รายงานฉบับสมบูรณ์

ทุนอุดหนุนการวิจัยจากเงินงบประมาณ

ประจำปีงบประมาณ พ.ศ. 2548

(โครงการวิจัยต่อเนื่องระยะเวลา 2 ปี)

ชื่อโครงการ

ภาษาไทย :

วิธีการใหม่ของการนำส่งยา propranolol ผ่านทางผิวหนังโดยการใช้ระบบควบคุมการปลดปล่อยที่เลือกเฉพาะอิแนนทิโอเมอร์

ภาษาอังกฤษ :

A novel approach for transdermal delivery of propranolol by the use of enantioselective-controlled release system

ชื่อหัวหน้าโครงการ:

รศ. ดร. รุ่งนภา ศรีชนะ (ชื่อดี)

ภาควิชาเภสัชเคมี คณะเภสัชศาสตร์

ผู้ร่วมโครงการ:

1) รองศาสตราจารย์ ดร. ธีระพล ศรีชนะ

2) ผู้ช่วยศาสตราจารย์ ดร. เสน่ห์ แก้วนพรัตน์

3) ดร. ศรัณยู สงเคราะห์

บทคัดย่อ

ในงานวิจัยนี้ได้พัฒนาเมมเบรนสำหรับการเลือกนำส่งเอส-อิแนนทิโอเมอร์ของยาบีต้าบร็อคเคอร์ พรอพราโนรอลด้วยการเติมพอลิเมอร์ที่มีรอยพิมพ์ประทับโมเลกุลเข้าไปในรูพรุนของแบคทีเรียล เซลลูโลสเมมเบรนที่ใช้วิธีพอร์ฟังก์ชันนัลไลเซชั่นด้วยไซลาไนซ์คลัปปลิ้ง และพอลิเมอร์ที่มีรอยพิมพ์ประทับนี้ได้ถูกสังเคราะห์ขึ้นให้มีความสามารถจดจำต่อเอส-พรอพราโนลอลด้วยปฏิกิริยาโคพอลิเมอไรเซชันของเมธาคริยลิกแอซิดกับเอธิลีนกลัยคอล ไดเมธาคริยเลท โดยมีเอส-พรอพราโนลอลเป็นตัวพิมพ์ หลังจากนั้นทำการสกัดตัวพิมพ์ออกด้วยตัวทำละลายที่เหมาะสม และจากการตรวจสอบพบว่าเซลลูโลสเมมเบรนที่ประกอบด้วยพอลิเมอร์ที่มีรอยพิมพ์ประทับที่เตรียมขึ้นมาสามารถเลือกนำส่งเอส-พรอพราโนลอลได้จำเพาะดีมากและผลการเลือกนำส่งเอส-พรอพราโนลอลที่เกิดขึ้นเนื่องจากการทำงานของเซลลูโลสเมมเบรนตั้งต้นเป็นหลักบวกกับการการทำงานที่เสริมกันของพอลิเมอร์ที่มีรอยพิมพ์ประทับที่รูของเมมเบรน เมมเบรนดังกล่าวยังให้ผลการเลือกนำส่งอิแนนทิโอเมอร์กับโปรดรักของพรอพราโนลอลอีกด้วย ซึ่งแสดงให้เห็นว่ารูปร่างและการจัดเรียงของหมู่ฟังก์ชันที่คล้ายคลึงกับโมเลกุลตัวพิมพ์มีความสำคัญต่อการเลือกจดจำสารของเมมเบรน และการเลือกนำส่งอิแนนทิโอเมอร์ของเมมเบรนก็ปรากฏผลดีด้วยในการทดลองแบบนอกกายซึ่งใช้ผิวหนังหนู นอกจากนี้ได้เตรียมเจลขึ้นมา 2 ชนิด คือ พอลอกซาเมอร์และไคโตแซน สำหรับเป็นสูตรตำรับของราซิมิคพรอพราโนลอลที่จะบรรจุไว้ในแผ่นแปะของเซลลูโลสเมมเบรนที่มีพอลิเมอร์ที่มีรอยพิมพ์ประทับ ซึ่งเมื่อตรวจประสิทธิภาพของเจลทั้ง 2 ชนิดในการเป็นสูตรตำรับสำหรับนำส่งเอส-อิแนนทิโอเมอร์ของพรอพราโนลอลจากเซลลูโลสเมมเบรนที่ประกอบด้วยพอลิเมอร์ที่มีรอยพิมพ์ประทับด้วยการทดลองแบบนอกกายซึ่งใช้ฟรานซ์ดิฟฟิวชันเซลล์ พบว่าเอส-อิแนนทิโอเมอร์ถูกเลือกนำส่งออกมาด้วยการควบคุมของเมมเบรน และอัตราเร็วของการเลือกนำส่งก็ขึ้นกับชนิดของเจลที่ใช้ นอกจากนี้ยังพบว่าชนิดของเจลส่งผลต่อประสิทธิภาพในการเลือกนำส่งอิแนนทิโอเมอร์พรอพราโนลอลด้วย เจลพอลอกซาเมอร์ซึ่งมีความหนืดสูงกว่าเจลไคโตแซนไม่ให้การเลือกปลดปล่อยอิแนนทิโอเมอร์ของพรอพราโนลอลซึ่งแสดงให้เห็นว่าโครงสร้างของเจลอาจจะรบกวนกระบวนการนำส่งที่ผิวของรูของเมมเบรน ขณะที่เจลไคโตเซนให้ประสิทธิภาพในการเลือกนำส่งเอส-พรอพราโนลอลที่ดีกว่ามาก นอกจากนี้ได้ทดลองเตรียมแผ่นแปะของเซลลูโลสเมมเบรนที่มีพอลิเมอร์ที่มีรอยพิมพ์ประทับและมีไคโตเซนเจลเป็นสูตรตำรับของราซิมิคพรอพราโนลอล และเมื่อตรวจความสามารถในการนำส่งผ่านผิวหนังหนูด้วยการทดลองแบบในกาย ผลปรากฏว่าแผ่นแปะให้การเลือกนำส่งเอส-พราพราโนรอลดีเช่นเดียวกับผลการทดลองที่ได้จากการทดลองแบบนอกกาย

Abstract

A composite membrane for transdermal delivery of *S*-propranolol enantiomer was developed based on the controlled pore functionalization of bacterial cellulose membranes using a molecularly imprinted polymer (MIP) layer synthesis. The reactive pore-filling of an asymmetric porous cellulose membrane with a MIP thin-layer was effected using a silanised coupler as an additional anchor for the MIP. MIP thin-layers with specific binding sites for *S*-propranolol were synthesized by copolymerisation of methacrylic acid with a cross-linker, ethylene glycol dimethacrylate in the presence of *S*-propranolol as the template molecule and the latter was subsequently extracted. Selective transport of *S*-propranolol through the MIP composite membrane was obtained, although this was determined mostly by the parent cellulose membrane with some ancillary contributory effect from the MIP layer. In addition, an enantioselectivity in the transport of propranolol prodrug enantiomers was found, suggesting that the shape and functional groups orientation, which are similar to that of the print molecule was essential for enantiomeric recognition of the MIP composite membrane. The enantioselectivity of S-MIP membranes was also shown when the release of propranolol enantiomers was studied *in vitro* using rat skin, with racemic propranolol contained in the donor compartment. Further, two gel formulations, containing either poloxamer or chitosan were selected as drug reservoirs for the formulation of racemic propranolol within the MIP thin-layer composite CM. The ability of the fabricated devices to deliver the *S*-enantiomer of propranolol was tested by conducting release studies *in vitro* using modified Franz diffusion cells. The enantiomer release profiles showed that the *S*-enantiomer delivery is completely controlled by the composite MIP membrane. The rate of drug release was demonstrated to be dependent on the type of gel used. The gel type was found to affect the enantioselective transport of propranolol, with the more rheologically structured poloxamer gel formulation providing no selective release of *S*-propranolol. The greater gel structure may have interfered with the enantioselective controlling process at the pore surface of the composite MIP membrane. In contrast the chitosan gel was found to allow excellent selectivity for delivery of the *S*-propranolol enantiomer. Also, the results observed were reproduced *in vivo* using rat.

