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^{1}H Nuclear magnetic resonance study of the molecular imprinting of (−)-nicotine: template self-association, a molecular basis for cooperative ligand binding

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Abstract

In the present study, the interactions of components in a (−)-nicotine molecular imprinting polymerization mixture have been studied by ^{1}H NMR spectroscopy. The dissociation constants for complexation of template by a functional monomer analogue, acetic acid, have been determined. Nicotine was shown to self-associate at concentrations comparable to those used in previous molecular imprinting studies (app K_{diss} = 0.082 M in $CDCl_3$ at 298 K). The extent of self-association was enhanced by the presence of acetic acid. Previous studies on (−)-nicotine–imprinted methacrylic acid–ethylene dimethacrylate co-polymers suggested the involvement of recognition sites for template–template complexes. Collectively these results provide the first direct evidence for the presence of template–template complexes, and support the previously hypothesized basis for cooperative ligand recognition events in this polymer system.

Keywords: Molecular imprinting; Self-association; Ligand binding; Nuclear magnetic resonance spectroscopy; Cooperative binding; Nicotine

1. Introduction

Molecular imprinting is a technique for the synthesis of polymeric materials with predetermined ligand selectivity [1–6]. The generally accepted basis for the observed selectivities resides in the formation of reversible interactions, covalent or non-covalent, between the specific functionalities present in a target structure (template) and those of suitably functionalized monomers. It is perceived that the coordinating effect of the template results in the formation of cavities in the resultant polymer with functionalities and topographies complementary to the template. A vast number of template structures and monomer functionalities have been employed in molecular imprinting studies, though the majority of reports have utilized non-covalent template–monomer interactions. In general, this has been due to the greater diversity of non-covalent interactions suitable for use in molecular imprinting protocols, and the relative ease with which non-covalent interaction based molecularly imprinted polymers can be synthesized.

The presence of non-covalent interactions between template structures and functional monomers or functional monomer analogues has been previously described, and the strengths and degrees of complex formation have been examined using NMR and optical spectroscopic techniques [7–11]. Collectively these studies have demonstrated that the degree of template–monomer complexation is directly dependent upon the types of interactions employed and the chemical composition of the polymerization reaction mixture. To this point in time, relatively few studies have addressed the mechanisms underlying the molecular imprinting polymerization, and the fate of template–monomer complexes during the polymerization process has not been examined.

The biological activity of nicotine [12] (Fig. 1) has made it of interest to us, and others, for use in molecular imprinting studies [13–20]. In a recent paper from our laboratory we described anomalous chromatographic recognition behavior observed in a series of (−)-nicotine-imprinted methacrylic acid–ethylene dimethacrylate (MAA–EDMA) co-polymers prepared using a range of template concentrations and various solvents [20]. The efficiency of racemic resolution demonstrated by these polymers varied as a function of

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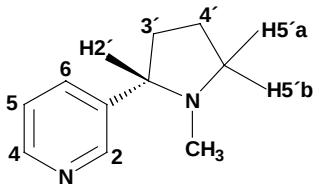

Fig. 1. Structure and atomic labeling of (−)-nicotine.

sample load. Increased sample load resulted in increased retention time and peak asymmetry. Moreover, this non-linear behavior was found to vary, depending upon the concentration of template employed in the polymer synthesis. This observed chromatographic response was hypothesized to be indicative of cooperative binding phenomena [21] and suggested the presence of recognition sites selective for template–template complexes.

In the present study we have investigated the interactions between the template (−)-nicotine and acetic acid, which behaves analogously to MAA in template recognition in organic media [8], in order to: (i) study the extent of monomer–template interactions; (ii) investigate the dependence of these interactions on solvent and the influence of the cross-linking agent used, EDMA; (iii) determine whether template–template interactions can be of significance to the imprinting process and subsequent recognition characteristics of the polymers; and (iv) examine whether monomer–template interactions survive the polymerization process, and thus provide physical proof of the molecular imprinting paradigm, i.e. that the template–functional monomer complexes formed in solution give rise to the ligand-selective sites present in the resultant polymer.

