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Research Paper

Uptake of nicotine from suspension culture of *Nicotiana tabacum* by molecularly imprinted polymers

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Abstract

Objectives The aim was to use molecularly imprinted polymers (MIPs) for the selective recovery of nicotine in plant cell cultures. MIPs can selectively uptake nicotine from suspension cultures of *N. tabacum*, and therefore may be useful for improving levels of secondary metabolites in plant cell cultures.

Methods Suspension cultures of *N. tabacum* were initiated from callus and maintained in liquid Murashige and Skoog (MS) media containing 3% w/v sucrose, 0.1 mg/l α-naphthaleneacetic acid acid (NAA) and 0.25 mg/l kinetin. Tween 80 at 1% was used for permeabilisation of cell cultures. Pre-weighed XAD-2 and two types of synthesized polymers, MIPs (A and B with one and two functional monomers, respectively) and corresponding non-imprinted polymers (NIPs), A and B, were introduced aseptically into the permeabilised suspension cultures of *N. tabacum,* the nicotine contents of polymers were determined by gas chromatography and the adsorption yield of polymers were determined.

Key findings Cell cultures of *N. tabacum* accumulated nicotine alkaloid intracellularly in varying levels, 6.8–14.9 mg/l fresh weight. MIPs were able to uptake 50–70% of released nicotine in suspension cultures of *N. tabacum*, whereas XAD-2 recovered only 30–40%. The total levels of accumulated nicotine were enhanced up to 20 mg/l by simultaneous use of Tween 80 and MIPs.

Conclusions The findings indicate the potential use of MIPs to uptake nicotine from suspension cultures of *N. tabacum*, and increase productivity of secondary metabolites in plant cell cultures.

Keywords molecularly imprinted polymer; *Nicotiana tabacum*; nicotine; permeabilisation; plant tissue cultures

Introduction

Molecularly imprinted polymers (MIPs) represent a class of materials that have artificially created receptor structures. MIPs are cross-linked polymers with specific binding sites for a target molecule; they are synthetic polymers which are commonly prepared by polymerisation of functional and cross-linked monomers in the presence of a certain analyte as a molecular template.[1] MIPs possess many outstanding advantages, such as high affinity and selectivity, unique stability and relative ease and low cost of their preparation.[2]

Two imprinting approaches are commonly used to prepare MIPs and have been reviewed and compared recently.[3] The non-covalent method is most widely used due to the ease of preparation, ease of removal of template and wide application to different molecules. This method depends on the self-assembly between the template and functional monomers and formation of host–guest complexes by non-covalent interactions such as hydrogen bonding, ionic forces and hydrophobic interactions. The second type is covalent imprinting, in which the complex is formed by covalent interaction between template and functional monomer before polymerisation. In both methods, after the removal of template by extraction or chemical reaction, binding sites are created which match the template in size, shape and position of the functional groups. These binding sites have the ability to rebind the template with high affinity and selectivity.

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Recently, MIPs have attracted much interest and have been used successfully in many applications, including separation, binding assays, sample pre-treatment and sensors,[4,5] the most common application being solid phase extraction. Molecularly imprinted solid phase extraction has been applied in the extraction and analysis of target analytes in different samples. These include environmental, biological, pharmaceutical and food samples. These applications have been recently reviewed.[6,7] Although MIPs show many advantages, they also possess some limitations, such as low yield of binding sites, non-specific binding and leaching of the template from the polymer, which interferes with trace analysis.[2]

Many studies have reported on the design of MIPs for solid phase extraction and determination of nicotine in many biological samples (e.g. determination of nicotine in the urine of tobacco smokers,[8] and analysis of nicotine in the hair of smokers[9] and in nicotine chewing gum[10]). Another interesting study concerned the use of MIPs for selective removal of nicotine from tobacco smoke, and it was found superior to that of a commercial filter tip in terms of affinity to nicotine.[11] The different MIPs used for extraction and determination of nicotine in different areas encouraged us to examine these types of sorbent materials for take-up of nicotine from suspension cultures and the possibility of enhancing secondary metabolite production.

