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A comparison of covalent and non-covalent imprinting strategies for the synthesis of stigmasterol imprinted polymers

Shima N.N.S. Hashim, Reinhard I. Boysen, Lachlan J. Schwarz, Basil Danylec, Milton T.W. Hearn*

Centre for Green Chemistry, Monash University, Monash 3800 Victoria, Australia

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ABSTRACT

Non-covalent and covalent imprinting strategies have been investigated for the synthesis of stigmasterol imprinted polymers. The synthesized molecularly imprinted polymers (MIPs) were then evaluated for their recognition and selectivity towards stigmasterol *via* static and dynamic batch-binding assays and their performance measured against control non-imprinted polymers (NIPs). MIPs prepared using the conventional non-covalent imprinting method displayed little to no binding affinity for stigmasterol under various conditions. In contrast, the application of a covalent imprinting approach using the novel post-synthetically cleavable monomer-template composite stigmasteryl-3-*O*-methacrylate resulted in the fabrication of a MIP that successfully recognized stigmasterol in both organic and partially aqueous environments. The affinity and selectivity of the covalently prepared MIP was enhanced when undertaken in a partially aqueous environment consisting of an acetonitrile/water (9:1, v/v) solvent mixture. These features have been exploited in a molecularly imprinted solid-phase extraction (MISPE) format, wherein the preferential retention of stigmasterol (with an imprint factor of 12) was demonstrated with 99% recovery in comparison to cholesterol (imprint factor of 6) and ergosterol (imprint factor of 4) while in the presence of several closely related steryl analogues.

1. Introduction

Sterols are important components of cell membranes and play a significant role in the physiology of eukaryotic organisms [1]. Human health-beneficial effects have been attributed to plant sterols, also called phytosterols [2–4]. It has been suggested that a substantial consumption of phytosterols in the diet could reduce the absorption of cholesterol into the bloodstream and lead to a lower risk of cardiovascular disorders and other diseases [5,6]. Phytosterols are found in vegetable oils, nuts, grains and seeds [7]. Grape seeds, the abundant by-products of wine making, have been shown to contain phytosterols with an average total sterol composition of 571 mg/100 g [8]. The analysis of natural phytosterols in plant-based foods requires the development of fast, inexpensive and reliable extraction methods to replace more time consuming and labor intensive conventional sample extraction procedures.

Molecularly imprinted polymers (MIPs) are an effective way to extract or pre-concentrate target analytes from a complex matrix prior to analysis [9]. MIPs are synthetic materials designed to have a predetermined selectivity for defined molecular targets, and can be synthesized by non-covalent self-assembly [10] or covalent assembly [11] of pre-polymerization complexes between a molecular template and suitable functional monomer(s) in an appropriate porogen. The pre-polymerization complex is then polymerized in the presence of a cross-linking agent, after which the template is removed. Template removal can be achieved either by a simple extraction procedure, or chemical cleavage, depending on the type of interaction between the template and monomer(s). This affords a porous polymer with specific recognition sites, complementary in shape, size and functionality to the template molecule, or closely related structural analogues [12].

Owing to the amphipathic nature of sterols, having a single hydroxyl head group (–OH) at the C_3 position in the A-ring and a relatively large hydrophobic backbone, the design of synthetic, molecular recognition-based polymer networks for sterol compounds has hitherto proved to be challenging. In the past, several strategies to apply MIP technology for target capture have focused either on the pre-polymerization stages by choosing the appropriate functional monomers and stabilizing the template-monomer assemblies [13] or on post-polymerization procedures by modifying the distribution of binding sites *via* physical [14] or chemical approaches [15].

* Corresponding author. Tel.: +61 3 9905 4547; fax: +61 3 9905 8501.
E-mail address: milton.hearn@monash.edu (M.T.W. Hearn).

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Several cholesterol imprinted polymers have been reported based on such non-covalent and covalent strategies using cholesterol or a cholesteryl variant as the molecular template, respectively. In the case of non-covalent imprinting, methacrylic acid [16,17] and 4-vinyl pyridine [18] have typically been employed as the functional monomers at low temperature under photo-irradiation, although higher temperature polymerizations have also been reported [19].