2. Experimental section

2.1. Materials

d_3-Acetonitrile (99.6%) and d_4-acetic acid (99.9%) was supplied by Aldrich (USA). d_1-Chloroform (99.8%) was obtained from Riedel-de Haën (The Netherlands), while deuterium oxide (99.9%) and d_4-methanol (99.0%) were purchased from Cambridge Isotope Laboratories (USA). (−)-Nicotine (>98% e.e.), EDMA (>98%) and MAA (>99%) were obtained from MERCK-Schuchardt (Germany). 2,2′-Azobis-(2-methylpropionitrile) (AIBN, 98.0%) was supplied from Janssen Chimica (The Netherlands). Sodium-2,2-dimethyl-2-silapentane-5-sulphonate (DSS) and tetramethylsilane (TMS) were purchased from Novachem (Australia).

2.2. NMR titrations

Acetic acid titrations: a solution of (−)-nicotine (30 mM) in the relevant solvent was titrated with consecutive additions of a solution, in the same solvent, containing acetic acid (2.67 M) and (−)-nicotine (30 mM). The solvents used were CD_3CN and $CDCl_3$. ^{1}H NMR spectra were recorded on a Bruker ARX spectrometer operating at 500 MHz at 298 K. For the nicotine–nicotine titrations, a solution of (−)-nicotine (10 mM) was titrated with (−)-nicotine solutions ranging from 0.50 to 6.23 M in strength (15 concentrations per titration). Acetic acid was present at a concentration of 20 mM for two of the nicotine–nicotine self-association studies. Apparent dissociation constants were calculated with non-linear regression using the software package GraphPad Prism (version 3.02, GraphPad Software, USA).

2.3. Job plot

Samples were prepared in $CDCl_3$ containing different molar fractions of (−)-nicotine and acetic acid, from 0 to 1.0, with a constant total concentration of 94 mM.

2.4. NMR studies of the polymerization process

EDMA (3.30 mmol) was dissolved in $CDCl_3$ (1000 μl) together with (−)-nicotine (0.16 mmol) and MAA (0.63 mmol). Aliquots (600 μl) were transferred to Eppendorf tubes containing 0.016 mmol AIBN. The tubes were shaken, purged with dry nitrogen then sealed and kept on ice at 273 K. Aliquots were transferred to NMR tubes containing a trace of TMS. An initial spectrum was recorded at 273 K. The NMR tubes where then either placed in a waterbath at 333 K, or under a UV source (Camag UV cabinet II, 366 nm) at 277 K. The tubes were periodically cooled on ice (1 min) before spectra were recorded.

3. Results and discussion

Molecular imprinting dogma dictates that the presence of specific monomer–template interactions is the basis for ligand (template) selective recognition sites in the resultant polymer. Whilst much effort has been directed towards the interpretation and optimization of ligand–polymer interactions, relatively little effort has been focused upon elucidating the mechanisms underlying the formation of the ligand-selective sites during the polymer synthesis.

In the case of molecularly imprinted polymers prepared using non-covalent interactions, the extent of template complexation in the pre-polymerization mixture is a consequence of a series of equilibria. Being a system in equilibrium, the nature and extent of template complexation in the pre-polymerization mixture can be influenced by a number of factors, e.g. concentration and nature of the interacting structures, solvent and temperature. Despite the inherent complexity of this situation, the relative simplicity associated with the use of non-covalent interactions for molecular imprinting is a key factor in the attractiveness of the technique. In comparison, reversible covalent

interactions provide superior monomer–template coordination complexation, though this approach is limited by the availability of compatible functionalities. It is worth noting that for some types of non-covalent functionalities it has been shown that essentially stoichiometric complexation can be achieved [22,23]. Nonetheless, for a number of application purposes, e.g. chromatography, fast off-rates and relatively mild elution conditions are most often required, for which weaker types of interactions are desirable. It should be noted, however, that stationary phase structure and binding site accessibility can play a prominent role in determining template elution times. In the current report we have selected to study the non-covalent molecular imprinting of (−)-nicotine in order to characterize the interactions between monomer and template and to establish whether template self-association can provide an explanation for the recognition behavior observed in these systems.