Keywords: Molecularly imprinted polymer; Cellulose membrane; Propranolol; Transdermal delivery; Enantioselectivity

Introduction

The efficiency of delivery of propranolol, a nonselective β-adrenergic receptor antagonist, *via* the oral route is affected by the low absorption [1] and extensive first-pass metabolism of the drug [2] whereas the transdermal delivery of propranolol enables the hepatic first-pass metabolism to be avoided, potentially improving bioavailability. The absorption of propranolol hydrochloride, which is the commercially available form of propranolol, through skin is very poor. A prodrug strategy has been used with some success where the drug after absorption through the stratum corneum is subsequently metabolized to the parent compound [3-5]. Propranolol possesses one chiral center and the *S*-enantiomer is 100-130 times more pharmacologically active than the *R*-enantiomer [6]. Propranolol is marketed as racemate and no stereoselective permeation of propranolol and propranolol ester prodrug was observed *in vitro* [7-8]. Thus, if the *S*-enantiomer were to be selectively transported across the skin, a better therapeutic response might be expected relative to that obtained using a racemic mixture of the drug.

A molecularly imprinted polymer (MIP) is a synthetic polymer possessing selective molecular recognition properties because of recognition sites within the polymer matrix that are complementary to the analyte molecule in the shape and positioning of functional groups. The use of MIPs as new sorbents for affinity membrane extraction is becoming more widespread, due to their potentially high selectivity [9-10]. MIP membranes utilising such an approach may comprise either membranes composed of a MIP or alternatively membranes containing a MIP. A composite MIP membrane is an optimised porous support membrane which has been functionalised with a suitable thin selective layer to yield a membrane with appropriate transport properties for the target compound [9]. A number of composite membranes functionalized with thin MIP layers have been previously developed to attain adsorption specificity [11-13]. The preparation of MIP composite membranes can allow the transport of drugs to be controlled by both membrane pore structure and MIP recognition. The structure of the base membrane in MIP composite membranes may be used as a means to adapt pore size, permeability, internal surface area and binding capacity to the desired application with synthetic membranes often being used.

Cellulose is a naturally occurring linear β-1,4-linked glucose polymer synthesized by plants and some species of bacteria. In contrast to some synthetic membranes, cellulose membrane exhibits a high resistance to corrosive chemicals but is biodegradable and hence eco-friendly. The unique properties of the bacterially-derived cellulose synthesized by *Acetobacter xylinum* have inspired a number of attempts to use it in a number of commercial products. Proposed applications include its use as a temporary skin substitute, a dressing material, a material for surgery or microsurgery and as membranes [14-15]. For example Biofill® is a specially prepared membrane which can be used as a temporary skin substitute

for patients with burns and ulcers. Bacterially-derived cellulose can be easily processed into a porous membrane possessing good mechanical strength, unlike the cellulose derived from plant biomass. These unique features make such bacterially-derived membranes potentially suitable for use as a component of transdermal delivery system although to date no studies have reported the use of such cellulose membranes in controlled drug delivery systems.

In a previous study, cellulose triphenylcarbamates were shown to have a good potential for controlling transport of propranolol enantiomers through a silastic membrane [16]. Cellulose and derivatives have proven stereoselectivity to propranolol in adsorption chromatography both in column chromatography [17] and thin layer chromatography [18], where they demonstrate a high affinity to the S-enantiomer of propranolol.

In this study, MIPs and bacterially-derived membranes were combined to investigate their potential as a transdermal delivery system of *S*-propranolol. Through optimisation of MIP affinity and ensuring a uniform distribution of the polymer within cellulose membrane pores as well as minimizing non-selective diffusion by pore filling, a selective absorption and subsequent release of the eutomer of propranolol through skin might be achieved. Accordingly MIP membranes selective for S-propranolol were developed and their enantioselective-controlled release was examined *in vitro*. Microporous cellulose membranes were generated by bacterial-cultivation and those were used as a matrix for a two-step grafting procedure to yield a MIP by *in situ* copolymerisation within the thin barrier layer of the base material. Using a silanisation procedure, supramolecular complexes of template molecules and suitable functional isopropenylates were fixed onto pore-containing surface of cellulose membranes. MIP synthesis mixtures comprising methacrylic acid as a functional monomer and ethylene glycol dimethacrylate as a crosslinker, in the presence of the template enantiomer were successfully polymerized, which allowed the generation of MIP composite membranes which included a thin MIP layer that might act as a selective barrier.

2. Experimental

2.1. Chemicals and reagents

Ethylene glycol dimethacrylate (EDMA), methacrylic acid (MAA) and propranolol hydrochloride, *R*-propranolol HCl, *S*-propranolol HCl and 3-methacryloxypropyltrimethoxysilane (3-MPS) and valeryl chloride were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2'-Azobis-(isobutyronitrile) (AIBN) was obtained from Janssen (Geel, Belgium). Yeast extract and peptone were obtained from Difco Laboratories, USA. Succinic anhydride and cyclopropanecarbonyl chloride were supplied by Fluka Chemie AG (Buchs, Switzerland). EDMA was purified by extraction with 10% (w/v) $CaCO_3$, washing with water, drying over anhydrous sodium sulfate and subsequent distillation under reduced pressure. MAA was purified by distillation under reduced pressure prior to use. All solvents used were of analytical grade and were dried with molecular sieves before use. Working standard solutions were prepared daily. The stability of *R*- and *S*-propranolol in pH 5.5 citrate and pH 7.4 phosphate buffer solutions was investigated at both room temperature (30°C) and 37°C, since the propranolol enantiomers were used under these conditions. The results showed that more than 99% content of both *R*- and *S*-propranolol was found after 7 days incubation at either temperature, indicating good stability of the enantiomers under these conditions.

2.2. Preparation of bacterial cellulose membranes

Cellulose membrane was obtained by incubating *Acetobacter xylinum* TISTR 975 in coconut juice supplemented with 4% sucrose (w/v) at pH 5.0. A stainless steel round shallow tray of 39 cm diameter was used to grow the cellulose-producing bacteria at 30 °C at the surface of culture medium under static conditions. The culture volume was 500 ml and the effective area for membrane growth was 20 cm^2. Buffered Schamm & Hestrin's medium (BSH medium) was employed as the pre-culture medium, composed of 2.0% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.033% (w/v) $Na_2HPO_4.2H_2O$ and 0.011% (w/v) citric acid.H_2O pH 5.0. *Acetobacter xylinum* TISTR 975 was grown in 50 ml of BSH medium for 3 days to use as a pre-culture. The pellicles of bacterial cellulose (0.01 mm thickness) formed on the surface of this medium surface were harvested aseptically.

Two 'thin' cellulose membranes were prepared with two different quantities of bacterial culture, comprising suspensions $1x10^8$ $cfu.ml^{-1}$ and suspensions $3x10^8$ $cfu.ml^{-1}$ while one 'thick' cellulose membrane was prepared by placing 4 pieces of cut pellicle (1x1 cm) in the tray, approximately 10 cm from the edge of the tray and an equal distance from each other. For cellulose membrane production, the pre-culture of bacteria was inoculated into 500 ml of the culture media and the tray was covered with a linen cloth, followed by incubation for 1 day. The cellulose pellicle formed on the surface of the media was removed and discarded.

The remaining culture was further incubated under the same conditions for a further day. The formed cellulose membrane was harvested and transferred to 1% (w/v) NaOH at 80°C for 24 h and then thoroughly washed with distilled water to remove any remaining associated microorganisms and proteins. The pure cellulose sheets were dried at 37° C overnight and kept in a dust free atmosphere until required for use.

