stationary phases and solid phase extraction devices, sensors, catalysts, membranes, etc. [1–5].

The most widely used method for making molecularly imprinted polymers (MIP) is the non-covalent method. It consists of polymerizing suitable monomers in the presence of a template compound. The template interacts with the monomers in the prepolymerization mixture by non-covalent forces like van der Waals forces, hydrogen bonding or ionic attraction. The polymerization mixture typically includes also a porogenic solvent which assists in obtaining a large surface area, porous polymer. The monomers used are responsible for the non-covalent interactions with the template, and also for making a highly cross-linked, stiff network. After polymerization the template is extracted from the polymer. Subsequently, the polymer may be used for (re)binding the template or its chemical analogs from various media [1–6].

The main idea of imprinting is, that by procedures like the one just described, polymers can be prepared which can rebind the template or its close analogs efficiently and selectively. It is generally assumed that imprinting, as the name suggests, creates chemical imprints of the template molecule, i.e., binding sites, binding cavities or binding pockets which are complementary to the template in shape and chemical functionality.

This paper presents a surprising novel method of non-covalent imprinting which has been found as a result of a directed screening with polymers of different compositions. While other known instances of non-covalent imprinting rely on the formation of prepolymerization complexes from the template and the monomers, the successful prepolymerization mixtures presented in this work exclude virtually any possibility of forming a prepolymerization complex. The template is cholesterol (Fig. 1), a hydrocarbon which has only two functional groups, a double bond and an alcoholic OH group. As monomers three simple diesters have been tested: ethyleneglycol dimethacrylate (EDMA), 1,4-butanediol dimethacrylate (BDMA) or 1,6-hexanediol dimethacrylate (HDMA). Three different porogens have been used: 2-propanol (iPrOH), acetonitrile (ACN), and chloroform, but as will be seen, only iPrOH leads to successful imprinting. This is surprising because the only likely specific interaction between the alcoholic cholesterol and the diester monomers would be hydrogen bonding, which may be supported by the aprotic porogens acetonitrile and chloroform, respectively, but not by iPrOH.

As noted, cholesterol lacks the typical functional groups which have proved useful in other cases of non-covalent imprinting. The difficulties of imprinting with low functionality templates, including steroids, have been convincingly demonstrated by several groups [7–11]. Imprinting for steroids, and in particular for cholesterol has attracted continuous interest [12–15], with the earlier literature having been reviewed by

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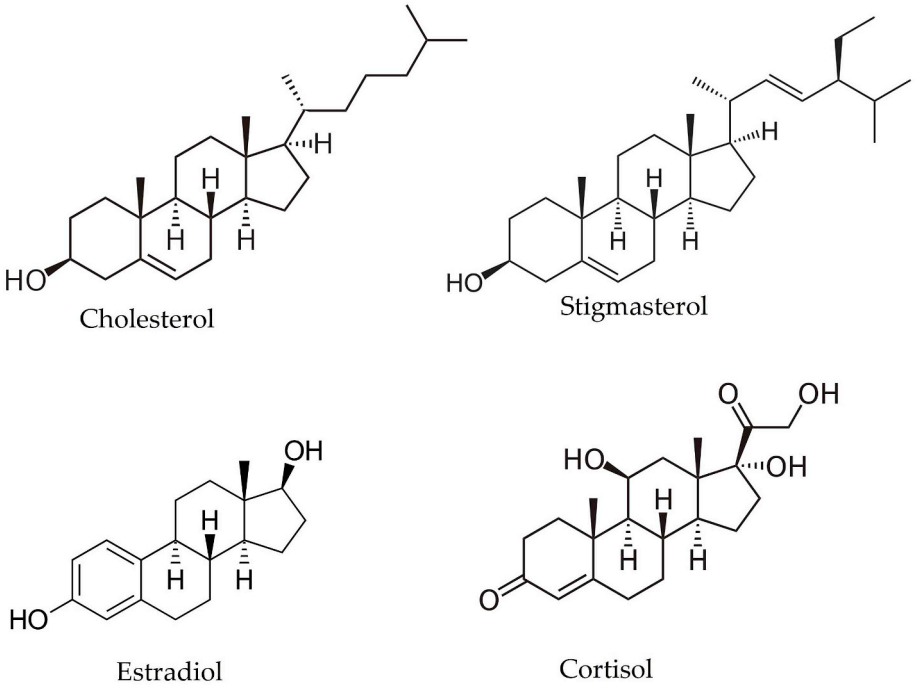

Fig. 1. Structures of the steroids used.