Materials and Methods

Cell cultures

Seeds of *Nicotiana tabacum* from Thailand were surface-sterilised by shaking them in hydrogen peroxide (30% w/v, BDH) containing two or three drops of Tween 80 for 3–4 min, and then incubated in the dark at 25 ± 2°C until the seeds germinated. The callus was obtained from the seedlings in solidified, 1% w/v agar (Sigma), Murashige and Skoog (MS) medium[12] containing sucrose 3% w/v (BDH), 1 ppm α-naphthaleneacetic acid (NAA) and 0.25 ppm kinetin. The suspension cultures were initiated from the fourth generation of callus and maintained in liquid MS medium containing sucrose 3% w/v (BDH), 0.1 ppm NAA and 0.25 ppm kinetin. These were incubated at a temperature of 25 ± 2°C under a 16-h light/8-h dark photoperiod on an orbital shaker (100 rev/min). The suspension cultures were maintained by subsequently transferring 5 ml old suspension into 50 ml fresh liquid medium every three weeks.

Extraction of alkaloids from suspension cultures of *Nicotiana tabacum*

Suspension cultures of *N. tabacum* were extracted wholly or after they had been separated into cells and liquid medium by filtration. The extraction process was based on a method reported earlier.[13] The whole suspension culture and the filtrate were extracted directly, while cells were suspended in sufficient sterilised water. The cultures were basified to pH 9–10 by the addition of a few drops of concentrated ammonia and then extracted three times using 20 ml redistilled dichloromethane (DCM) containing internal standard (*n*-heptadecane 2 mg/l). The suspension was shaken for 10 min, and then centrifuged at 3000 rev/min for 5 min. Then the DCM layer was passed through a Whatman No. 1 filter paper, and dried over anhydrous sodium sulfate (Na_2SO_4). The DCM extracts were evaporated under nitrogen flow to a volume of 100 μl, and kept at 5°C before analysis using GC and GC-MS.

Analysis and identification of nicotine using gas–liquid chromatography (GLC)

GC analyses of the concentrated extracts were carried out using a Finnigan Focus Gas Chromatography, fitted with a flame ionisation detector (FID): an Rtx-1 fused-silica column was used (30 m × 0.25 i.d., film thickness 0.25 μm). The results were recorded on a Chrom Card integrator. The oven temperature was maintained at 100°C for 3 min, subsequently programmed at 8°C/min up to 320°C with isothermal for 5 min; injector and detector temperatures were 250°C and 280°C, respectively. Helium was the carrier gas at a flow rate of 1 ml/min; 1 μl of samples were injected using the split-mode technique at 1 : 50 ratio. Each sample was injected twice. Identification of nicotine was confirmed by running standards and comparing their retention times and also by GC-MS. The concentrations were calculated from a standard curve constructed with respect to *n*-heptadecane (2 mg/l) as internal standard.

Gas chromatography–mass spectrometry

A Thermo Finnigan MAT 95 XP mass spectrometer equipped with an Xcalibur data system was used to perform GC-MS. The column used was HP5 fused silica capillary column (30 m length × 0.32 mm internal diameter × 0.25 μm film thickness. Helium was the carrier gas at a flow rate of 1.5 ml/min. The oven temperature program was identical to that used in GC. Electron impact–mass spectrometry (EI-MS): electron energy 60 eV; ion source temperature 200°C; resolution 1000; scan speed 1 s/decade. Identification of nicotine was based on matching of its mass spectra with those stored in the spectrometer database using NIST mass spectral library, with matching 97% and comparing mass spectra of authentic samples.

Permeabilisation of *Nicotiana tabacum* suspension cultures

Three types of chemicals were tested as permeabilising agents at different concentrations, and the effects of them on cell growth and alkaloid production were studied; these included dimethyl sulfoxide (DMSO), Tween 20 and Tween 80. At harvesting time the concentration of alkaloids in filtrates and cells were calculated.

