Nicotine-Selective Polymeric Adsorbent Obtained by Molecular Imprinting with Excess Use of Itaconic Acid

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Abstract
Currently, nicotine is mostly analyzed by chromatography with pretreatment such as solid phase extraction (SPE). One of effective pretreatment techniques would be affinity extraction; however, there is no practical biomolecular affinity media available for SPE of nicotine. Molecular imprinting has been studied as a methodology for producing nicotine-selective synthetic affinity media, which relies on the formation of complex species between a target molecule (as template) and functional monomer; therefore, the selection of functional monomer is greatly important for obtaining molecularly imprinted polymers (MIPs) with high affinity and selectivity. In this study, itaconic acid (IA), which bears two carboxyl groups, was used as functional monomer in the synthesis of nicotine selective-MIPs. Other acidic monomers, such as methacrylic acid (MA), 2-(trifluoromethyl)acrylic acid (FM) and methyl itaconate (MI) were compared with IA to evaluate their usefulness as functional monomer. In chromatographic tests, the retention factor for nicotine on a MIP synthesized with itaconic acid (IP-IA16) was 40.4, which was 3.1 times that on a non-imprinted polymer (BP-IA16), while the retention factor on MIPs with the other monomers was 21.6 or less and was 1.1 times that on corresponding non-imprint blank polymers (BPs). In the selectivity test using cotinine, 3-methylpyridine and N-methylpyrrolidine as reference compounds, IP-IA16 showed the largest retention factor for nicotine, which was more than 4.4 times that of the other compounds, suggesting that nicotine-recognition sites were formed in MIPs by the molecular imprinting using IA as functional monomer.

Keywords: Molecular imprinting; Molecular recognition; Polymer; Nicotine; Itaconic acid

1. Introduction
Nicotine, the principle addictive component in tobacco, is a compound that has a strong neurotoxic activity and has found application in various fields such as agrochemicals [1]. Therefore, nicotine from various sources, such as cigarettes [2,3], urine [4], nicotine patches [5] and plants [6], is required to be analyzed. Currently, most of the analytical protocols rely upon sample pretreatment procedures (e.g., SPE) followed by GC or HPLC. Although affinity-type adsorbent would be helpful for efficient sample enrichment and purification, only a limited number of nicotine-selective biomaterials have been reported. For instance, anti-nicotine antibodies have drawn much attention in terms of a therapeutic application for tobacco smoking cessation [7-10]. A nicotine-degrading enzyme from Pseudomonas putida has also been reported [11]. Application of these materials to SPE, however, does not seem practical due to drawbacks such as their fairly high cost and difficulty with mass production.

Molecularly imprinted polymers (MIPs) selective for nicotine have been recognized as replacement of such biomolecules, which can be easily synthesized in gram scale at low costs [12-19].

The principle of molecular imprinting relies upon the complexation of functional monomers with a template [20, 21], which is usually an analyte in question (Fig. 1). The complexes are subsequently immobilized in network polymer by co-polymerization with crosslinker, preserving the three-dimensional arrangement of functional monomers complementary to the template. After removal of the
template, the network polymer is expected to possess a template-shaped cavity with the functional monomer-derived moieties that have been preorganized for recognizing the template.

Synthesis of nicotine-selective MIPs has been previously reported, where methacrylic acid (MA) and/or 2-(trifluoromethyl)acrylic acid (FM) were used as functional monomer(s) [12-19]. These acidic monomers had been expected to form complexes with nicotine via hydrogen bonding and/or electrostatic interaction with the two basic nitrogen atoms. According to the successful results with MA and FM, it was considered promising to test other acidic monomers.

Thus, we focus on itaconic acid (IA), which has two carboxyl groups, as functional monomer for imprinting nicotine. IA has been demonstrated to be useful for molecularly imprinting of several basic compounds such as a β-adrenergic blocker [22], an anti-HIV drug [23], a neurotransmitter [24,25], creatinine [26] and an adrenocortical hormone [27]. Molecular imprinting of nicotine with itaconic acid has been also reported; however, no detailed investigation on the synthesis conditions has been made [28]. Furthermore, no discussion is made about putative merits of the two carboxyl groups of IA or the manner of intermolecular interaction between nicotine and IA; it is still unclear whether nicotine and itaconic acid forms a 1:1 complex or a 1:2 complex (Fig. 1). In this study, we synthesized nicotine-imprinted polymers (IPs) with various molar ratios of IA as functional monomer, and chromatographically assessed their retention behaviors. The IA-based IPs were compared with other acidic monomer-based MIPs for elucidating a unique feature of IA as functional monomer. Also, NMR spectroscopy was employed to obtain a clue for understanding the manner of intermolecular interaction between nicotine and IA.