Gore et al. [16] in 2004. An early and very important paper by Whitcombe et al. [17] demonstrated in 1995 that cholesterol can be efficiently imprinted with the then novel semicovalent imprinting method. Although the cited work was very efficient, the results did not provide clues for the possibility of non-covalent imprinting with cholesterol. One may also note that rebinding was studied only in a nonpolar solvent, hexane. Sellergren et al. [7] and Gore et al. [16] made non-covalent imprinting for cholesterol by employing monomers covalently decorated with cholesterol. The idea behind this method was that cholesterol easily forms dimers in suitable solvents. The selectivity improvement of the MIP relative to the non-imprinted control polymer (NIP) in Sellergren et al.'s work was good against ergosterol but not against testosterone. The selectivity of Gore et al.'s MIPs against testosterone was at best 2.1 times higher than that of the NIP, and against several other compounds it was even less than with the NIP. When the steroids contained several hydrophylic groups, unlike cholesterol, which has only one such group, good imprinted selectivity could be demonstrated, using acidic functional monomers. For example, steroids with alpha and beta 17-OH group, respectively, could be very nicely separated in two papers (with separation factors of 2.5 and 2.1, respectively) on MIP HPLC columns imprinted with one of the two isomers (or with an analogous compound) [18,19]. Several workers had screened, similarly to the present work, a series of monomers for the non-covalent imprinting with steroids. Baggiani et al. [20] combined 10 different functional monomers, 7 crosslinkers and 5 porogen solvents. Kopperi and Riekkola [9] screened with three functional monomers, three crosslinkers and four porogens.

All of the above cited non-covalent imprinting studies employed some functional monomer like methacrylic acid (MAA), vinylpyridine (VP), 2-hydroxyethyl methacrylate, acrylamide, or monomers decorated with steroids. In the present work imprinting with the low functionality steroid, cholesterol, was successful without such functional monomers, as only ester type crosslinking monomers were used. Despite of this, increased template binding and increased selectivity due to imprinting could be demonstrated. One should mention that Spivak and coworkers [21] succeeded in making many non-covalent imprints by using merely a crosslinking monomer, but that monomer was a half amide half ester, and thus could utilize the known advantageous properties of amides as functional monomers in non-covalent imprinting. The group of Tanaka observed [22] that non-imprinted polymers made from crosslinkers alone had a memory for the porogenic solvent used. This observation was similar to the first known instance of molecular imprinting, when Polyakov [23] noticed the adsorptive memory of silica gels dried in different solvents. The group of Tanaka went a step further and utilized also the cross-reactivity of the porogen-imprinted polymers [24]. However, an attempt to imprint with a template dissolved in their non-polar porogens was apparently not leading to improved selectivity for the template (Fig. 1 in Ref. [25]). As presented below, we could imprint for a substance dissolved in the porogen.

Polymers imprinted with cholesterol can be used mainly as solid phase extraction sorbents [7,12–17]. Milk and dairy products are important cholesterol sources and therefore there is continued interest in the determination of cholesterol in these matrices [26–29], also with MIPs [13,30]. Cholesterol oxidation products in milk products [27,28] and cholesterol metabolites in blood [31] also need to be analyzed. The cholesterol MIP may then be used as a class selective sample pretreatment sorbent.