A variation on the conventional non-covalent approach has been employed to generate cholesterol MIPs that demonstrate host-guest recognition in aqueous media driven by hydrophobic interactions between the steroid backbone of the cholesterol template and the cholesteryl containing hydrophobic functional monomers, 3β-methacryloylcholesterol [20] or cholesteryl (triethoxysilyl)propyl carbamate [21]. In the later example, a sol–gel methodology was employed to reduce the amount of non-specific interactions by replacing the typical acrylic cross-linker with an ortho-silicate scaffold [21].

An alternative hybrid covalent/non-covalent approach, reviewed elsewhere [22], has been employed by Whitcombe *et al.* [23] wherein a "sacrificial spacer" was introduced by the polymerization and subsequent hydrolysis of a cholesteryl (4-vinyl)phenyl carbonate ester with the loss of CO_2. The resulting recognition sites, housing a phenolic functional group, were able to form non-covalent hydrogen bonding interactions when interrogated with cholesterol. Hwang and Lee later compared this strategy with non-covalently prepared cholesterol imprinted polymers wherein they observed similar cholesterol uptake regardless of preparation strategy [18]. The chromatographic efficiency, however, was observed to be significantly enhanced when employing covalently prepared MIPs, resulting in a fivefold increase in theoretical plate number as well as considerably reduced peak broadening and tailing.

While there has been extensive interest in the development of cholesterol selective separation and sequestration media, the literature addressing similar developments for structurally related phytosterols is rather sparse [22,24,25]. This is surprising, given their documented health-beneficial effects, although their prohibitive cost and availability may be a factor in this regard [23]. Therefore, we have selected one of the most prevalent plant sterols, stigmasterol, as the molecular template. Hitherto, the design and preparation of novel stigmasterol imprinted polymers *via* the non-covalent and covalent imprinting synthetic strategies adopted in this investigation have not been described. In addition to the previously mentioned health-beneficial cardiovascular effects, stigmasterol has also been reported to have potent anti-inflammatory activity [26] and protective effects towards Alzheimer's disease [27]. The selectivity of these new MIPs in solid-phase extraction formats for stigmasterol *vis-à-vis* other steroidal compounds has also been investigated.

2. Materials and methods

2.1. Reagents

Reagent grade acetic acid (AcOH), formic acid (FA) and hydrochloric acid (HCl) were purchased from Ajax Finechem (Melbourne, Australia). Ethanol (EtOH), acetonitrile (ACN), methanol (MeOH), chloroform ($CHCl_3$) and sodium hydroxide (NaOH) were purchased from Merck (Melbourne, Australia). All solvents used for the MIP preparations and solid-phase extraction evaluations were HPLC grade. Water was purified using a Cascada™ IX water purification system (Pall Corp., Melbourne, Australia) to a resistivity of 18.2 MΩ cm. For the preparation of MIPs, methacrylic acid (MAA), 4-vinyl pyridine (4-VP), ethyleneglycol dimethacrylate (EGDMA) and 2,2′-azobis(2-methylpropionitrile) (AIBN) were purchased from Sigma-Aldrich (Sydney, Australia).

2.1.1. Standards

Commercial standards of sterols including stigmasterol, **1** (Sigma, Melbourne, Australia), cholesterol, **2** (Aldrich, Melbourne, Australia) and ergosterol, **3** (Fluka Chemicals, Melbourne, Australia) were used in these experiments with purity of ≥95%. A stock solution of stigmasterol was prepared in ACN/H_2O (9:1, v/v) at a concentration of 1.0 mM. The calibration curve for stigmasterol was prepared by serial dilution of the stigmasterol stock solution with ACN/H_2O (9:1, v/v) over a concentration range of 5.0×10^{-2} to 5.0×10^{-1} mM. The same procedures were implemented for the construction of the stigmasterol calibration curve using ACN (99.9% purity) as diluent.

