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Stoichiometric molecularly imprinted polymers for the recognition of anti-cancer pro-drug tegafur

Paula Mattos dos Santos[a], Andrew J. Hall[b], Panagiotis Manesiotis[a,*]

[a] School of Chemistry and Chemical Engineering, Queen's University Belfast, David Keir Building, Stranmillis Road, BT9 5AG Belfast, Northern Ireland, United Kingdom

[b] Medway School of Pharmacy, Universities of Greenwich and Kent at Medway, Anson Building, Central Avenue, Chatham Maritime, Chatham, Kent, ME4 4TB, United Kingdom

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ABSTRACT

Molecularly imprinted polymers (MIPs) targeting tegafur, an anti-cancer 5-fluorouracil pro-drug, have been prepared by stoichiometric imprinting using 2,6-bis(acrylamido)pyridine (BAAPy) as the functional monomer. Solution association between tegafur and BAAPy was studied by ^{1}H NMR titration, which confirmed the formation of 1:1 complexes with an affinity constant of $574 \pm 15\ M^{-1}$ in $CDCl_3$. Evaluation of the synthesised materials by HPLC and equilibrium rebinding experiments revealed high selectivity of the imprinted polymer for the pro-drug *vs.* 5-fluorouracil and other competing analytes, with maximum imprinting factors of 25.3 and a binding capacity of $45.1\ \mu mol\ g^{-1}$. The synthesised imprinted polymer was employed in solid-phase extraction of the pro-drug using an optimised protocol that included a simple wash with the porogen used in the preparation of the material. Tegafur recoveries of up to 96% were achieved from aqueous samples and 92% from urine samples spiked with the template and three competing analytes. The results demonstrate the potential of the prepared polymers in the pre-concentration of tegafur from biological samples, which could be an invaluable tool in the monitoring of patient compliance and drug uptake and excretion.

1. Introduction

Since its introduction in 1957, 5-fluorouracil (5-FU) remains an essential part of the treatment of a wide range of solid tumours, particularly those found in the gastrointestinal tract, breast, head and neck. However, as with all anti-cancer medications, 5-FU is quite toxic, due to phosphorylation in the digestive tract, with myelotoxicity and gastrointestinal disorders being its major side-effects [1,2]. Furthermore, the activity of 5-FU is restricted by rapid degradation of up to 85% of the drug within the first minutes of entry to the blood stream, thus leaving a very small amount for anti-cancer action. The above limitations, as well as the need for patients to regularly visit the hospital for intra-venous administration of the drug, with the associated increased risks of thrombosis or infection, have driven research into the development of orally administered pro-drugs of 5-FU, usually in the form of substituted fluoropyrimidines. These include floxuridine (5-fluoro-2′-deoxyuridine), doxifluridine (5-fluoro-5′-deoxy uridine) and tegafur or ftorafur (1-(2-tetrahydrofuryl)-5-fluorouracil), the latter being one of the most studied orally administered 5-FU pro-drugs. Tegafur is often co-formulated with uracil or other active ingredients that enhance its bioavailability and reduce its toxicity [3–5]. It is metabolised to 5-FU in the body by cytochrome P450 2A6 and its terminal elimination half-life is 11 h [6]. Despite its several benefits over 5-FU, orally administered tegafur needs to be taken twice or thrice daily and up to 20% of the pro-drug is excreted in urine un-metabolised. Thus, monitoring patient compliance, as well as drug uptake and excretion, is of vital importance, and tools facilitating analysis of the drug in complex biological samples are essential.

Here, we wish to report, for the first time, on the design, development and application of a synthetic affinity separation phase exhibiting high selectivity for tegafur and capable of extracting the pro-drug from aqueous and biological samples. Our strategy was based on the technique of molecular imprinting, according to which a three-dimensional synthetic polymer network is formed in the presence of a target substance, thus embedding binding pockets complementary to the latter in terms of size, shape and functional group orientation. The resulting materials are capable of reversibly binding the so-called template or closely related substances from complex mixtures, rendering them robust, reusable and inexpen-

* Corresponding author. Fax: +44 28 9097 6524.
E-mail address: p.manesiotis@qub.ac.uk (P. Manesiotis).

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sive alternatives to affinity phases based on natural receptors, such as enzymes and antibodies [7–9].

The design of imprinted polymers for tegafur presented here was based on our prior experience in the development of recognition elements for uracil derivatives, which contain a hydrogen bond acceptor–donor–acceptor (A–D–A) imide moiety [10,11]. Our previous work, as well as that of others' [12–15], has shown that polymerisable functional monomers with complementary donor–acceptor–donor (D–A–D) arrays result in highly selective imprinted polymers, capable of recognising the template substance even in the presence of strongly competing analytes. Thus, of the previously reported custom-made functional monomers, including polymerisable adenine, purine and pyrimidine derivatives, 2,6-bis(acrylamido) pyridine (BAAPy) has been shown to be the most successful receptor and was used in the extraction of riboflavin from highly complex samples, such as beer and milk [16,17]. More recently, BAAPy was used in the development of molecularly imprinted polymers for the recognition of pyrimidine nucleosides [18], and in the preparation of composite beads for the enantiomeric resolution of the metastatic breast cancer drug aminoglutethimide by RAFT polymerisation [19]. Lastly, composite poloxamer nanomaterials containing *R*-thalidomide molecularly imprinted polymers based on BAAPy were recently used in enantioselective controlled release and targeted drug delivery [20]. The above examples highlight the versatility of the molecular imprinting technique, as well as the potential of BAAPy, and by extension other custom-made functional monomers, in the preparation of affinity separation phases with high selectivity for their respective targets, even when applied in highly competitive media.