The present work appears to be the first instance when non-covalent molecular imprinting has been successful with a single monomer, without specific interactions between the template and the monomer, and with a template other than a porogenic solvent. The imprinting method employed in the present work may also be extended to other templates which have poor functionality for the usual prepolymerization complex formation.

2. Materials and methods

Cholesterol, stigmasterol, estradiol, cortisol (all four being shown in Fig. 1), ethylene glycol dimethacrylate (EDMA), 1,4-butanediol dimethacrylate (BDMA) and 1,6-hexanediol dimethacrylate (HDMA) were purchased from Sigma Aldrich (USA). HPLC solvents (methanol (MeOH), 2-propanol (iPrOH), acetonitrile (ACN), n-hexane) were purchased from Merck (Germany). Azobisisobutironitrile (AIBN) was purchased from Fluka (Switzerland). Water was purified with a Milli-Q (Millipore) system. Chloroform was purchased from Macron (Poland).

Prior to use EDMA, BDMA and HDMA were all purified from inhibitors using commercial inhibitor remover column (Sigma Aldrich).

2.1. Instrumentation

Grant-Bio PTR-35 multirotator, Eppendorf Minispin centrifuge, Biosan TS-100 Thermo Shaker, PerkinElmer Series 200 HPLC, Nova2000e (Quantachrome) gas sorption device and Zeiss LEO 1540 XB scanning electron microscope were used.

2.2. Polymer preparation

All polymers were prepared by bulk polymerization.

General procedure for imprinted polymers: Inhibitor free monomers (2.00 mL) were mixed in a glass vial with porogen (4.00 mL iPrOH or chloroform, or 2.00 mL ACN) and the template cholesterol (1.0000 g, except in the case of ACN porogen, which was saturated with cholesterol). Then initiator AIBN (50.0 mg) had been added and, if necessary, the mixture was heated until the template completely dissolved. The mixture was purged with argon for 5 min and heated in a water bath at 60 °C for 24 h. Imprinted polymers were washed with MeOH, and the template extracted in a Soxhlet apparatus using chloroform as solvent until no more template was detected to 'bleed' from the polymer (often 4-5 days). Finally, the polymers were dried overnight.

General procedure for non-imprinted polymers: Non-imprinted polymers were prepared as the imprinted ones, but with the omission of the template.

Imprinted and non-imprinted polymers P13-P18 (Table 1) with the porogen ACN were prepared at room temperature (25 °C) under UV light (366 nm) for 24 h.

The components of all prepolymerization mixtures are listed in Table 1.

2.3. Binding experiments

30.0 ± 0.1 mg of polymer was weighed in a 1.5 mL Eppendorf tube and 300.0 μL of 0.1 mM steroid solution was added. Polymer and steroid solution were mixed using a rotator for 2 h, centrifuged for 10 min (13400 rpm) and the supernatant was transferred to a 0.5 mL Eppendorf tube and centrifuged again (10 min, 13400 rpm). Supernatant free of polymer particles was analyzed using HPLC.

When measuring binding in hexane, after second centrifugation, 100.0 μL of supernatant was evaporated until dryness and reconstituted in 100.0 μL of MeOH, after which HPLC determination of cholesterol (or stigmasterol) was conducted.

The measurement of the unbound steroids occurred typically with 1% standard deviation by the HPLC methods presented below.

Table 1
Components of the prepolymerization mixtures.

Polymer	Functional monomer	Crosslinker	Template	Initiator	Porogen
P1	/	EDMA	/	AIBN	iPrOH
P2	/	BDMA	/	AIBN	iPrOH
P3	/	HDMA	/	AIBN	iPrOH
P4	/	EDMA	Cholesterol	AIBN	iPrOH
P5	/	BDMA	Cholesterol	AIBN	iPrOH
P6	/	HDMA	Cholesterol	AIBN	iPrOH
P7	/	EDMA	/	AIBN	Chloroform
P8	/	BDMA	/	AIBN	Chloroform
P9	/	HDMA	/	AIBN	Chloroform
P10	/	EDMA	Cholesterol	AIBN	Chloroform
P11	/	BDMA	Cholesterol	AIBN	Chloroform
P12	/	HDMA	Cholesterol	AIBN	Chloroform
P13	/	EDMA	/	AIBN	ACN
P14	/	BDMA	/	AIBN	ACN
P15	/	HDMA	/	AIBN	ACN
P16	/	EDMA	Cholesterol	AIBN	ACN
P17	/	BDMA	Cholesterol	AIBN	ACN
P18	/	HDMA	Cholesterol	AIBN	ACN