2.1.2. Synthesis of stigmasteryl-3-O-ferulate, *4*, stigmasteryl-3-O-(4-acetoxyferulate), *5*, and stigmasteryl-3-O-acetate, *6*

These stigmasterol ester analogues were all prepared from stigmaster-3-ol. Stigmasteryl-3-*O*-ferulate, **4,** was quantitatively generated by K_2CO_3 hydrolysis of **5,** at 65 °C in a 2:1 $CHCl_3$/MeOH (v/v) solution. The stigmasteryl-3-*O*-(4-acetoxyferulate), **5**, was prepared in 76% yield using a *N*,*N*′-dicyclocarbodiimide and 4-dimethylaminopyridine promoted coupling of stigmaster-3-ol with *trans*-4-*O*-acetylferulic acid in CH_2Cl_2. *Trans*-4-*O*-acetylferulic acid was prepared in 87% yield by acetylation of ferulic acid with acetic anhydride in pyridine catalyzed by 4-dimethylaminopyridine. A similar acetylation procedure generated stigmasteryl-3-*O*-acetate, **6**, in 77% yield.

2.1.3. Synthesis of stigmasteryl-3-O-methacrylate, *7*

The compound, stigmasteryl-3-*O*-methacrylate, **7** was prepared by first dissolving 4-dimethylaminopyridine (80 mg, 0.654 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (1.349 g, 6.539 mmol) in anhydrous dichloromethane (25 mL). Methacrylic acid (555 μL, 6.539 mmol) was then added and the mixture stirred for 10 min, followed by the addition of stigmasterol (2.064 g, 5.000 mmol), together with further dichloromethane (20 mL). The reaction was left to stir overnight at room temperature. The resultant dicyclohexylurea was filtered off and washed in the funnel with dichloromethane (3 × 10 mL). The combined filtrate volume was increased to approximately 150 mL with the addition of dichloromethane and this filtrate was successively washed with water (100 ml), 5% aq. AcOH (100 mL), saturated aq. $NaHCO_3$ (100 mL), saturated aq. NaCl (100 mL) and water (2 × 100 mL). The organic layer was then dried (anhydrous Na_2SO_4), filtered and rotary evaporated to dryness to return a white powder (2.853 g). This material was dissolved in $CHCl_3$ (5 mL) and column chromatographed (SiO_2, 35 mm i.d. × 120 mm length using linear gradient elution starting with *n*-hexane (100%) and finishing with 19:1 *n*-hexane/EtOAc and the product collected in the fourth column volume) to give 1.783 g (74.2% yield) of stigmasteryl-3-*O*-methacrylate, **7** (Fig. 1) as a white powder. The analytical characteristics of **7** were: R_f 0.64 (19:1 *n*-hexane/EtOAc on a Merck NP thin-layer plate (20 cm × 20 cm) with silica gel 60 F_{254}, part number 1.05554.0001); mp. 145.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.70 (s, 3H, CH_3), 0.79–0.86 (m, 9H, 3 × CH_3), 1.02 (d, 3H, J = 6.8 Hz, CH_3), 1.04 (s, 3H, CH_3), 1.94 (s, 3H, methacrylate CH_3), 0.95–2.29 (broad overlapping multiplets corresponding to the steryl core, 29H), 2.33–2.40 (m, 2H, H-4a, H-4b,), 4.65–4.71 (m, 1H, H-3α), 5.12 (dd, 1H, J = 8.4, 15.2 Hz, H-23), 5.17 (dd, 1H, H-22), 5.38 (d, 1H, H-6), 5.53 (bs, 1H, methacrylate vinyl H); 6.08 (bs, 1H, methacrylate vinyl H); ^{13}C JMOD NMR (100 MHz, $CDCl_3$): δ 12.39, 12.59, 18.66, 19.34, 19.70, 21.43, 21.57 (7 × CH_3), 21.38, 24.71, 25.75, 28.13, 29.26, 32.23, 32.26, 36.99, 37.37, 38.48, 39.99, 40.84, 42.57, 50.42, 51.59, 56.30, 57.15, 74.56, 122.97, 125.24, 129.64, 137.23, 138.65, 140.05, 167.20; ESI-MS; m/z at 503 ($[M + Na]^+$, 100%), 504 (36%).