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Molecularly imprinted polymers for the isolation of bioactive naphthoquinones from plant extracts

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ABSTRACT

Molecularly Imprinted Polymers (MIPs) targeting shikonin, a potent antioxidant and wound healing agent, have been prepared using methacrylic acid (MAA) and 2-diethylaminoethyl methacrylate (DEAEMA) as functional monomers. An investigation of solution association between shikonin and both acidic and basic functional monomers by UV–vis titrations, suggested stronger affinity towards the basic functionality. Strong inhibition of the co-polymerisation reaction of such basic monomers was observed, but was overcome by reduction of the amount of template used during polymer synthesis. Polymer morphology was severely impacted by the template's radical scavenging behaviour as demonstrated by solid state NMR spectroscopy measurements. HPLC evaluation of the final materials in polar conditions revealed limited imprinting effects and selectivity, with the MAA polymers exhibiting marginally better performance. During application of the polymers as MI-SPE sorbents in non-polar solvents it was found that the DEAEMA based polymer was more selective towards shikonin compared to the MAA counterpart, while shikonin recoveries of up to 72% were achieved from hexane solutions of a commercial sample of shikonin, hexane extract of *Alkanna tinctoria* roots and a commercial pharmaceutical ointment.

1. Introduction

Alkannin and its enantiomer shikonin, are naturally occurring isohexenylnaphthazarins, found in the external layer of the roots of numerous plant species that belong mainly to the genera *Alkanna, Lithospermum, Echium, Onosma* and *Anchusa* of the Boraginaceae family [1,2]. They are potent pharmaceutical substances with a wide spectrum of antimicrobial [3], anti-inflammatory [4], antioxidant [5] and antitumor activity [6]. Shikonin, alkannin and related naphthazarin derivatives (Fig. 1) are also established as strong wound healing agents and are nowadays commercially available in the form of a wound healing pharmaceutical ointment under the trademark HELIXDERM®.

Extraction of bioactive constituents, such as alkannin, shikonin and their esters, from medicinal plants is typically performed by Soxhlet extraction, ultrasound-assisted extraction, maceration extraction using organic solvents and accelerated solvent extraction either at ambient temperature or at reflux conditions [7]. Techniques that enable faster extraction, higher sample throughput and require less organic solvent such as microwave-assisted extraction and rapid solid-liquid dynamic extraction have recently been proposed [8,9]. Nonetheless, these techniques are energy intensive and contribute to sound pollution, especially in industrial scale. Furthermore, active ingredients of plant origin are in many cases thermally labile. All these techniques require multiple further steps and time for isolating and purifying alkannin, shikonin and their derivatives, resulting in their extraordinarily high price.

In order to address the above limitations we employed molecular imprinting as an alternative extraction technique, aiming to isolate shikonin from natural extracts and to purify commercial samples. Molecular imprinting is a technique that introduces specific binding sites in the matrix of a synthetic polymer, by co-polymerisation of appropriate functional and cross-linking monomers in the presence of a target substance, the so-called template [10,11]. These binding sites are complementary to the template in terms of size, shape and functional group orientation and are able to rebind and extract it from complicated samples. Thus, such materials have been extensively used as sorbents in solid-phase extractions (MI-SPE) [12], chiral separations [13], catalysis [14] and sensing [15]. In contrast to natural receptors, imprinted polymers are stable in a wide range of temperatures,

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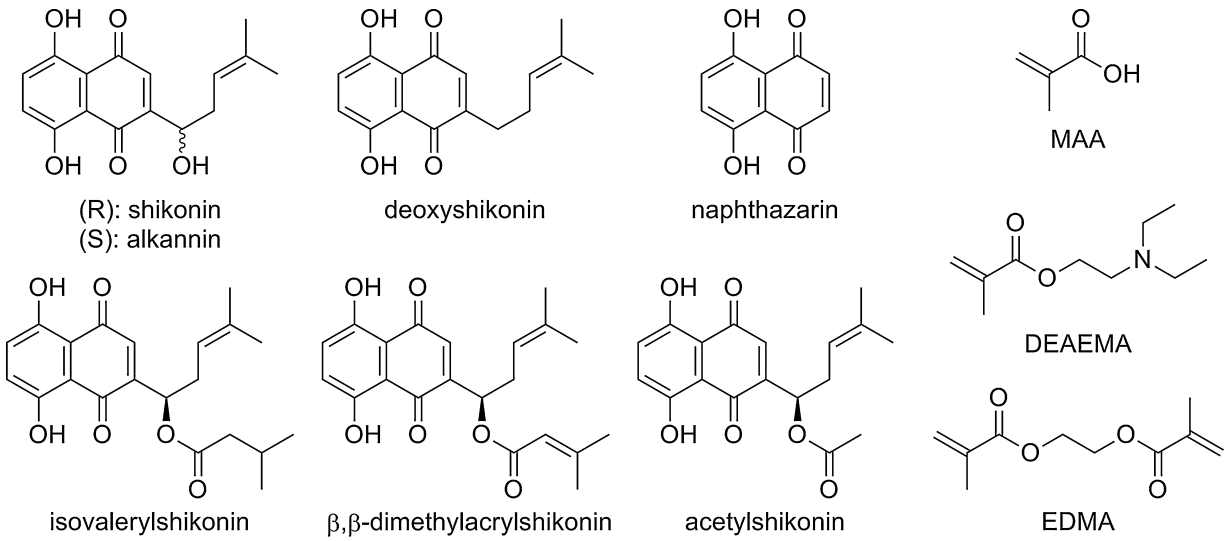

Fig. 1. Chemical structures of shikonin and related substances, functional monomers and cross-linker used in this study.

pH and organic solvents, while their preparation is rapid and inexpensive, rendering them a valuable class of affinity sorbents [16].