2.4. HPLC methods

Method for cholesterol and stigmasterol determination: Isocratic elution using MeOH/iPrOH 65/35 (v/v) as solvent with a flow rate of 0.6 mL/min at 25 °C. Injection volume was 10.0 μL, and detection wavelength 205 nm. Retention time was 2.3 min for cholesterol and 2.5 min for stigmasterol using Zorbax Eclipse XDB-C18, 50 × 4.6 mm, 1.8 μm particle size column (Agilent Technologies).

Method for estradiol determination: Isocratic elution using MeOH/water 50/50 (v/v) as eluent with a flow rate of 0.75 mL/min at 25 °C. Injection volume was 10.0 μL, and detection wavelength 280 nm. Retention time was 6.7 min using Hypersil GOLD, 50 × 3 mm, 5 μm particle size column (Thermo Scientific).

Method for cortisol determination: Isocratic elution using MeOH/water 50/50 (v/v) as eluent with a flow rate of 0.5 mL/min at 25 °C. Injection volume was 10.0 μL, and detection wavelength 242 nm. Retention time was 2.9 min using Hypersil GOLD, 50 × 3 mm, 5 μm particle size column (Thermo Scientific).

2.5. Nitrogen adsorption

Nitrogen adsorption/desorption isotherms were measured at −196 °C with a Nova2000e (Quantachrome) computer controlled apparatus. Transformation of the primary adsorption data and pore size analysis were performed with the QuantachromeASi Qwin software (version 3.0). The apparent surface area S_{BET} was calculated using the Brunauer–Emmett–Teller (BET) model. The pore volume at relative pressure 1 (V_{tot}) and at relative pressure 0.96 ($V_{0.96}$) was derived from the amount of nitrogen adsorbed at the corresponding relative pressure, assuming that the pores are then filled with liquid adsorbate. The micropore volume (W_0) was derived from the Dubinin–Radushkevich (DR) plot.

2.6. Scanning electron microscopy (SEM)

10 μL of 1% w/v methanolic suspension of finely powdered polymer (NIP P1 and MIP P4, respectively) was dropped onto gold plated silicon chips which had been pre-cleaned with ethanol. After evaporation of MeOH the chips were kept under argon until the SEM measurement. Pictures were taken with a Zeiss LEO 1540 XB Crossbeam Scanning Electron Microscope with aperture hole 20.00 μm. The EHT (electron high tension) value was 1.5 kV. The chamber vacuum was $7*10^{-7}$ mBar, the gun vacuum $2.2*10^{-10}$ mBar.

3. Results

In search for the optimal conditions of the novel imprinting method, a series of non-imprinted and cholesterol imprinted polymers (Table 1) has been tested for the binding of some representative steroids, which have varying levels of functionality. The polymers did not contain any special functional monomers, but consisted only of ester type crosslinkers. The three crosslinkers tested, EDMA, BDMA and HDMA, are all methacrylate diesters of diols, differing only in the length of the alkyl chains between the terminal diol groups. Three different porogens were tested (iPrOH, ACN and chloroform, respectively). Thus altogether nine crosslinker/porogen combinations were investigated.