2.3. Preparation of MIP composite membrane

The grafting procedure of ethylene glycol dimethacrylate and methacrylic acid copolymer onto the pore-containing surface of the bacterially-derived membrane. The grafting was carried out in the presence of the propranolol enantiomer template. A cellulose membrane was reacted with 3-MPS (10% w/w in toluene) at 80 °C for 5 h. The resulting membrane was then thoroughly washed in methanol and dried. The reacted cellulose membrane was then placed in crystallizing dish, 18 cm in diameter. A solution containing 12 mmol of MAA as a functional monomer, 0.05 mol of EDMA as a cross-linking monomer, 2 mmol of *R*- or *S*-propranolol as template and 0.7 mmol of AIBN as a radical polymerization initiator in DMF (2 ml) was poured onto the surface of cellulose membranes. The dish was then purged with nitrogen for 2-3 min to remove oxygen (which acts as a radical scavenger) before closure and the temperature maintained at 60 °C for 18 h. After polymerisation, membranes were transferred to a Soxhlet extractor and extracted with 10% (w/v) acetic acid in methanol for at least 72 h before further extraction with methanol for 72 h to remove any non-grafted polymer, monomer, residual initiator and template molecule. The prepared membranes were dried *in vacuo* overnight. The complete removal of the template molecule from the polymer was confirmed by its absence from the methanol extract of the polymer, as verified by assay (see Section 2.9) and the absence of nitrogen content in elemental analysis results obtained by using a JSM-5800 LV electron microscope (Jeol, MA, USA) equipped with an Oxford Instruments LINK-ISIS 30 x-ray detector and microanalysis system. The membrane prepared with an imprint of *R*-propranolol is referred to as R-MIP membrane and the membrane having the imprint of *S*-propranolol is referred to as S-MIP membrane. Blank composite membranes (non-imprinted polymer (NIP) cellulose membranes), for control experiments were prepared in the same manner as MIP modified cellulose membrane but with the template molecule omitted (these membranes are referred to as NIP membranes). The change in weight of the membrane induced by the grafting procedure was measured using a microbalance (Precisa 300 A, USA: capacity 305 g, readability 0.1 mg). The degree of modification (DM) was calculated from the difference in weight between the sample modified with a deposited MIP layer and the initial unmodified sample.

2.4. Characterisation methods

2.4.1. Surface morphology and cross-section study

The membrane morphology of the initial and modified cellulose was studied by scanning electron microscope observation of the cross-section and inner and outer surfaces at an accelerating voltage of 15 kV with the samples being sputter-coated with gold before imaging (Jeol serie JSM 5800LV, CA, USA). The morphology was further examined with an atomic force microscope (AFM) using a Digital Instruments NanoScope IIIa scanning probe microscope (Veeco Instruments GmbH, Germany). AFM observations were carried out at room temperature without any previous treatment, using rectangular silicon nitride cantilevers with pyramidal tips.

2.4.2. Pore size measurements

The pore size of membranes was estimated from surface pictures of the membrane obtained by SEM with the aid of a Carnoy computer imaging program (Lab of Plant Systemics, Belgium).

2.4.3. IR spectral analysis

IR spectroscopy (FT-IR 1600, Perkin-Elmer, CT, USA) was used to confirm the attachment of MAA/EDMA copolymer to the cellulose polymer. In addition, ATR-FTIR spectra of cellulose membranes and MIP-modified cellulose membranes were determined using an Equinox 55 FT-IR spectrometer (Bruker, Switzerland). One hundred scans were obtained at a resolution of $\pm$ 2 cm^{-1}. A ZnSe internal reflection element was used in the ATR at an incident angle of 45 °, giving an IR penetration depth of 0.5 - 5 μm.

2.4.4. Electrical resistance measurements

The degree of membrane fibrillation was assessed from the value obtained for the resistance of membrane. Also, impedance measurement was used to confirm the deposition of a MIP layer onto the surface of the cellulose membranes. Electrochemical resistance measurements of membranes were carried out by short-circuit current technique using a Revision G Voltage-Current Clamp, Model VCC 600 (Harvard Apparatus, CA, USA). Ussing chambers having effective area of 1 cm^2 were used. The test membrane was mounted in the measuring cell and a current established across it using a potentiostat *via* an amplifier with high-resistance inputs. The four electrode potentiostat assured a passage of current between the two calomel electrodes in such a manner as to hold constant amplitude of voltage between the two identical reversible silver-silver chloride electrodes and the intensity and phase of current in the circuit. A 60 μA current was applied and the membrane potential

difference, *PD* (mV) and the short circuit current, I_{sc} (A) were recorded simultaneously. The membrane resistance, R_m ($\Omega.cm^2$) was calculated from PD/I_{sc}, based on the Ohm's law. These were corrected by eliminating the offset voltage between the electrodes and solution resistance, which was determined prior to each experiment using identical bathing solutions. All experiments were carried out at 25±1°C.

2.4.5. Mechanical properties measurements

The mechanical strength of the membranes was measured using a Universal testing machine (Lloyd, UK) with an operating head load of 100 N. The membranes were then placed between the grips of testing machine. The grip length was 2.5 cm and the speed of testing was set at the rate of 30 mm/min. Tensile strength was calculated according to the equation: tensile strength (kN/m^2) = max load (kN)/cross sectional area (m^2).

2.4.6. Thermogravimetric analysis

Thermogravimetric analysis (TGA) of membranes was carried out using a Perkin-Elmer DTA7 analyzer (Perkin Elmer, CT, USA). A portion of membrane (0.5 mg) was heated from 50 to 800°C at 10 °C/min in nitrogen gas.

2.4.7. Degree of swelling of membranes

The degree of swelling of cellulose and modified cellulose membranes was evaluated in pH 5.5 and pH 7.4 buffers, since for some applications membranes were used in these conditions. All polymer membranes were vacuum-dried at room temperature for at least 3 days before testing. The membrane samples were weighed and then soaked in individual tubes containing phosphate buffer pH 7.4 or citrate buffer pH 5.5 at room temperature (~30°C). The membranes were incubated in the medium until the weight of wet membranes remained stable, which usually occurred after approximately 7 h incubation. Before measuring the weight of the wet membrane, surface water was gently removed with a tissue. The wet films were dried under vacuum at room temperature to a constant weight (over a minimum of 2 days) and stored in a dessiccator at room temperature before measurements. The degree of swelling of the membrane (%) was calculated from the equation: $(W_{wet}-W_{dry})/W_{dry}$ x 100% where W_{dry} and W_{wet} are the weights of dried and wet samples, respectively. Each test was carried out in sets of three.

2.5. Stability Studies

Samples of *R*- and *S*-propranolol (50 mg) were incubated separately with pH 5.5 citrate or pH 7.4 phosphate buffer solution (1 ml) at both room temperature (30°C) and 37°C

for 7 days. The same quantities of enantiomer (50 mg) were incubated also at 60°C in 1 ml of DMF (or chloroform) for 24 h. Samples were removed at the end of the incubation times and assayed for enantiomer content according to the method described in section 2.9.

2.6. Measurement of partition coefficient

The membrane-pH 5.5 or pH 7.4 buffer partition coefficient was evaluated by equilibrating racemic propranolol solutions with a membrane. The difference between the initial and equilibrium concentrations of each enantiomer in the aqueous phase was determined (Section 2.8) and hence the amount of propranolol enantiomer sorbed to the membrane was calculated. In a typical binding assay, the membrane ($1cm^2$) was added to 5 ml of an aqueous solution containing 100 $\mu g.ml^{-1}$ of racemic propranolol, and stirred overnight at room temperature for equilibrium to be established. The partition coefficient (K) was calculated from the equation: $K = C_p/C_s$, where C_p is the concentration of the analyte associated with the membrane, and C_s is the concentration of the analyte in the solution. The selectivity factor representing the effect of the imprinting process was the ratio of K of the *S*-isomer to K of the *R*-isomer.