In this study, two sets of polymers were prepared by bulk polymerisation using methacrylic acid (MAA), a commonly used acidic functional monomer, and 2-diethylaminoethyl methacrylate (DEAEMA), a basic monomer. Polymer morphology was characterised by solid state NMR spectroscopy and nitrogen sorption porosimetry and the materials were subsequently evaluated as stationary phases in HPLC. Finally, solid phase extraction experiments were performed aiming to isolate shikonin from plant extracts and a commercial pharmaceutical ointment.

2. Experimental

2.1. Materials and methods

Commercial shikonin (Ichimaru Pharcos, Japan), alkannin (Ikeda Corporation, Japan), isovalerylshikonin, deoxyshikonin (TCI, Belgium), acetylshikonin, β,β-dimethylacrylshikonin (ABCR GmbH & Co., Karlsruhe, Germany) and naphthazarin (Fluka, Switzerland) were used as received. Hexane extracts of *Alkanna tinctoria* roots (Kremer Pigmente GmbH & Co. KG, Germany) were prepared by Soxhlet extraction according to a published procedure [17]. The hexane extract (3.5 wt.% /root) contains a mixture of pigments which include small amounts of esterified alkannin/shikonin derivatives and free alkannin/shikonin, whose concentration depends on the type of root sample. Free-radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV), cross-linking monomer ethyleneglycol dimethacrylate (EDMA), 2-diethylaminoethyl methacrylate (DEAEMA) and methacrylic acid (MAA) were from Sigma-Aldrich (Wicklow, Ireland). Chloroform, acetonitrile, hexane, methanol, acetic acid and water were of HPLC grade. All monomers were purified by distillation prior to use.

UV–vis spectra were recorded using a Cary 60 UV-vis Spectrometer (Agilent Technologies). HPLC measurements were performed using an Agilent 1100 HPLC system equipped with a photodiode array detector and a Synergi column (4 μm, max-RP 80A, 150 mm × 4.60 mm, Phenomenex, Torrance, CA, USA). Surface area analysis was performed using a Micromeritics Gemini VI Nitrogen sorption analyser (Particular Sciences, Dublin, Ireland). ^{13}C cross-polarisation magic angle spinning (CP-MAS) solid-state NMR measurements were performed using a Jeol ECX 400 spectrometer (Tokyo, Japan) using a 3.2 mm broadband probe and SiN_3 rotors, while sample spinning was maintained at 10 kHz.

2.2. UV–vis titration experiments

Monomer–template complexation was studied prior to polymer synthesis by means of UV–vis titrations in chloroform, in order to establish the strength of interactions present in the pre-polymerisation solution. Hence, to a solution of shikonin (0.1 mmol L^{-1}) increasing amounts of MAA or DEAEMA, up to 25 equivalents, were added sequentially. The change in absorbance at the characteristic wavelength of shikonin (520 nm) was recorded after each addition, and the corresponding titration curves were constructed. The raw titration data was fitted to the 1:1 binding isotherm and association constants were obtained by nonlinear regression [18].

2.3. Preparation of imprinted polymers

A molar ratio of shikonin:MAA:EDMA of 1:4:20 was used in the synthesis of P1; this was modified to 0.25:4:20 for P2, where DEAEMA was used as the functional monomer. This was due to the observation that higher amounts of shikonin inhibited the co-polymerisation reaction in the preparation of P2. A typical preparation procedure was as follows: 0.288 g (1 mmol) of shikonin and 0.342 mL (4 mmol) of MAA were dissolved in 5.6 mL of chloroform followed by addition of 3.8 mL (20 mmol) of EDMA in a 20 mL glass ampule. Respectively, 0.072 g (0.25 mmol) of shikonin and 0.810 mL (4 mmol) of DEAEMA were dissolved in 5.6 mL of chloroform followed by addition of 3.8 mL (20 mmol) of EDMA. The solutions were degassed using a stream of N_2 while cooling in an ice-bath for 5 min. Finally, 0.04 g of ABDV were added and the ampules were sealed and placed in a water-bath at 40 °C for 24 h. The corresponding non-imprinted polymers, PN1 and PN2, were synthesised in the same manner with omission of the template. The resulting polymer monoliths were smashed to coarse particles and washed with a mixture of chloroform/acetic acid (9:1 v/v) for 24 h using a Soxhlet apparatus. The polymers were manually ground and sieved, fine particles were removed by repeated sedimentation in methanol–water (4:1, v/v) and the 25–38 μm fraction was collected for packing into HPLC columns and SPE cartridges.

2.4. Chromatographic evaluation

Imprinted or non-imprinted particles of 25–38 μm were slurry packed in 30 mm × 4.6 mm i.d. stainless steel columns using a 4:1