Adsorption of four steroids was studied: cholesterol, stigmasterol, estradiol and cortisol (Fig. 1). The steroids were adsorbed either from MeOH/water 9/1 or 8/2 (v/v), or from hexane. The starting solution concentration was always 0.1 mM. Not every possible combination of the mentioned factors was tested, partly because of solubility problems, partly because some combinations were not deemed interesting. All

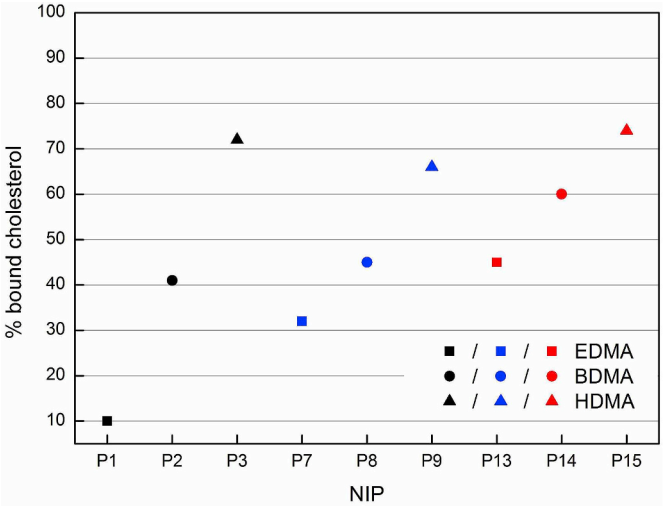

Fig. 2. Cholesterol binding on NIPs from MeOH/water 9/1 (v/v). Porogens used in NIP preparation are represented by different point colors: black – iPrOH, blue – chloroform, red – ACN; crosslinkers used in NIP preparation are labeled on the graph. The polymer codes of the horizontal axis are used according to Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

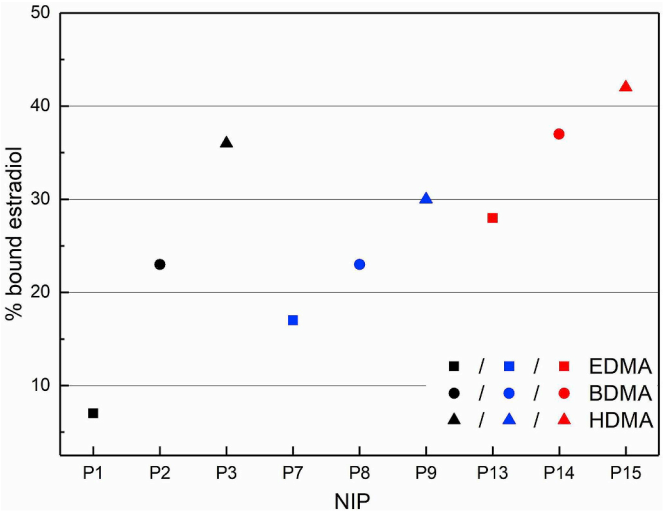

Fig. 3. Estradiol binding on NIPs from MeOH/water 9/1 (v/v). Porogens used in NIP preparation are represented by different point colors: black – iPrOH, blue – chloroform, red – ACN; crosslinkers used in NIP preparation are labeled on the graph. The polymer codes of the horizontal axis are used according to Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

polymer compositions are given in Table 1, and representative measurement results are given in Figs. 2–4 and in Tables 2 and 3.

Nitrogen adsorption experiments have been done with the MIP P4 and its NIP counterpart NIP P1, since MIP4, prepared in iPrOH from EDMA, has shown the best features in the binding experiments. The results are presented in Table 4. Scanning electron microscopy (SEM) has been done on the same two polymers and representative pictures are shown in Fig. 5.