Synthesis of molecularly imprinted polymers

For the preparation of polymer, 5 mmol nicotine as template, 15 mmol 2-acrylamido-2-methylpropane sulfonic acid (AMPS), 5 mmol styrene (Sty) (only in polymer B) as functional monomers, 20 mmol ethylene glycol dimethacrylate (EGDMA) as cross linker monomer, 1 mmol α,α'-azoisobutyronitrile (AIBN) as initiator and 65 mmol (polymer A) or 60 mmol (polymer B) DMSO as solvent were mixed in a 25-ml ampoule. The mixture was degassed by purging with nitrogen gas for 10 min. The ampoule content was polymerised at 5 cm distance from the UV irradiation

(maximum spectral intensity at 356 nm; F8T5/BLB, 8 Wattage (W) fluorescent black light, diameter 5/8″, length 12″; Sylvania, USA). Following polymerisation, the bulk polymers were forced through a sieve (mesh No. 60, 250 μm). The polymer particles then were extracted by DMSO and washed with a gradient of methanol : acetic acid and water to remove residual nicotine. A non-imprinted polymer (NIP) was prepared in the same way but without nicotine.

Uptake of nicotine by different types of polymers

XAD-2 (Aldrich) commercial polymer was used to compare the absorption capacity with that of the synthesised polymers. Pre-weighed XAD-2 polymer and different types of synthesised polymers were transferred to a dialysis tube and sealed with thread before they were autoclaved in double-distilled water. At 14 days after subculture of *N. tabacum* suspension cultures, the pre-autoclaved dialysis tubes containing different types of polymers were aseptically introduced into the suspension cultures, which were maintained as usual until the harvesting time. After six days, the dialysis tubes were washed in distilled water three times and their contents of nicotine were extracted with DCM and analysed using GC and GC-MS.

The extraction yield percentage was calculated according to the following equation:

$$\text{Extraction yield}\,(\%) = [\text{Cp}/(\text{Cp} + \text{Cm})] \times 100 \qquad (1)$$

Where Cp is the amount of nicotine measured in the polymer and Cm is the amount of nicotine measured in the medium after the incubation period.

Statistical methods

Statistical analysis of the effect of polymer type on extraction yield and effect of permeabilisation (Tween 80 1%) and different types of polymers on total nicotine levels was performed using the Kruskal–Wallis test. Individual differences between the various polymers were tested using Dunn's post-test. The effect of incubation time of polymers on nicotine levels was statistically analysed using the Mann–Whitney *U*-test. $P < 0.05$ indicated significance in all cases. Data are usually represented by the average of at least three replicates.

Results

Alkaloid production in *Nicotiana tabacum* suspension cultures

GC and GC-MS of DCM extracts of *N. tabacum* suspension cultures showed the presence of only nicotine alkaloid. The levels of nicotine were detected at varying concentrations over the range 6.8–14.9 mg/l. All of the nicotine contents were intracellular, no nicotine was detected in the medium before 17 days after subculture, and only 11.5% of the total nicotine content was detected extracellularly at 21 days. The time-course accumulation of nicotine is shown in Figure 1; the highest levels were obtained at days 14–21 (8.4–10.6 mg/l),

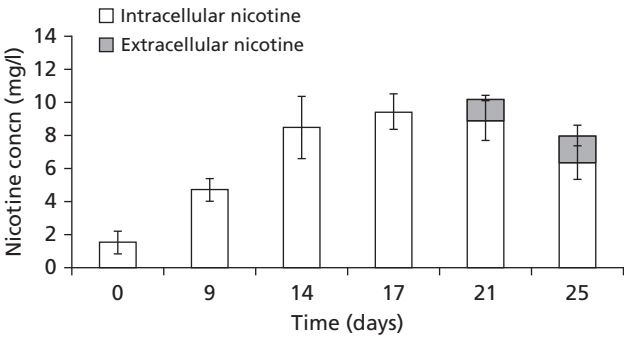

Figure 1 Time-course for nicotine accumulation in suspension cultures of *N. tabacum*. Data are the means ± SD of three experiments

and declined afterwards as the media became exhausted and the growth was inhibited.