2.7. Permeation determination

The enantioselective transport of the cellulose and modified cellulose membranes (NIP, R-, and S-MIP membranes) was evaluated by a dialysis method using a vertical Franz-type diffusion cell. A membrane (exposed area 0.78 cm^2) was mounted between the two chambers of the diffusion cells, the volumes of the donor and receptor chambers being 1.0 ml and 2.5 ml, respectively. The required amount of racemic propranolol was dissolved in either pH 5.5 or 7.4 phosphate buffer solutions (μ = 0.2) to obtain the donor solutions. Buffer (pH 7.4) was introduced into the receiver compartments of the diffusion cells. Drug release was measured by the removal of samples (250 μl) from the receiving chamber at appropriate time intervals over 6 h. The volume of the sample withdrawn was replaced by the same volume of the medium. Each test was carried out in sets of six. MIP membranes were reusable at least twice after regeneration by incubating with water to release sorbed propranolol.

The diffusion of each propranolol enantiomer was determined using the chiral-HPLC analytical method outlined in section 2.9. The cumulatively permeated amounts (μg) were calculated and plotted as a function of time. The flux J ($\mu g\ cm^{-2}\ h^{-1}$) is defined by:

$$J = Q^* A^{-1} * t^{-1}$$

where Q (μg) is the amount of analyte permeated, A (cm^2) is the effective membrane area and t (h) is the time. The selectivity of the membrane was defined as the ratio of the permeation flux of *S*-isomer to that of the *R*-isomer.

2.8. In vitro percutaneous penetration study

Propranolol was used as the hydrochloride salt. Racemic propranolol hydrochloride was dissolved in phosphate buffer saline (PBS pH 7.4) to produce a range of drug concentrations; 100, 200, 300, 400 $\mu g.ml^{-1}$.

The *in vitro* percutaneous penetration study of S-MIP membrane was performed with a Franz-type diffusion cell (see section 2.7). Adult male, Wister rats weighing 230-250 g were sacrificed by snapping the spinal cord at the neck. Rectangular sections of dorsal skin were shaved and excised from the animal using surgical scissors. Adhering fat and other visceral debris were removed from the undersurface with tweezers. The excised skin was immediately mounted between the half-cells, with and without a coupled test membrane, such that the dermal surface was in contact with the receptor fluid and the epidermal side in contact with the test membrane (if present). Drug solution (1 ml) was applied to the membrane surface and the cell was maintained at 37°C by an external circulating water-bath. The receptor phase was stirred constantly at 250 rpm with a magnetic bar. An aliquot (250 μl) of receptor fluid was collected at set time intervals over 2 days and replaced with the same volume of fresh PBS. The concentration of propranolol in the collected sample was determined by HPLC (Section 2.9).

To clean-up the receptor phase samples, liquid-liquid extraction method was employed as follows. 250 μl of receptor phase samples was added to 1 ml of 0.1 M phosphate buffer pH 4 that was saturated with NaCl. Propranolol enantiomers were extracted into 2 ml diethyl ether by shaking for 10 min, using mutually pre-saturated phases. Organic phase (2 ml) was evaporated to dryness under reduced pressure and dissolved in 125 μL of HPLC mobile phase, in readiness for assay.

The *in vitro* permeation data were obtained from the equation: $J_{ss} = K_p C_s$ where J_{ss} is steady state flux measured as the slope of the profile after regression analysis. K_p is the apparent permeability coefficient through the skin, calculated by dividing J_{ss} by the starting concentration C_s. The x-intercept of the extrapolated linear region of the curve was employed to obtain a lag time (η).

2.9. Stereospecific HPLC method

The analysis of the propranolol enantiomer content of the samples was performed directly using a chiral-HPLC method. The HPLC system (Shimadzu Corporation, Kyoto, Japan) comprised with a Shimadzu LC-10AD pump, FCL-10AL gradient valve, DGU-14A in-line solvent degasser, SCL-10A system controller, SIL-10AD auto injector (20 μl injection

loop), equipped with a Shimadzu SPD-10A UV-Vis detector set at 290 nm. Data were collected and analysed on a personal computer using Class VP software (version 4.2, Shimadzu). The column employed was a 250 mm x 4.6 mm I.D., particle size 10 μm Chiralcel OD-R column (Daicel, Chemical Industries Ltd, Japan). The mobile phase was 40:60 (v/v) 1 M sodium perchlorate:acetonitrile and a flow rate of 1.0 ml/min was employed. Typical retention times were 10.0 min for *R*-propranolol and 14.7 min for *S*-propranolol. Correlation coefficients for the calibration curves in the range 2-25 $\mu g.ml^{-1}$ for *R*- and *S*-propranolol enantiomers were greater than 0.999. The sensitivity of detection was 1.3 $\mu g.ml^{-1}$ and the reproducibility of the peak areas of both enantiomers was more than 95%.

Both the cumulative percentages of each enantiomer released and permeated were plotted as a function of time. Results of controlled membrane and skin permeation experiments were expressed as a mean ± SE (n=6). The paired two sided *t-test* was performed and a significance limit of 5% level was applied, where appropriate.

2.10. Method validation

It was necessary to confirm that the clean-up procedure used for treatment of the receptor phase samples removed any interfering residues derived from the excised rat skin. Rat skin exudate was obtained by incubating a rectangular section (2 x 2 cm^2) of shaved, excised rat skin (with fat removed) in 10 ml PBS at room temperature for 24 h. Racemic propranolol was spiked into the rat skin exudate to give a range of enantiomer concentrations: 5, 10 and 20 $\mu g.ml^{-1}$. The intra- and inter-assay variability of propranolol enantiomer assay after liquid-liquid extraction was determined (by assaying three replicates) on the same day of drug analysis and on 3 sequential days, respectively. The accuracy (%) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: %accuracy = $[(C_{obs}-C_{nom})/C_{nom}]$ x 100. The precision indicated by the relative standard deviation (RSD) value for the lowest acceptable reproducibility concentration was pre-defined as ± 30% for *R*- and *S*-propranolol enantiomers.

The intra- and inter-assay precision and accuracy of propranolol enantiomer were both well within the pre-defined limits of acceptability. The average recovery of *R*- and *S*-propranolol enantiomers was found to be 110.3±33.1% and 84.87±28.7%, respectively. The lowest acceptable reproducible concentration of *R*-propranolol and *S*-propranolol was found to be 3.8 and 2.8 $\mu g.ml^{-1}$, respectively.

2.11. Molecular modelling

The 3-D chemical structures of *S*-propranolol and *S*-prodrugs have been modelled with molecular mechanics (Chem3D Ultra 8.0, CambridgeSoft, MA, USA) followed by energy minimisation using Truncated Newton (MM2 force field).

2.12 Physico-chemical properties of the propranolol prodrugs

To study the pysico-chemical properties of propranolol prodrugs, the methods of Ahmed et.al. were modified. The physico-chemical properties in this study all included solubility, dissociation constant, partition coefficient and rate constant of hydrolysis.

-Solubility determination

An equilibrium solubility determination was undertaken in 0.01 M acetate buffer (pH 4, 37°C). The prodrug was added to 1 ml of buffer. The sample were shaken mechanically in a temperature-controlled water bath at 37°C for 24 h. In case of propranolol and SN-P, the added drugs were needed. The supernatant was then filtered and after dilution, the solubility of each drug was determined by UV-Vis.

-Dissociation constant (p*K*a) Determination

The p*K*a value of propranolol prodrugs were determined by acid-base titration method. The solution of propanolol prodrugs (5.4 mM,25 ml). The drug solutions were titrated with 1 M NaOH in 10 μl. The changes in pH were recorded and smaller volumes of the titrants (10 μl) were added. The data was plotted, the first derivative was calculated and plotted, and the equivalence point and p*K*a values were determined graphically.

-Measurement of partition coefficient

The partition coefficient of propranolol and the prodrugs were determined in 1-octanol – pH 4 phosphate buffer system. The buffer solution and 1-octanol were saturated at 25°C before use. The concentration of each drug in pH 4 buffer was measured by UV-Vis. The partition coefficients were determined as the ratio between the concentration measured in 1-octanol and that of in the buffer.