4. Discussion

The goal of this work has been to develop a novel, very simple method of non-covalent imprinting, which would be capable of imprinting even with the most difficult templates, which have almost no

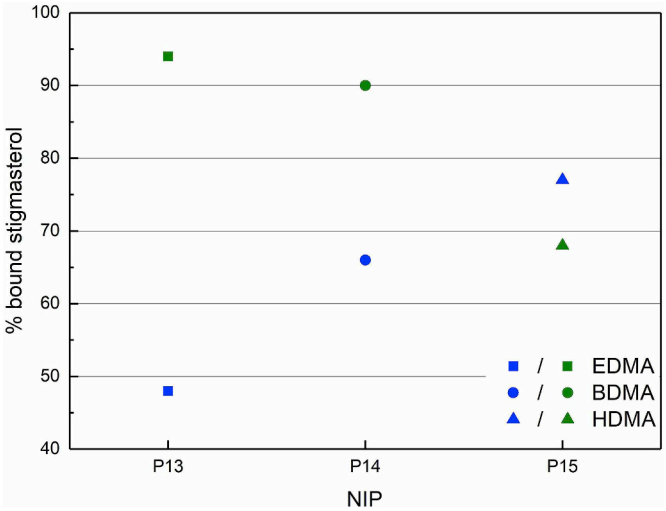

Fig. 4. Stigmasterol binding from MeOH/water 9/1 (v/v) (blue points) and from hexane (green points), respectively, on NIPs made in ACN. Crosslinkers used in NIP preparation are labeled on the graph. The polymer codes of the horizontal axis are used according to Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Imprinting factors (IF) for cholesterol imprinted polymers prepared of EDMA, BDMA and HDMA in iPrOH (The polymer codes are used according to Table 1). The notations 9/1 and 8/2, respectively, denote the MeOH/water volume ratios of the respective rebinding media.

MIP/NIP	IF_{chol} 9/1	IF_{chol} 8/2	Monomer
P4:P1	1.9	3.4	EDMA
P5:P2	1.4	1.4	BDMA
P6:P3	1.5	1.1	HDMA

Table 3
Selectivity of the MIP, made from EDMA in iPrOH, for cholesterol as against other steroids. Selectivity values are the ratios of distribution factors for two compared compounds. Media: MeOH/water 9/1 for stigmasterol, MeOH/water 8/2 for estradiol and cortisol. Improvement factors against the NIP are the ratios of the selectivities measured with the MIP and the NIP, respectively.

Compound	Selectivity	Improvement vs. NIP
Stigmasterol	1.1	1.0
Estradiol	6.5	1.7
Cortisol	10.6	2.1

Table 4
Nitrogen adsorption results for NIP P1 and MIP P4, respectively, both made from EDMA in iPrOH.

Polymer	S_{BET} m^2/g	$V_{0.96}$ cm^3/g	V_{tot} cm^3/g	W_0 cm^3/g
P1 NIP	205	0.147	0.167	0.089
P4 MIP	223	0.155	0.161	0.093

functionality for specific interactions. If the template lacks such functionality there is no reason to use so-called functional monomers. Therefore, it has been decided to screen polymers consisting of crosslinkers only, and these crosslinkers should contain only ester groups. The template chosen has been cholesterol (Fig. 1) as it has only an alcoholic OH group available for specific interaction. The porogens have been chosen partly for their capability to dissolve cholesterol, partly to cover a wide range of porogen properties. Thus acetonitrile was selected as a dipolar aprotic porogen, isopropanol as a protic polar

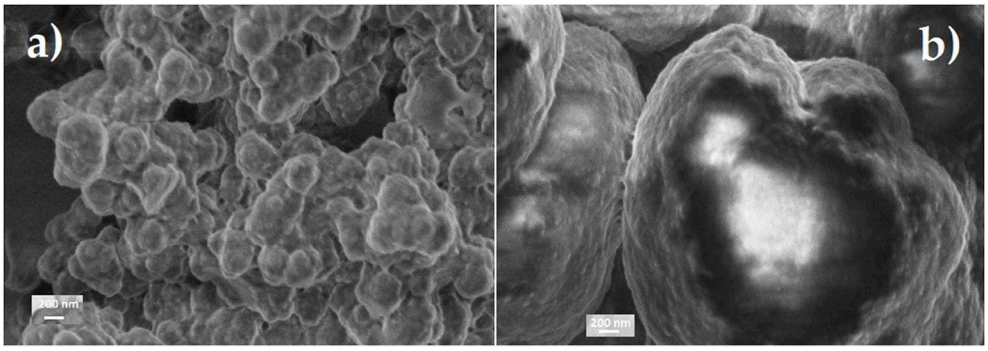

Fig. 5. SEM pictures of NIP P1 (a) and MIP P4 (b), respectively, at 50,000x magnification.

porogen and chloroform as it tends to form gels rather than macroporous polymers.