Selection of permeabilising agent

From the preliminary results, Tween 80 at a concentration of 0.5 and 1% was the best both in terms of releasing the intracellular nicotine into the medium and also the effects on cell growth of suspension cultures. No toxicity was observed from Tween 80 even at the high concentration of 1%, so Tween 80 was selected as the permeabilising agent and it was used in the subsequent experiments. Tween 20 and DMSO both at a concentration of 1% reduced cell viability and seriously inhibited cell growth; the cultures turned dark brown even at lower concentrations, and hence no data or results were recorded for them. Further experiments are needed to optimise the use of Tween 80 in the permeabilisation of *N. tabacum* suspension cultures, such as the effect on the growth, and the effect of a different range of concentrations on the release pattern of nicotine during the growth cycle of cultures. However, the aim of this experiment was to investigate the MIPs' capability to take up nicotine from suspension cultures of *N. tabacum*, and the previously reported use of Tween 80 in permeabilisation of different suspension cultures[14] decreased the potential to do these further experiments.

Effect of Tween 80 on nicotine production

As shown in Figure 2, the addition of Tween 80 1% after 14 days of subculture, the same time for addition of the polymers, enhanced the levels of extracellular nicotine from 1.2 to 7.3 mg/l (60.8% of total amount) and also enhanced the total nicotine levels from 12 to 17.5 mg/l at the harvesting time of 20 days.

Extraction yield of different types of polymers

The extraction yields of different types of polymers used are shown in Figure 3. Two types of synthesised polymers were used; MIPs (A and B) and non-imprinted polymers (NIPs; A and B) and the commercial XAD-2 polymer for comparison. All the synthesised polymers, MIPs and NIPs, had a higher extraction yield than that of the XAD-2 polymer. The extraction yields of MIP B were the highest, at 70%, whereas the extraction yields of MIP A, NIP A, NIP B and XAD-2 were 63, 51, 53 and 31%, respectively. There

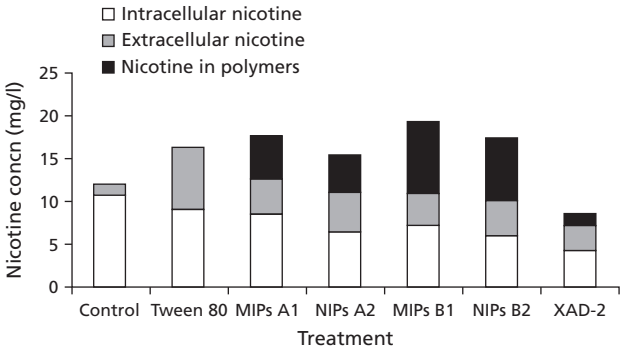

Figure 2 Effect of permeabilisation (Tween 80 1%) and different types of polymers on nicotine levels in suspension cultures of *N. tabacum* after six days incubation time. Data are the means ± SD of three experiments

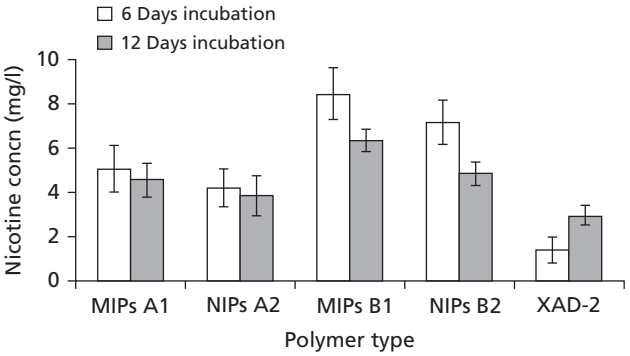

Figure 4 Effect of the incubation time of the different polymer types on the amount of nicotine taken up from suspension cultures of *N. tabacum*. Data are the means ± SD of three experiments

was no significant difference between the extraction yields of MIPs A and B and those of NIPs A and B ($P > 0.05$), but there was a significant difference between the extraction yields of MIPs A and B and that of XAD-2 ($P < 0.05$).

Effect of incubation time of different polymers on levels of nicotine

In the study to determine the effect of incubation time of polymers on the absorption of nicotine, the different types of polymer were incubated into suspension cultures for six and 12 days. Apart from XAD-2 polymer, higher concentrations of nicotine were obtained after six days incubation time than after 12 days (Figure 4). This is probably due to the detrimental effect of different types of polymers on the cell growth with the long incubation period. A significant difference in nicotine levels was obtained between incubation time of six and 12 days with MIP B, NIP B and XAD-2 polymer.