2. Experimental

2.1. Materials and methods

Tegafur was purchased from Tokyo Chemical Industry UK Ltd. (Oxford, UK). 2,6-Bis(acrylamido) pyridine (BAAPy) [21] and 9-isobutyladenine [22] were synthesised as described previously. All other tested analytes, shown in Fig. 1, ethyleneglycol dimethacrylate (EDMA), HPLC grade solvents and deuterated solvents were purchased from Sigma Aldrich (Gillingham, UK). 2,2′-Azobis(2,4-dimethylvaleronitrile) (ABDV) was purchased from Wako Chemicals GmbH and was used as received. Polymerisation inhibitors were removed from EDMA by filtration through a basic alumina column. ^{1}H NMR spectra were collected on a Bruker ECX 400 MHz NMR spectrometer. An Agilent 1100HPLC instrument equipped with photodiode array detector and a Phenomenex Kinetex^{TM} C_{18} column (5 μm, 150 mm × 4.6 mm i.d.) was used for all chromatographic separations.

2.2. ^{1}NMR titration experiments

Monomer–template complexation was studied prior to polymer synthesis using ^{1}H NMR titrations in order to establish the type and strength of interactions present in the pre-polymerisation solution. Thus, to a solution of BAAPy (0.001 mol L^{-1}) in $CDCl_3$ were added increasing amounts of tegafur, until at least a 10-fold excess was reached. The complexation-induced shifts (CIS) of the BAAPy amide protons were followed and a titration curve was constructed. The stoichiometry of the monomer-template complex was confirmed using Job's method of continuous variation. Hence, equimolar solutions (0.01 mol L^{-1}) of BAAPy and tegafur were mixed in different ratios and a plot of $\Delta\delta$ against the molar fraction of monomer multiplied by the CIS ($X_i \times \Delta\delta$) was constructed [23,24].

2.3. Preparation of imprinted polymers

The imprinted (P_{TGF}) and corresponding non-imprinted polymer (NIP) used in this study were prepared by thermally initiated free-radical polymerisation. Briefly, tegafur (0.202 g, 1 mmol) and BAAPy (0.219 g, 1 mmol) were transferred into to a glass vial and mixed with 5.6 mL of chloroform. Upon complete dissolution, 3.8 mL (20 mmol) of EDMA were added followed by 0.04 g of ABDV. The pre-polymerisation solutions were ultra-sonicated for 5 min, purged with N_2 and then hermetically sealed. The vials were placed in a water-bath thermostated at 40 °C for 24 h. The resulting rigid monoliths were smashed and washed with methanol in a Soxhlet apparatus in order to remove the template and any unreacted monomers. The coarse polymer particles were ground using a mortar and pestle, wet-sieved with acetone and the 25–38 μm fraction was collected, dried and stored at room temperature for further experiments. The corresponding non-imprinted polymer was prepared in the same manner, but without addition of the template in the pre-polymerisation mixture.

2.4. Chromatographic evaluation

Chromatographic evaluation of the prepared polymers was performed using LC columns (50 mm × 4.6 mm i.d.) manually packed with the 25–38 μm fraction of each material. The columns were then connected to a HPLC instrument and equilibrated with 1% acetic acid in acetonitrile until a stable baseline was obtained. Analyses were performed by injecting 5 μL of 1 mmol L^{-1} solution of each analyte in acetonitrile and recording its elution profile at a flow-rate of 1 mL min^{-1} and a wavelength set accordingly for each tested analyte. Retention factors ($k = (t_R - t_o)/t_o$), where t_R is the retention time of each analyte (average of three injections) and t_o the retention time of the void volume marker (acetone), as well as imprinting factors (IF = k_{MIP}/k_{NIP}), were calculated.

2.5. Equilibrium rebinding experiments

The polymers' affinity and capacity for tegafur and 5-FU were estimated using equilibrium rebinding experiments performed in acetonitrile. Thus, 10 mg of each polymer were transferred in 2 mL glass vials and equilibrated with 1 mL of analyte solution of increasing concentration (0–5 mmol L^{-1}) for 24 h. All samples were prepared in triplicate. The supernatants were then analysed by HPLC using a mixture of water/acetonitrile 80:20 as the mobile phase. The flow-rate was 1 mL min^{-1} and the detection wavelength was set at 270 nm. The amount of analyte bound to each polymer was calculated by subtracting the amount determined after the experiment from the starting amount of the drug. The averaged results ($n = 3$) were plotted as concentration of free analyte in solution *vs.* the amount of analyte bound to the polymer to produce binding isotherms that were fitted by non-linear regression to the appropriate binding model.

2.6. Solid phase extractions (MI-SPE)

50 mg of P_{TGF} and NIP particles were dry packed in 3 mL SPE cartridges using 20 μm porous polyethylene frits. The developed SPE protocol consisted of an initial aqueous conditioning step followed by loading of 1 mL of aqueous sample. Initially, a screening study was performed whereby 1 mL of water/acetonitrile mixtures ranging from 0:100 to 100:0 was percolated through the drug loaded cartridges. This was later replaced with an aqueous wash (1 mL), followed by drying of the SPE cartridges on the vacuum manifold by allowing air to flow through for 5 min, and a second washing step with 1 mL of chloroform. Finally, the cartridges were eluted with 1 mL of a 5% acetic acid in methanol solution. SPE analysis of urine