The expected result from imprinting has been, as always, that the MIPs will bind more template (cholesterol) than the NIPs, and that the selectivity of the MIPs will be better for cholesterol than that of their NIP counterparts. For this reason, the NIPs have been studied first.

4.1. Binding of steroids by the NIPs

The adsorption pattern of steroids on the non-imprinted control polymers has been established by static (batch) binding experiments. Two types of binding media were investigated: polar MeOH-water mixtures and nonpolar hexane, respectively. Binding results are presented in Figs. 2–4.

One can see some clear trends of the binding of steroids on NIPs made of diester crosslinkers alone, as demonstrated by Figs. 2–4. The three series of NIPs made with different porogens (P1–P3 with iPrOH, P7–P9 with chloroform and P13–P15 with ACN) each show that steroid binding from aqueous MeOH is increased by increasing the chain length of the crosslinker (Figs. 2 and 3). The increase of steroid adsorption with monomer chain length was observed both with the very hydrophobic cholesterol (Fig. 2) and the less hydrophobic estradiol (Fig. 3).

One can also notice that the less hydrophobic estradiol is much less bound from the partly aqueous medium than cholesterol (note the different scales of Figs. 2 and 3, respectively.). Similar trends have been observed with the very hydrophobic stigmasterol (Fig. 4) and the least hydrophobic cortisol. All this indicates that the binding from the partly aqueous medium is strongly influenced by the hydrophobicity of the adsorbed compound.

If the rebinding trend between the polymers having different chain length is studied in the nonpolar solvent hexane, as opposed to the MeOH/water mixtures above, the trend becomes reversed, i.e, the binding is stronger on the polymer with shorter chain (Fig. 4). This occurs presumably because in hexane the hydrophobicity of the steroids is not relevant for binding. It is much more the functional groups (in the case of Fig. 4 the alcoholic OH group of stigmasterol) which can interact with the ester groups of the crosslinkers. Since the concentration of ester groups in the polymers is higher when the chain length is shorter, one does indeed expect that stigmasterol binding from hexane will be highest with EDMA and lowest with HDMA.

The results with the NIPs as shown in this section serve partly the subsequent comparison with MIPs, but they indicate also that steroids which are not too similar to each other, may be effectively differentiated by the non-imprinted polymers themselves.

4.2. Binding of steroids by imprinted polymers

Imprinting with steroids is difficult, due to their low functionality. Estradiol as a phenolic compound is more likely to promote imprinting, but cholesterol or stigmasterol seem to offer little useful interaction possibility for non-covalent imprinting. The reason for choosing cholesterol as template was just this property. We wanted to see if non-covalent imprinting with such a weakly interacting template was at all possible when using weakly interacting monomers.

When ACN or chloroform were used as porogens, imprinting with cholesterol remained unsuccessful. The imprinting factors of the MIPs imprinted for cholesterol in ACN and also in chloroform were with all three crosslinkers close to 1, when tested in MeOH/water mixtures. (The imprinting factor, IF, has been defined here as the ratio of the respective distribution coefficients of cholesterol measured with the MIP and the NIP, respectively. A value of IF = 1 means that imprinting has not increased the binding of the template by the polymer.)

We found, however, appreciable IF values (Table 2) when cholesterol imprinting occurred in iPrOH and when using EDMA (and to a lesser extent when using BDMA or HDMA) as the sole monomer. Table 2 shows that the highest observed IF is 3.4. The imprinting efficiency depended on the crosslinker chain length as well as on the MeOH/water ratio of the medium used for testing.