-Preparation of Skin homogenate

All operations were carried out at 0-4°C. After sacrificing the mouse by snapping the spinal cord at the neck, the skin was removed from back. The fat and the muscular tissues as well as capillaries adhering to the dermis were removed. The skin was quickly minced, mixed with five volumes of cold Tris-HCl buffer (pH 7.4) containing 0.15 M KCl, and subjected to four separate bursts of a tissue homogenizer (Ultrasonic Liquid Processing Vibra Cell, SONICS, Sonics&Materials, Inc., USA). There was a pause of 1 min between each burst to permit cooling of tissue. The whole homogenate was filtered with a funnel through cotton soaked in the buffer and centrifuged at 15000 rpm for 30 min at 0°C with HERMLE z 323k centrifuge (HERMLE LABORTECHNIK, Germany). The supernatant was stored at -80°C until used in the hydrolysis experiments.

-Preparation of Liver Homogenate

The liver was removed from the animal and thoroughly washed with 0.15 M KCl and then minced, added to three volumes of cold 0.15 M KCl, and subjected to four separate bursts of a tissue homogenizer. There was a pause of 1 min between each burst to permit cooling of tissue. The whole homogenate was filtered with a funnel through cotton soaked in the buffer and centrifuged at 15000 rpm for 30 min at 0°C. The supernatant was stored at -80°C until used in the hydrolysis experiments.

-Plasma

The blood was collected in heparinized tube and centrifuged at 3000 rpm, and the plasma was collected. Plasma was diluted to 5% with Tris-HCl buffer (pH7.4) containing 0.15 M KCl and used immediately.

-Hydrolysis of prodrugs in skin preparation.

All kinetic measurements were carried out at 37°C in a shaking thermostatic waterbath. The reaction was initiated by addition of 4 μl of stock solution of prodrug in dimethylsulfoxide (DMSO; 0.05 M) to 4 ml of the skin preparation, which has been preincubated for 10 min. At appropriate intervals, 100 μl aliquots were withdrawn and then extracted with 1 ml of diethyl ether. The organic phase was dried and after that dissolved in 125 μl of mobile phase. The residual prodrug was analysed by Chiral-HPLC. First order rate constants for the hydrolysis were determined from the slopes of linear plots of the logarithm of residual prodrug against time.

The liver hydrolysis study was performed as described for skin preparation using stock solutions that were diluted to 0.5% with Tris-HCl buffer (pH 7.4) containing 0.15 M KCl. Hydrolysis of prodrugs in plasma, the reaction was initiated as just described using 5.0% (v/v) plasma in Tris-HCl buffer (pH 7.4) containing 0.15 M KCl. Hydrolysis of prodrug in buffer. The study on the hydrolysis of the prodrugs was also performed in Tris-HCl buffer (pH 7.4) containing 0.15 M KCl according to the procedure just described for plasma.

2.13 Preparation of the gel formulations

Chitosan gel was prepared by the reported method of Thacharodi and Rao. Briefly, chitosan (2.5g) was dissolved in 250 ml of 10% (w/w) aqueous acetic acid to form a viscous solution. Ten percent (w/w) aqueous NaOH was added to this solution until a white cloudy precipitate appeared. It was incubated at 37°C overnight. The gel thus formed was washed three times with distilled water. For preparation of poloxamer gel, the dry powder of poloxamer 407 (4.5 g) was added to iced distilled water (24.5 g) in a capped test tube and the mixture was kept in the refrigerator (temp ~ 4°C) overnight.

2.14 Evaluation in stereoselective release of the gel formulations

Propranolol HCl and valeryl propranolol HCl were dispersed in chitosan gel and poloxamer gel to make gel formulation, 300 μg/ml for propranolol HCl and 400 μg/ml for valeryl propranolol HCl.

The gelling effect and release study of cellulose membrane, NIP membrane and S-MIP membrane were determined by using a vertical Franz-type diffusion cell that have the receptor volume of 25 ml and the diffusional area of 3.803 cm^2. The release experiment was carried out six for each experiment and the average release was calculated for each release point. A membrane was mounted between the two chambers of the diffusion cells. The receptor chamber was filled with phosphate buffer solution pH 7.4 (PBS) and the solution was stirred at 500 rpm with magnetic bar. The drug formulation (0.5 ml) was placed in the donor compartment. The aliquots (250 μl) of sample were withdrawn from the receiver periodically up to 72 h. The equal volume of fresh PBS was replaced to maintained the sink condition of the receiver. The samples were analyzed for propranolol enantiomer and valeryl propranolol enantiomer by means of chiral-HPLC analytical method. The diffusion parameters were determined.

2.15 Evaluation in stereoselective release of the gel formulations across rat skins

Adult male Wistar rat were sacrificed by snapping the spinal cord at the neck. The hair of the back area of the rat was gently removed by shaving and the full thickness skin was carefully excised by using surgical scissors. Adhering subcutaneous tissues and blood vessels were removed. The prepared skin was then washed with saline and used within 1 week.

In vitro percutaneous penetration of S-MIP membrane was studied with a Franz-type diffusion cell. Each study was carried out four and the average release was calculated for each release point. The skin samples were mounted between the half-cells, with and without a coupled test membrane (cellulose membrane, NIP membrane and S-MIP membrane). Propranolol gel or valeryl propranolol gel (300 and 400 μg/ml, respectively, 0.5 ml) was applied to the membrane surface and the receptor solution was maintained at 37±0.5 °C by surrounding water jacket and stirred constantly at 500 rpm with a magnetic bar. The aliquots (250 μl) of receptor fluid were collected at appropriate intervals over 48 h and replaced with the same volume of fresh PBS.

Liquid-liquid extraction was used to clean up the sample. To 250 μl sample, 50 ml of L-phenylephrine was added as internal standard and then extracted with 1 ml diethyl ether by shaking for 10 min. Organic phased was evaporated to dryness under reduced pressure and dissolved in 125 μl of mobile phase. The samples were analyzed for propranolol enantiomer and valeryl propranolol enantiomer by means of chiral-HPLC analytical method.

2.16 Preparation of the selective patch

The simple transdermal patches were prepared for evaluating the efficacy of the S MIP membrane *in vivo* study. All of special membranes were the gifts from 3M Company (USA and Thailand). Foam tape (3M™ Foam Tapes 9773) was cut to make an effective area for 16 cm^2 and the S-MIP membrane was placed between the foam tape and the release liner (1022 Scotchpak™ Liner). The racemic propranolol in chitosan gel (1.5 mg/ml, 0.5 ml) was uniformly spread over the effective area. The backing membrane (3M™ Scotchpak™ 9723) was stuck with adhesive onto the foam tape. The devices were kept in dry and cool place.

2.17 In vivo study

The hair on the dorsal side of the male Wistar rats (230-250g) was removed by gentle shaving. The animals were anaesthetized with urethane 30% (0.9g/kg, i.p.).In this study, the effective of the transdermal patch (S MIP membrane) were evaluated compared with the control (racemic propranolol gel and *S*-propranolol gel. The patches or the gel were then applied on hair free dorsal skin. Blood samples (250 μl) were collected at 0, 3,6,9,12,18 and 24 h from femoral vein. The plasma was separated immediately and frozen at -20^{0}C until analysis.

To clean up the sample 50 ml of L-phenylephrine , as internal standard was added to 200 μl plasma and then extracted with 1 ml diethyl ether by shaking for 10 min. Organic phased was evaporated to dryness under reduced pressure and dissolved in 125 μl of mobile phase. Propranolol enantiomers were estimated using chiral-HPLC equipped with fluorescence detector with 290 and 340 nm as excitation and emission wavelength, respectively. The lower detection limit was 5 ng/ml. Correlation coefficient of *R*- and *S*-propranolol were greater than 0.995.