^{1}H NMR titration experiments were used to determine the strength of monomer–template interactions in the pre-polymerization mixture. Interactions between functionalities on the template and the functional monomer(s) can result in chemical shift changes that may be used to determine the dissociation constants of the complexes formed. Importantly, NMR provides a means of identifying the specific sites in interacting structures that engage in the complexation. The sequential addition of acetic acid to solutions of (−)-nicotine, prepared in chloroform or acetonitrile (ACN),

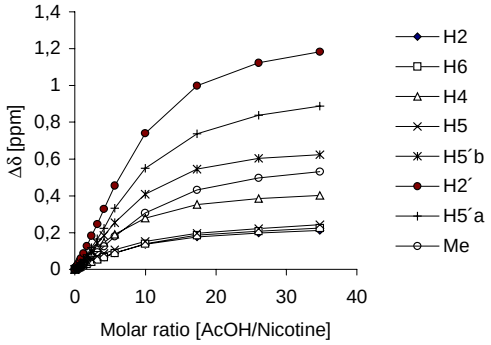

Fig. 3. Binding isotherms from a nicotine/acetic acid titration in acetonitrile ((−)-nicotine concentration 30 mM).

resulted in up to ∼1.2 ppm (in ACN) shifts of a number of resonances arising from protons in the vicinity of the basic pyridyl and pyrrolidinyl nitrogens (Fig. 2). Acetic acid has previously been demonstrated to be a suitable analogue for the functional monomer methacrylic acid. Generally speaking, the extent of the observed shifts was proportional to their proximity to these sites, and related to the nature of the interaction (Fig. 3). The relatively large shifts of the protons close to the more basic pyrrolidinyl nitrogen indicate that protonation of the tertiary amine and subsequent interaction, presumably by ion pairing with the carboxylate anion, results in a significant change in the magnetic environment

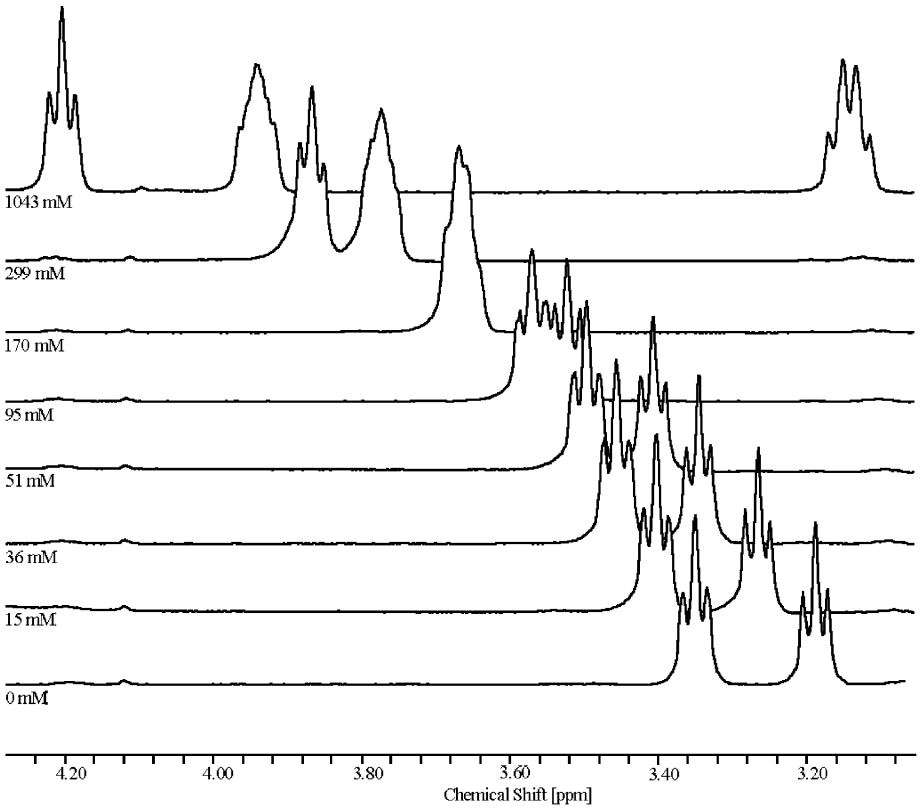

Fig. 2. Portion of the ^{1}H NMR spectra of (−)-nicotine, showing the change in chemical shifts of protons H5′b (3.35 ppm) and H2′ (3.18 ppm) upon subsequent additions of acetic acid (concentration indicated under each spectra) to a 30 mM solution of (−)-nicotine in chloroform.