Effect of permeabilisation and molecularly imprinted polymers on nicotine levels

Figure 2 shows total nicotine levels, extracellular, intracellular and that absorbed by the polymer by simultaneous use of Tween 80 1% as a permeabilising agent and MIP and NIP. The concentration of total nicotine was increased from 10–12 mg/l in the control suspension culture to 15–19 mg/l upon using of Tween 80 1% and the synthesised polymers. Meanwhile, low total nicotine levels were obtained in suspension cultures treated with XAD-2 polymer (8–10 mg/l). A significant difference in total nicotine levels was obtained by using Tween 80 and MIPs B compared with control cultures ($P < 0.05$).

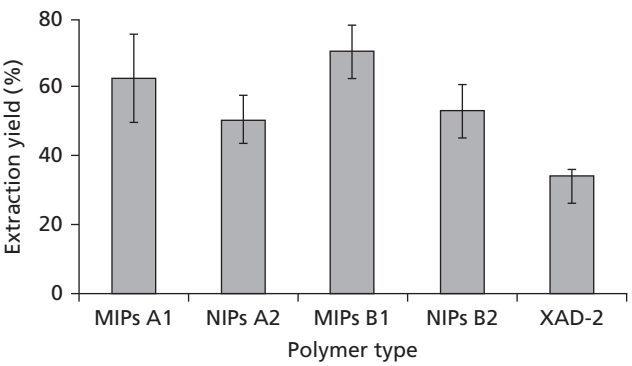

Figure 3 Extraction yield percentage of different types of polymer after six days incubation in suspension cultures of *N. tabacum*. Data are the means ± SD of three experiments

Discussion

MIPs have been widely used for solid phase extraction of certain analytes in different samples such as environmental, biological, food and pharmaceutical samples.[7] Their applications in plant tissue cultures are few; selective uptake of certain compounds from plant cell culture may be useful for improving levels of secondary metabolites. Adsorption of products once released could decrease feedback inhibition and enhance levels of secondary metabolites.

The accumulated nicotine in suspension cultures of *N. tabacum* was mainly intracellular, therefore permeabilisation of cell culture was necessary to excrete nicotine into the medium and hence the introduction of different types of polymers were applied as solid phase extractors of nicotine.

Initial work regarding the selectivity of the MIPs showed that nicotine was exclusively taken up into the polymers with concomitant exclusion of all similar plant cell culture components in MS medium,[12] namely nicotinamide, thiamine, nicotinamide-*N*-oxide, pyridoxine and nicotinic acid.

The extraction yields of MIPs were higher than those of corresponding NIPs, but this increment was not significant. The non-significant difference between the extraction yield of MIPs and that of NIPs indicated that the absorption of nicotine was not only due to molecular imprinting but also was due to hydrophobic absorption and ionic interaction by the functional monomers of polymers (AMPS and Sty). The differences between the absorption capacity of polymers A and B are attributed to the difference in polarity, measured by the ratio of functional monomers, [AMPS] / ([AMPS] + [Sty]). This ratio is 1 and 0.75 for polymers A and B, respectively, which means that there is a higher content of the hydrophobic styrene residues in polymer B than that in polymer A.

In terms of nicotine productivity, high levels of total nicotine, extracellular, intracellular and that absorbed by the polymer, were obtained by the simultaneous use of Tween 80 1% as a permeabilising agent and MIPs and NIPs as shown in Figure 2. The concentration of total nicotine was increased from 10–12 mg/l in the control suspension culture to 15–19 mg/l on simultaneous use of Tween 80 and the synthesised polymers. What is not explained are the low levels of total nicotine obtained from the use of XAD-2 polymer, lower than that of the control culture; this could be due to the absorption of growth hormone and the nutrients from the medium and hence the growth is severely inhibited.

Conclusions

The synthesised MIPs were able to successfully uptake nicotine from suspension cultures of *N. tabacum*, and increase productivity of secondary metabolites. This allows the possibility of using MIPs to improve alkaloid production and collection in plant cell cultures. MIP (B) is clearly the most beneficial polymer in terms of total nicotine levels, and uptake by the polymer, while extracellular nicotine is mildly variable with all of the MIPs and NIPs.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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