2. Experimental
2.1. Materials and instruments
Nicotine, itaconic acid (IA), methacrylic acid (MA), 2-(trifluoromethyl)acrylic acid (FM), methyl itaconate (MI), dimethyl itaconate (DI), 2,2′-azobisisobutyronitrile (AIBN), acetonitrile, acetic acid, DMSO-d6, ethylene glycol dimethacrylate (EDMA), tetrahydrofuran (THF) and chloroform were purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-Methylpyridine, cotinine and N-methylpyrrolidine were purchased from Tokyo Chemical Industry (Tokyo, Japan). Nicotine, MA, EDMA, THF and chloroform were purified prior to use by distillation with drying agents. A Waters e2695 HPLC system (Milford, MA, USA) with a Waters 2489 UV/Visible detector and a Waters 2424 ELS detector was used for HPLC analysis. Nitrogen NMR spectra were recorded with a JEOL JNM-ECA500 NMR spectrometer (Tokyo, Japan).

2.2. Synthesis of nicotine-imprinted polymers (IPs) and non-imprint blank polymers (BPs)
A typical procedure for the synthesis of nicotine-imprinted polymers (IPs); into THF (25 mL) were added nicotine (1.67 mmol), itaconic acid (IA) (1.0 - 32 eq. to nicotine), ethylene glycol dimethacrylate (EDMA) (47.1 mmol) and AIBN (0.73 mmol). The mixture was sparged with nitrogen gas and heated at 60 °C for 12 h. Non-imprint blank polymers (BPs) were synthesized similarly without adding nicotine into the polymerization mixture.

As listed in Table 1, the obtained imprinted polymers were named as IP-(functional monomer)(molar equivalence of the functional monomer to the template); for example, IP-IA16 stands for the polymer synthesized with 16 eq. of
itaconic acid (IA). The blank polymers were named in the same fashion using “BP” instead of “IP”.

Imprinted polymers were also synthesized using methacrylic acid (MA), 2-(trifluoromethyl)acrylic acid (FM), methyl itaconate (MI) and dimethyl itaconate (DI) as functional monomer in the identical manner as the synthesis of IP-IA16, which were named as IP-MA16, IP-FM16, IP-MI16 and IP-DI16, respectively (Fig. 2). Corresponding non-imprint blank polymers were synthesized without addition of the template nicotine.

Imprinted polymers, IP-MA16-CHL and IP-FM16-CHL, were synthesized in the similar manner as the synthesis of IP-MA16 and IP-FM16, respectively, using chloroform in place of THF as porogen.

2.3. Chromatographic assessment of IPs
Obtained bulk polymers (IPs and BPs) were ground in a mortar, wet-sieved (63 μm, methanol) and packed in stainless-steel column tubes (50 x 4.6 mm, i.d.). After being washed with 300 mL of methanol – acetic acid (8:2, v/v) using a chromatographic pump, retention of nicotine was measured by HPLC using acetonitrile – acetic acid – water as the eluent at a flow rate of 1.0 mL min⁻¹. Selectivity was examined by measuring the retention of nicotine-related compounds, cotinine, 3-methylpyridine and N-methylpyrrolidine, using acetonitrile – acetic acid (9:1, v/v) as the eluent (Fig. 3 and Fig. 4). The sample concentration and volume were 1.0 mM and 20 µL, respectively. Retention factors (k') and imprint factors (IF) were obtained by equation (1) and (2), respectively, where t₀ is a retention time of a sample, t₀ is an elution time of a void marker acetone, and k'IP and k'BP are retention factors of an imprinted polymer (IP) and a blank polymer (BP), respectively. IF was used for the assessment of each imprinting system under the adopted synthesis conditions. Average data of three measurements were used for obtaining k' and IF.

\[ k' = \frac{(t_R - t_0)}{t_0} \text{ ... (1)} \]
\[ \text{IF} = \frac{k'_{\text{IP}}}{k'_{\text{BP}}} \text{ ... (2)} \]

2.4. 15N NMR titration
Nitrogen NMR (15N NMR) spectra were recorded for nicotine (0.3 M) mixed with different ratios of itaconic acid (IA) in DMSO-d6.