2.18 Skin irritation study

Skin irritation studies were carried out to investigate the potential of the transdermal patches to cause irritation in the rat skin compared with that of the control (racemic propranolol gel and *S*-propranolol gel). After 24 h, patch or the gel residual was removed and skin erythema and skin edema were observed.

3. Results and discussion

3.1 Preparation of composite MIP membranes

Composite MIP membranes were prepared by reactive filling of the pores of the bacterial cellulose membranes with MIPs having recognition sites for the *S*-propranolol enantiomer. In order to achieve an additional anchor for the MIP, asymmetric porous cellulose membrane was treated with 3-MPS. Thin-layer MIPs were synthesised by a free radical copolymerization of MAA functional monomer with EDMA as a cross-linker in the presence of propranolol enantiomer (as a template molecule) in DMF, and this was followed by subsequent template molecule extraction. MAA was chosen as the functional monomer, because of the facility of the carboxylic group to interact with the hydroxyl group on the chiral carbon of the enantiomer template. The cross-linker, EDMA was selected due to the anticipated rigidity and compactness it might confer to the imprinted polymer. The absence of nitrogen content in the elemental analysis results confirmed that the template molecules had been removed from the MIP membrane by the washing procedure employed.

The porous composite cellulose membranes, evenly functionalised with thin MIP layers would be expected to act as high performance enantioselective-membrane adsorbers. Porous asymmetric cellulose membranes were obtained as a product of *Acetobacter xylinum* fermentation. The properties of prepared cellulose membrane such as thickness, degree of fibrillation, porosity and pore size can be adjusted by controlling the loading concentration of bacteria during culture as well as the time of cultivation. Three bacterial cellulose membranes having different thickness and electrical membrane resistance were used in this work for screening experiments to optimise the MIP affinity and distribution of the MIP within the membrane. The three lots of cellulose membrane displayed variation in membrane thickness and resistance respectively as follows: (1) 5 μm/1 $\Omega.cm^2$; (2) 10 μm/ 2 $\Omega.cm^2$; (3) 15 μm/4 $\Omega.cm^2$. Cellulose membranes were imprinted with *S*-propranolol enantiomer during pore-filling and their stereoselective sorption and transport properties determined. The same polymer mixtures were used in the pore-filling process of all three lots of cellulose membranes.

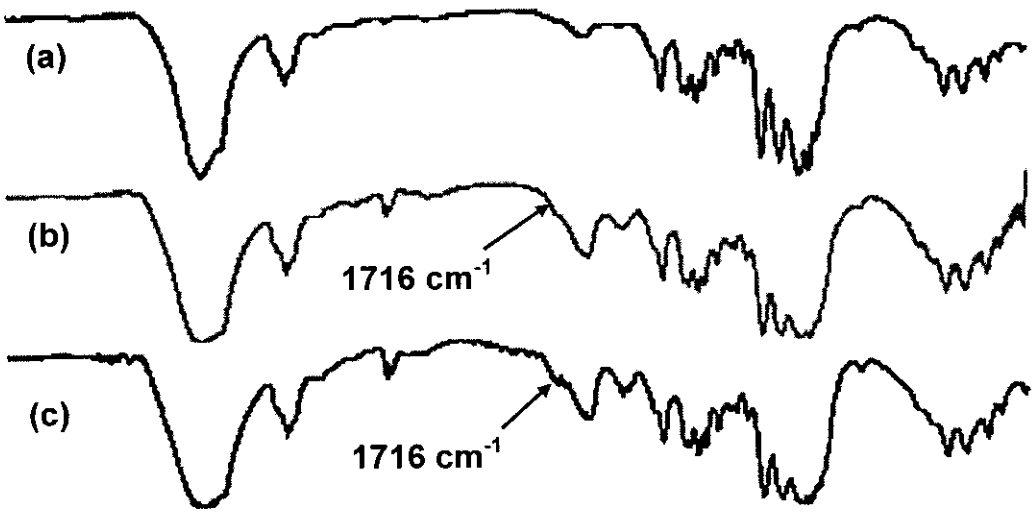


Figure 1: FT-IR spectra of (a) 5 μm cellulose membrane (b) 5 μm S-MIP membrane and (c) 10 μm S-MIP membrane. The peak at 1716 cm^{-1} correspond to the interaction of C=O groups in the grafted polymers.

An FT-IR spectroscopic method was employed to confirm that the deposition of new MIP layer had taken place on the smaller thickness cellulose membranes (5 μm and 10 μm membranes). IR spectra of both the parent cellulose and the MAA-EDMA copolymerized cellulose membranes are shown in Figure 1. In comparing these spectra, an absorption peak at 1716 cm^{-1}, was apparent in the MAA/EDMA grafted polymers which was assigned to the stretching of the introduced C=O of acid groups. FT-IR with ATR option mode was employed to determine the IR spectra of the thickest membrane (15 μm membrane) and the resultant spectra of the MAA-EDMA co-polymerized cellulose membranes, indicated the presence of carbonyl groups. The peak at 1720 cm^{-1} was not found in the IR spectrum of the parent cellulose membrane (see Figure 2).

The membrane thickness and pore diameter of cellulose membrane appeared to have no significant effect on degree of modification (DM). The DMs of the three modified membranes were the similar with an average DM of 0.80 mg/cm^2. The variation in the DM of the individual membranes was found to be less than 10%. The increase of membrane resistance (R_m) after the modification was found to be 0.7 $\Omega.cm^2$ for the 5 μm membrane, 1.3 $\Omega.cm^2$ for the 10 μm membrane and 2.2 $\Omega.cm^2$ for the 15 μm membrane. The tensile strengths of the cellulose membranes were found to increase when each was modified with MIP to form composite membranes.

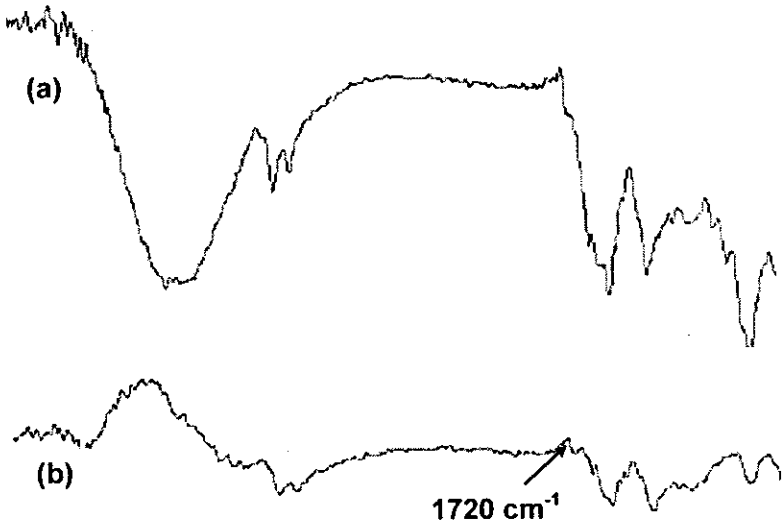


Figure 2: ATR-FTIR spectra of (a) 15 μm cellulose membrane and (b) 15 μm S-MIP membrane. The peak at 1720 cm^{-1} correspond to the interaction of C=O groups in the grafted polymers.

An increase from 3.16 kN/m^2 to 7.20 kN/m^2 occurred for the 5 µm membranes, from 6.60 kN/m^2 to 8.78 kN/m^2 for the 10 μm membranes and from 8.83 kN/m^2 to 21.45 kN/m^2 for the 15 μm membrane. SEM cross-sectional images of initial and modified cellulose membranes show the different membrane thickness (Figure 3). The cross-sectional view shows an array of closely packed MIP domains in the cellulose membranes. The thickness of the deposited MIP layer tends to increase as the thickness of cellulose membrane increases. However the relatively small thickness of the MIP layer would be expected to allow high fluxes through MIP composite cellulose membrane. The cellulose membranes varied in the degree of porosity and fibrillation (Figure 4). Fibers were still apparent after modification of the thinnest membrane (5 μm) with MIP but when the same amount of MIP was applied to the 15 μm membrane, it did not penetrate as effectively into the tighter meshed polymer chains, presenting a smoother, but thicker layer with a fibrous surface. The SEM micrographs would suggest that a greater amount of MIP is coupled to the outer surface of the 15 μm membrane compared to the 5 μm membrane.