4.3. MIP selectivity

The observation of IF values higher than 1 shows that the MIP can bind more template than the NIP. The selectivity of the MIPs is another important feature. Table 3 shows the selectivity of the MIP made with EDMA in iPrOH. With respect to stigmasterol, which is very similar to cholesterol (Fig. 1) the selectivity is 1.1, that is the MIP cannot significantly differentiate stigmasterol from cholesterol. The selectivity against two other steroids, estradiol and cortisol, is very high, however. As shown in Section 4.1., the NIP has also some selectivity for cholesterol in the partly aqueous media used here. This is due to the pronounced hydrophobicity of cholesterol. Nevertheless, the selectivity of the MIP is significantly higher against estradiol and cortisol than that of the NIP. This is shown by the respective improvement factors presented in Table 3. Note here for comparison, that one of the most studied MIPs, the propranolol MIP, behaves similarly. Its selectivity for propranolol against some other amines is better than the selectivity of the NIP, but the NIP already shows substantial preference for propranolol [6].

The imprinting factor and selectivity features of MIP P4 are very close to the values reported for other cholesterol imprinted polymers. For example, Puoci et al. [13] reported an imprinting factor of 4.7, and a selectivity against cortisol of 9.3, while Lee et al. [32] found a selectivity against estradiol of 3.1. Therefore, MIP P4 should be suitable for similar analytical applications in sample cleanup of dairy products and other biological samples, as some other reported cholesterol MIPs.

It is very surprising that among the tested porogens only iPrOH gave good imprinted polymers. This solvent likely prevents binding of the cholesterol OH group to the ester groups of the crosslinkers, while such interaction was thought more likely to lead to efficient imprinting in chloroform or ACN.

4.4. Nitrogen adsorption and SEM results with the optimal MIP and its NIP

The results of nitrogen adsorption measurements with the MIP P4, prepared from EDMA in iPrOH, and with the corresponding NIP P1, are summarized in Table 4.

The NIP and the MIP have similar surface area and pore volume values. This indicates that the increased cholesterol binding by the MIP (Table 2, column 3, line 1, IF = 3.4) is not due to a simple surface increase, and thus it is a real imprinting effect.

SEM pictures of the same two polymers, NIP P1 and MIP P4, respectively, are shown at 50,000x magnification in Fig. 5. The size and shape of the elementary particles of the two polymers are quite different. The structure of the elementary particles has also different character. This may be related to the presence of cholesterol during imprinting. The elementary particles of the MIP seem to have a layered structure, and the cholesterol adsorption may occur between these layers and/or at the filament-like structures connecting elementary particles. Both of these features are seen only on the MIP picture, but not on the NIP.

5. Conclusions

This work has shown that even in cases when imprinting was not earlier deemed feasible, there may be significant imprinting effects. With cholesterol, a known difficult template, imprinting was efficient without using any of the typical functional monomers, simply by employing diester crosslinkers, particularly EDMA. Surprisingly, the best porogen was iPrOH, a solvent which is likely to prevent hydrogen bonding between the template and the ester type monomer. The MIPs so obtained had shown increased binding of cholesterol and also increased selectivity for cholesterol against estradiol and cortisol. The obtained imprinting factors and selectivity improvements are remarkable even when compared to other non-covalent imprinting systems. This result projects the possibility of similar analytical applications of the novel MIP as with other cholesterol MIPs, e.g., in the analysis of dairy products. Additionally, the independence of the novel imprinting system from specific functional monomer - template interactions should afford successful imprinting with many other analytically relevant, but poorly functionalized templates.

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CRediT authorship contribution statement

Miloš P. Pešić: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization. **Miljana D. Todorov:** Conceptualization, Methodology, Investigation, Writing - original draft. **Gergely Becskereki:** Conceptualization, Methodology, Investigation, Writing - original draft. **George Horvai:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Tatjana Ž. Verbić:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Blanka Tóth:** Conceptualization, Methodology, Writing - original draft, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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