3. Results and discussion
3.1. Synthesis of nicotine imprinted polymers (IPs) using itaconic acid (IA)
Nicotine-imprinted polymers were synthesized using varied amounts (1.0 - 32 eq. to nicotine) of an acidic functional monomer, itaconic acid (IA), which is expected to form hydrogen bonding and/or electrostatic interaction with nicotine’s nitrogen atoms. The monomer mixed with nicotine as template in THF was copolymerized with ethylene glycol dimethacrylate (EDMA) as crosslinker to produce bulk network polymer. THF was used as the porogen according to a literature [22]. The obtained bulk polymers were ground in a mortar, sieved, and packed in stainless-steel columns, and subjected to chromatographic assessment of nicotine binding properties.

| Polymer   | Eluent (acetonitrile - acetic acid (AA) - water) | k' | IF | k' | IF |
|-----------|-----------------------------------------------|---|----|---|----|
| IP-IA1    | AA1% AA5% AA10% AA10% + DW10%                |    |    |   |    |
| BP-IA1    | 1.4                                          |    |    |   |    |
| BP-IA2    | 1.7                                          |    |    |   |    |
| IP-IA4    | 2.8                                          |    |    |   |    |
| BP-IA4    | 1.5                                          |    |    |   |    |
| IP-IA8    | 4.9                                          |    |    |   |    |
| BP-IA8    | 2.8                                          |    |    |   |    |
| IP-IA16   | 13.7                                         |    |    |   |    |
| BP-IA16   | 6.4                                          |    |    |   |    |
| IP-IA32   | 45.4                                         |    |    |   |    |
| BP-IA32   | 1.5                                          |    |    |   |    |

Table 1 summarized the retention factors (k') and imprint factors (IF) for nicotine exhibited by the nicotine-imprinted polymers (IPs) and the non-imprint blank polymers (BPs). IF values calculated by dividing k'IP by k'BP are used here as index to evaluate the effect of molecular imprinting on resultant nicotine-retaining ability under the adopted synthesis conditions.

While little imprint effect was observed when the stoichiometric amount of IA was used, IF enlarged in accordance with the increase in the amount of IA. When 16 molar equivalent of IA to nicotine was used, the imprint effect appeared to be maximal, exhibiting k' and IF as 40.4 and 3.1, respectively, using acetonitrile – acetic acid (9:1, v/v) as the eluent. The reason why the excess use of IA resulted in higher IF values could be explained by an equilibrium in the polymerization mixture; the excess IA could have shifted the equilibrium toward the nicotine – IA complex formation and increased the number of the nicotine binding sites. However, the amount of IA should be optimized because excess IA residues randomly located in the imprinted polymers would show the similar binding property as those in the non-imprint blank polymers, thus diminishing the imprint effect. In fact, IF value was significantly decreased when 32 eq. of IA was employed; IP-I16 and IP-I32 resulted in IF values as 2.8 and 1.5, respectively, when acetonitrile – acetic acid – water (8:1:1,
v/v/v) was used as the eluent.

The relatively low IF exhibited by the IA32 imprinting-system could also be explained by a cross-linking ratio; the higher content of the functional monomer decreases the cross-linking ratio of the resultant polymer, though it is known that highly cross-linked structure is necessary for imprinted polymers to preserve the template-shaped binding site.

3.2. Selectivity of IPs for nicotine

Selectivity of IPs was assessed using a metabolite of nicotine, cotinine, as a reference compound. 3-Methylpyridine and N-methylpyrrolidine were also tested as mimics of the partial structure of nicotine. As shown in Fig. 2, IP-IA16 retained nicotine the best among the tested compounds. Although nicotine was also retained by BP-IP16 to some extent, the comparison of the retention of nicotine with that of cotinine would highlight the imprint effect; nicotine was retained 6.9 times longer than cotinine on IP while 2.7 times on BP.

3-Methylpyridine and N-methylpyrrolidine resulted in significantly shorter retention, suggesting that both nitrogen atoms of nicotine were simultaneously engaged in the retention mechanism. Slightly longer retention of N-methylpyrrolidine could be attributed to the higher basicity of pyrrolidine’s nitrogen atom compared with that of pyridine’s. It could be noteworthy that both 3-methylpyridine and N-methylpyrrolidine were retained by IP longer than by BP, implying that the nicotine-selective binding sites could also recognize the partial structures of nicotine.

3.3. Comparison with IPs synthesized with other acidic monomers

Itaconic acid (IA) was compared with the previously used functional monomers; IPs and BPs were prepared with methacrylic acid (MA) and 2-(trifluoromethyl)acrylic acid (FM). Esters of IA, monomethyl itaconate (MI) and dimethyl itaconate (DI), were also tested as functional monomer, and the obtained polymers were chromatographically examined.

As shown in Fig. 3, none of them was comparable to IP-IA16 in terms of the retention factor ($k'$). The nicotine-imprinted polymer IP-FM16, which was synthesized with a monomer with a higher acidity, FM, showed retention to a certain degree; however, only a little imprint effect was exerted. The imprinted polymer synthesized with MA, IP-MA16, hardly retained nicotine under the same conditions. Because MA was successfully utilized for molecular imprinting of nicotine in the previous study, the quick elution of nicotine would be attributed to the adopted eluent, acetonitrile containing 10% acetic acid, which would severely interfere in hydrogen bonding and/or electrostatic interaction between nicotine and carboxyl.
moieties in the polymers.