Table 1
Dissociation constants (K_{diss} (M)) for complex formation in different solvent configurations

Proton	Nicotine/HOAc		Nicotine/nicotine			
	$CDCl_3$	CD_3CN	$CDCl_3$	CD_3CN	$CDCl_3$/HOAc	CD_3CN/HOAc
H2	0.28 ± 0.016	0.35 ± 0.017	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
H6	0.22 ± 0.010	0.39 ± 0.023	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
H4	0.15 ± 0.0072	0.28 ± 0.015	0.065 ± 0.013	0.90 ± 0.67	0.090 ± 0.019	0.13 ± 0.015
H5	0.12 ± 0.0077	0.28 ± 0.0052	0.035 ± 0.0074	2.1 ± 0.46	0.16 ± 0.033	0.37 ± 0.085
H5′b	0.19 ± 0.0053	0.40 ± 0.041	0.11 ± 0.027	1.5 ± 0.27	0.070 ± 0.019	0.18 ± 0.026
H2′	0.25 ± 0.0092	0.44 ± 0.042	0.12 ± 0.041	1.7 ± 0.38	0.070 ± 0.018	0.14 ± 0.016
H5′a	0.31 ± 0.0047	0.48 ± 0.056	0.073 ± 0.013	n.d.[a]	0.070 ± 0.016	0.13 ± 0.024
Me	0.35 ± 0.0089	0.55 ± 0.060	0.09 ± 0.019	1.2 ± 0.29	0.11 ± 0.023	0.15 ± 0.029

The dissociation constants were calculated using non-linear regression with the software package Prism (version 4, GraphPad software, USA). Each regression is based on 14–16 data points and is presented with the standard error.

[a] In some cases calculations were impossible due to the very small observed shifts.

of adjacent protons. The influence on the protons *ortho* to the pyridyl nitrogen was notably less, reflecting the weaker basicity of this site. The shifts were used to calculate apparent dissociation constants (app K_{diss}) for the observed interactions (Table 1). Although the magnitudes of the changes in chemical shift differed in relation to proximity to the site of interaction, the calculated apparent dissociation constants (app K_{diss}) were quite similar. Correlation with the stoichiometries used in previous studies on the synthesis of nicotine molecularly imprinted polymers indicates that 35% of nicotine was complexed. Titration of nicotine solutions, in chloroform or acetonitrile, with EDMA induced no significant changes (<0.004 ppm) in chemical shifts. This result confirms that the cross-linking agent used in this study does not interact significantly with the template. It is important to note that carboxylic acids such as acetic acid can undergo self-association, especially in non-polar media (K_{diss} 0.25 M in chloroform at 300 K) [24], and that the extent of template complexation is influenced by the relative strengths of the various species of complex possible.

To establish the extent of template–template self-association, nicotine–nicotine titrations were performed over a concentration range from 0.01 to 2.50 M both in the presence and absence of acetic acid in chloroform and acetonitrile. In the absence of the functional monomer analogue, the nicotine–nicotine complexes are some 18 times stronger in chloroform than in acetonitrile, based upon the observed average dissociation constants, which reflects the relative strengths of electrostatic interactions in these solvents (Table 1). However, and somewhat surprisingly, the influence of acetic acid in conjunction with acetonitrile is more profound than in the case of chloroform. A plausible explanation for nicotine–nicotine complexes being stronger in the presence of acetic acid is that protonation of the pyrrolidine nitrogen enables it to form stronger interactions with a second nicotine molecule. However, a molecular level explanation as to why acetonitrile, a more polar solvent, elicits a better relative enhancement of acetic acid–nicotine interactions than chloroform is still not clear. In summary, the results described indicate that nicotine is able to self-associate at the concentrations used for typical molecular imprinting. Moreover, the presence of acetic acid, an analogue for the functional monomer MAA, enhances nicotine–nicotine complex stability.

The stoichiometry of complexation was investigated using a Job-plot (Fig. 4). The preferred 1.5:1 complex (nicotine/acetic acid) stoichiometry can be explained by the proposed self-association as the nicotine molecules compete with the acetic acid for favorable interactions at higher concentrations.

In order to determine whether such complexes survive the polymer synthesis step, (−)-nicotine molecularly imprinted polymers corresponding to that described by Andersson et al. [20] as P1 were synthesized in NMR tubes, and the reaction was followed by a series of NMR experiments (Fig. 5). The presence of functional monomer induced changes in chemical shifts that are commensurate with the experiments described previously. The first spectrum (t = 0 min) was recorded prior to the start of the polymerization reaction. Polymerization was then commenced, by either thermal (333 K) or photochemical initiation (277 K, 366 nm), and the spectra were recorded at intervals. The concentration of (−)-nicotine in the polymerization mixtures, 0.094 M in chloroform, was higher than the corresponding apparent dissociation constants for nicotine–nicotine complexes in the presence of acetic acid in chloroform (0.08 M, based

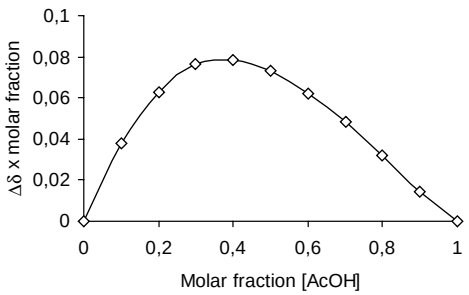

Fig. 4. ^{1}H NMR Job plot for the complexation of (−)-nicotine with acetic acid.