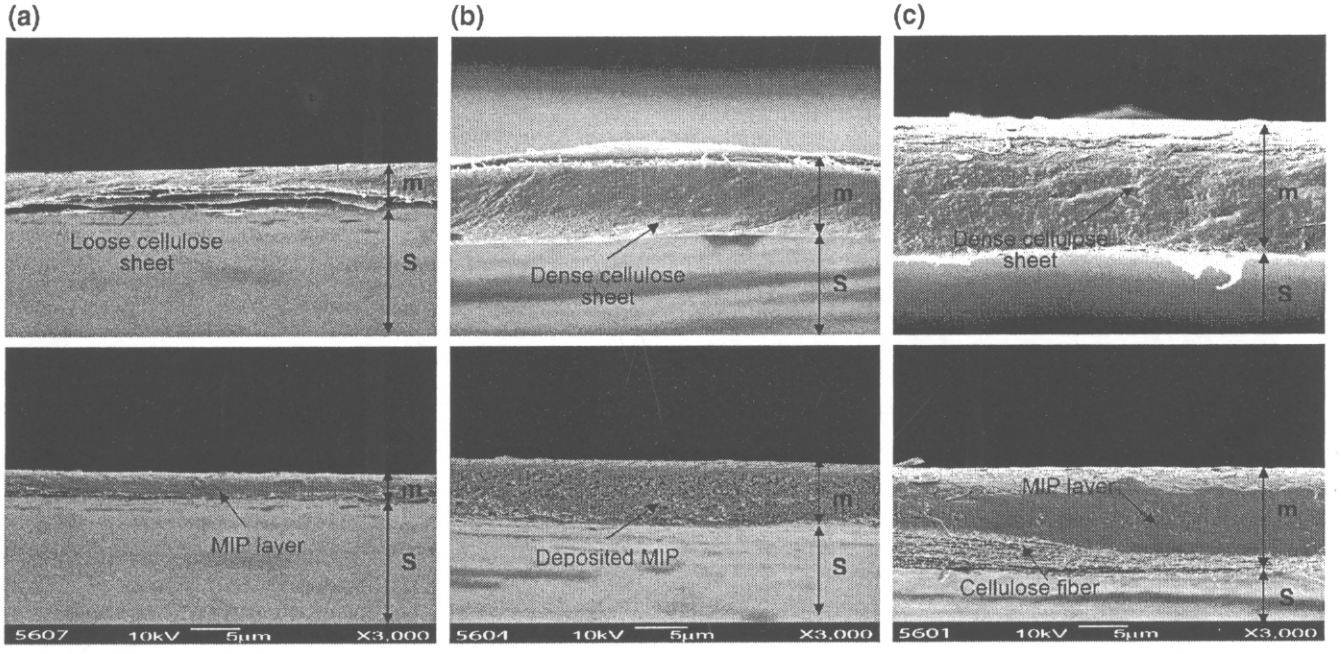


Figure 3: SEM-cross-section images of the initial cellulose membranes (top) and the MIP modified membranes (bottom), showing the different membrane thicknesses with differed membrane resistances. (a) 5 μm/1 $\Omega.cm^2$ membrane (b) 10 μm/2 $\Omega.cm^2$ membrane and (c) 15 μm/4 $\Omega.cm^2$ membrane. Picture designation: s = stub, m = membrane.

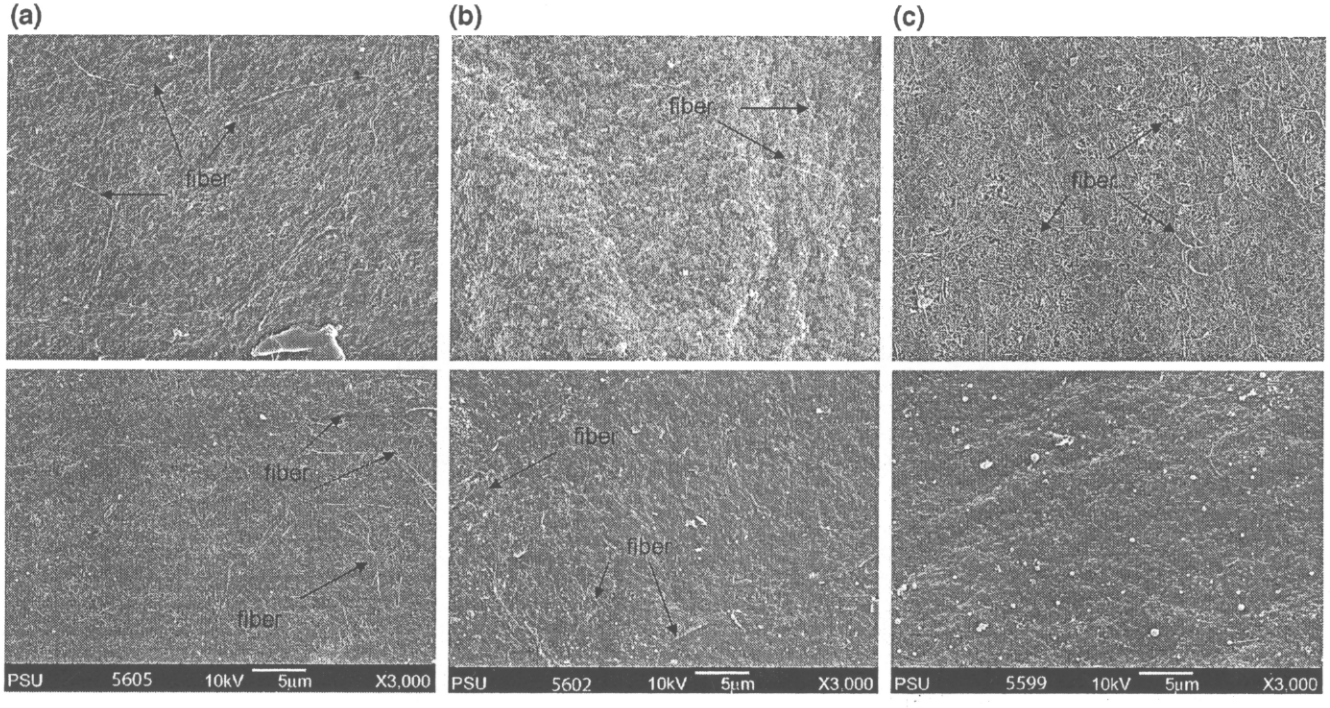


Figure 4: Surface morphology of the initial cellulose membranes (top) and the MIP modified membranes (bottom). (a) 5 μm/1 $\Omega.cm^2$ membrane (b) 10 μm/2 $\Omega.cm^2$ membrane and (c) 15 μm/4 $\Omega.cm^2$ membrane.

The AFM images of the membrane (Figure 5) show the vertical profile of the sample with the light regions representing the highest points and the darkest regions showing the pores. The pore size obtained from the AFM images of the MIP membrane indicated that this was smaller than that of the parent membrane. Also, the AFM images of the MIP membrane indicate that derivatisation induces an increase in the surface roughness and hence the total contact surface area of the membrane. The increase in the surface roughness and contact surface area can contribute to the rise of propranolol sorption.