MI and DI were also examined as functional monomer, in which either or both of the carboxyl groups of IA were esterified, resulting in almost no retention. From these results, it appeared that the presence of two carboxyl groups of IA was critical for the largest retention factor ($k'$) and imprint factor (IF). IP-IA8, which was supposed to possess the same number of carboxyl moieties as IP-MA16, still showed a greater $k'$ and IF values than IP-MA16, suggesting that the superiority of IA was not just due to the doubled concentration of carboxyl group.

3.4. Comparison with IPs synthesized in chloroform using methacrylic acid (MA) and 2-(trifluoromethyl)acrylic acid (FM)

MA and FM had been successfully utilized as functional monomer for nicotine imprinting in the previous studies; in this study, however, they resulted in poor IF values as shown in Fig. 2. The poor imprint effect would be attributed in part to THF used as the porogen for the polymerization. THF was adopted in this study for making fair comparison with IA, which only dissolves in relatively polar solvents like THF. However, such polar porogen would shift the equilibrium in the polymerization mixture toward dissociation of nicotine – functional monomer complexes, and therefore would decrease the number of binding sites and increase that of randomly located carboxyl moieties, thus reducing the IF value. Therefore, in the previous study on nicotine imprinting, less polar solvents like chloroform were adopted as porogen.

Thus, in this study, MA-based and FM-based nicotine-imprinted polymers (IP-MA16-CHL, IP-FM16-CHL) were also synthesized using chloroform as porogen for comparing IA with MA and FM under the optimal conditions for each. As a result, IP-MA16-CHL exhibited almost no retention ($k' = 0.21$), again suggesting the superiority of IA to MA. IP-FM16-CHL exhibited a significantly longer retention ($k' = 27.8$) than IP-FM16, and marked an imprint factor as 3.96. Compared with IP-IA16, the IF value of IP-FM16-CHL was higher than that of IP-IA16 (IF = 3.1), while its $k'$ value was significantly smaller than that of IP-IA16 ($k' = 40.4$).

Finally, IP-IA16 and IP-FM16-CHL were compared to each other from a viewpoint of selectivity. Fig. 4 shows the relative $k'$ value of cotinine when that of nicotine is taken as 100%. Nicotine was retained 6.9-times longer than cotinine on IP-IA16, while IP-FM16-CHL retained nicotine only 3.7-times longer than cotinine. Thus, it would be reasonable to conclude that IP would be the most suitable functional monomer among those examined in this study for nicotine imprinting, according to the results that IP-IA16 exhibited the longest retention and highest selectivity.

3.5. Nitrogen NMR titration

The results so far strongly suggest that the second carboxyl group of IA plays a critical role in nicotine imprinting. A possible contribution of the second carboxyl group would be stabilization of the template-functional monomer complex via cooperative double hydrogen bonding and/or electrostatic interaction, where the two carboxyl groups of IA simultaneously interact with the both nitrogen atoms of nicotine (Fig. 1 (b)). In this case, the expectable template effect is not to preorganize plural functional monomer molecules, but is limited to the formation of a nicotine-modeled cavity.

![Fig. 4. Comparison of the selectivity between IP-IA16 (blank) and IP-FM16-CHL (shaded). Acetonitrile – acetic acid (9:1, v/v) was used the eluent.](image)

![Fig. 5. Changes of chemical shift in $^{15}$N NMR spectra for nicotine upon addition of itaconic acid (IA). Dashed lines in graph c indicate linear approximation with data at 0-4 eq. of IA, which were drawn for assisting to compare profiles of the pyridine’s nitrogen and the pyrrolidine’s.](image)
To examine whether nicotine and IA forms such a 1:1 complex via the double interaction, $^{15}$N NMR titration study was conducted. As the concentration of IA increased, the peak attributed to the pyridine ring’s nitrogen showed an up-field shift (Fig. 5a), while that of pyrrolidine ring’s nitrogen showed a down-field shift (Fig. 5b), which agrees with the expected changes upon their involvement in hydrogen bonding and/or electrostatic interaction, according to a literature [29]. Fig. 5c shows the absolute peak attributed to the pyridine ring’s nitrogen showed an up-field shift (Fig. 5b), which agrees with the expected changes upon their involvement in hydrogen bonding and/or electrostatic interaction, according to a literature [29].

This can be confirmed by the fairly long retention of nicotine exhibited by non-imprint BP-I16. However, the fact that a more acidic FM was less suitable than IA suggests that the acidity can not fully explain the suitability of IA. We are currently conducting a further detailed $^{15}$N NMR study and in-silico simulation to elucidate the utility and mechanism of IA as functional monomer in molecular imprinting.

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