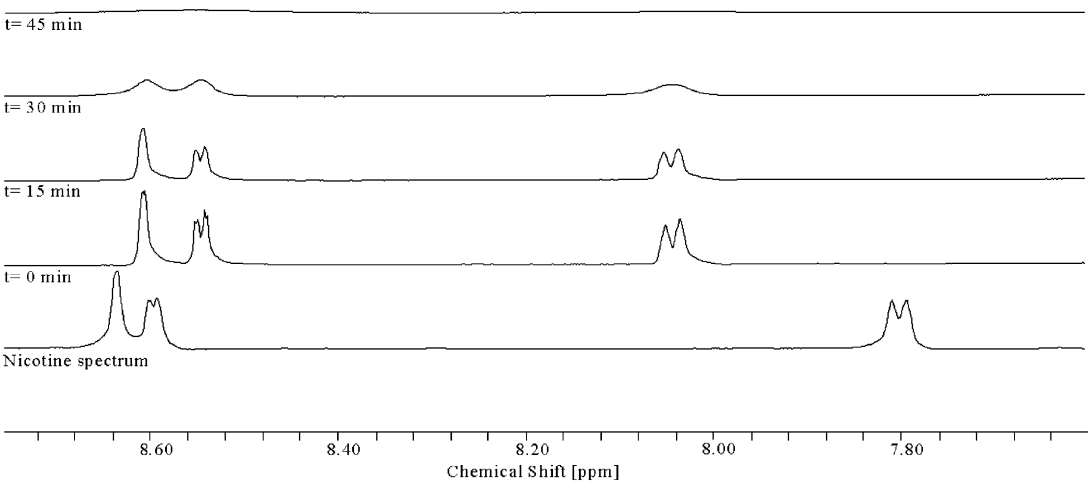

Fig. 5. ^{1}H resonances of the pyridine protons H2 (8.64 ppm), H6 (8.60 ppm) and H4 (7.81 ppm) monitored during the polymerization process.

upon the average shifts of the pyrrolidine ring protons), indicating that nicotine–nicotine–methacrylic acid complexes exist in these polymerization mixtures.

In the case of UV-initiated polymerizations, the chemical shifts induced in the ^{1}H NMR spectra of (−)-nicotine by the functional monomer MAA are clearly retained throughout the first 45 min of the polymerization reaction. Sample anisotropy results in severe line broadening as the reaction mixture approaches gel phase, and complete solidification was apparent after approximately 1 h. The heat-initiated polymerization experiments showed similar results, though gel phase was reached after 30 min, which suggests that, in this case, the polymerization reaction was somewhat more efficient. It has previously been demonstrated that molecularly imprinted MAA–EDMA co-polymer particles, synthesized under similar conditions and collected after gel phase had been reached (10 min), were able to show ligand selectivity [25]. Thus, in this study, we have been able to prove that the functional monomer–template complexes formed in a molecularly imprinted MAA–EDMA co-polymer pre-polymerization mixture are maintained through to the point where selective recognition can be demonstrated.

In conclusion, the results of this study indicate that, firstly, under the relative stoichiometries and concentrations employed for molecular imprinting polymerizations, the functional monomer methacrylic acid is capable of complexing the template (−)-nicotine. Secondly, the strength of template–monomer interactions is dependent upon solvent; however, the cross-linking agent, EDMA, does not perturb template–functional complexes significantly. Thirdly, template–template interactions are present during the polymerization process, and the functional monomer employed in this study, contributes to this self-association process. This result provides support for the hypothesis that co-operative effects underlie previously observed anomalous chromatographic behavior of (−)-nicotine MAA/EDMA co-polymers. Finally, the first direct physical evidence that non-covalent monomer–template interactions survive the polymerization process is presented, thus supporting the purported basis for the molecular imprinting technique.

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