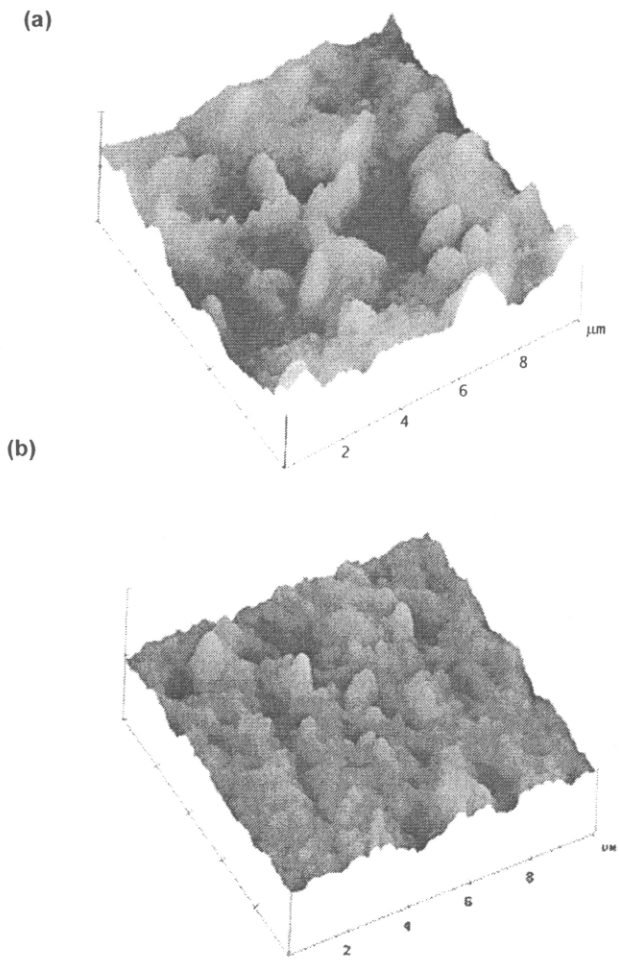


Figure 5: Three-dimensional AFM image of (a) 5 μm cellulose membrane and (b) 5 μm S-MIP membrane.

Table 1
Partition (K) and diffusion coefficients (D, $cm.h^{-1}$) of propranolol into/through MIP modified cellulose membranes of different thicknesses and membrane resistances at room temperature (mean ± SE, n=3).

Solute*	5 μm/1 Ω.cm² Membrane		10 μm/2 Ω.cm² Membrane		15 μm/4 Ω.cm² Membrane	
	$K \times 10^3$	D (x 10^{-6} $cm.h^{-1}$)	$K \times 10^3$	D (x 10^{-6} $cm.h^{-1}$)	$K \times 10^3$	D (x 10^{-6} $cm.h^{-1}$)
R	1.01±0.22	0.70±0.26	1.59±0.18	3.46±0.41	0.37±0.20	6.11±1.23
S	1.79±0.22	30.84±2.51	1.56±0.38	8.10±1.82	0.29±0.16	6.62±0.73
S/R	1.77±0.58	44.06±2.96	0.98±0.25	2.34±0.42	0.78±0.12	1.08±0.09

* Refer to *R*- or *S*-isomer of propranolol.

Table 1 shows that the partitioning of the propranolol enantiomers between S-MIP membranes and pH 7.4 buffer decreased with increase in the membrane thickness. The differences in the partition coefficients of *S*-enantiomer and *R*-enantiomer were greatest when the thickness of the composite membrane was smallest. Differential transport of the template enantiomer significantly increased with a decrease in the thickness of the membrane. The increase in the thickness of membrane resulted in a reduction in the specificity of S-MIP membrane. This phenomenon may be attributed to the effects of a gradient in DMF concentration being established in the monomer mixture near the surface of the membrane during the imprinting process. Such a gradient would be expected to lead to an alteration in the density of the created polymer network dependent upon depth of the grafted MIP layer. Supporting evidence for this can be seen from the SEM images (Figure 3) with much of the MIP not fully penetrating into the membrane pores of the thickest (15 μm) membrane but accumulating at the surface. Since the 5 μm MIP membrane enabled the highest diffusion rates of the enantiomers to be obtained with the greatest imprinting effect, this membrane was selected for further study as a basic membrane for the development of a composite MIP membrane, which might be applied to transdermal delivery.

It was necessary to confirm that the basic membrane prepared with the method described in Section 2.2 each time provided similar appearances and release properties. Therefore the thinnest cellulose membranes were prepared on a number of occasions and their morphology examined and the mechanical strength determined. The percentage coefficient of variation (CV) for the thickness of the non-modified cellulose membranes was found to be 5% (n = 6). Membrane porosity was found to vary from 1.4% to 2.4%, with the average pore size 170 nm. The %CV of the partition coefficients and enantioselectivity of the modified cellulose membranes obtained for three different batches of the initial membrane were found to be 5.0% and 10.0%, respectively. The mechanical stability of the

cellulose membranes was not damaged during grafting process. The stability of cellulose membrane after modification was good, since the membrane could be reused after washing and similar release characteristics and selectivity were obtained.

3.2. Characterisation of composite MIP membranes

The general properties of 5 μm S-MIP membranes were compared with those of NIP membrane and R-MIP membranes, which were all produced from the same basic cellulose membrane (Table 2). The values obtained for the electrical resistance of membranes provided an indication of the leakage of membrane and it was found that the electrical resistance of the cellulose membrane increased upon modification. This was presumably as a consequence of the potential occlusion of the pores due to modification with the copolymer. An increase in the tensile strength of the membranes after modification was also found, possibly as a consequence of the introduction of a rigid copolymer of MAA/EDMA within the pores of cellulose membrane. The average pore diameters obtained by SEM, however, proved to be very similar, within the range 150-200 nm. The results presented in Table 2 indicated that cellulose membranes swelled greatly in aqueous solvent confirming the hydrophilic and highly porous nature, which promotes water absorption capacity. Swelling of the non-modified cellulose membranes in citrate buffer (pH 5.5) is clearly inherently greater than when they are placed in phosphate buffer (pH 7.4). When EDMA-MAA based polymers are bound at the pore surface, this causes a decrease in the degree of swelling. It might also have been expected that the introduction of ionizable carboxylic acid groups would have resulted in an increased swelling of the composite membrane at the higher pH. However the (modified) cellulose membrane still shows a greater swelling at pH 5.5 than at pH 7.4. This suggesting that the pH-dependency in the swelling of the MIP composite cellulose membranes is governed predominantly by the cellulose membrane.

Table 2
Characteristics of cellulose and modified cellulose membranes (5 μm thickness).

Membrane	Membrane resistance ($\Omega.cm^2$)	Tensile strength (kN/m^2)	Pore size (nm)*	Degree of swelling (%)**	
				pH 5.5	pH 7.4
Cellulose	1.11	3.16	177.9±35.4	101.25±9.21	55.57±5.72
NIP	1.71	8.23	146.3±36.5	74.30±9.94	34.01±3.30
R-MIP	1.71	7.20	176.9±47.8	77.61±8.62	33.41±3.82
S-MIP	1.71	7.20	198.3±4.2	72.36±3.81	44.44±9.84

* Refer to mean pore size estimated from surface pictures of membrane obtained by SEM (± SE, n=10).
** mean ± SE, n=3.

3.3. The stability issues of propranolol enantiomers

The stability of *R*- and *S*-propranolol in pH 5.5 citrate and pH 7.4 phosphate buffer solutions was investigated at room temperature (30°C), 37°C and in DMF solvent at 60°C since the propranolol enantiomers were used under these conditions. The results showed that more than 99% content of both *R*- and *S*-propranolol was found after 7 days incubation in buffer solution at room temperature and 37°C, indicating good stability of the enantiomers under these conditions. At 60°C, where the polymerization procedure of the imprinting was effected, it was found that 14.7 ± 3.0% (n = 3) racemised when the pure *R*-enantiomer was incubated alone in DMF for 24 h. In contrast, under the same conditions only 1.5 ± 0.3% (n = 3) of the pure *S*-isomer was found to racemise. However when the individual *R*- and *S*-propranolol enantiomer was incubated in chloroform at temperature 60°C, both remained stable with no racemisation of enantiomer after 24 h.

This indicates that the type of solvent in which the enantiomers are dissolved affects the stability of the propranolol enantiomers. Therefore it is possible that if racemisation occurs during imprinting then the selectivity of the final MIP might be decreased. However the total recovery of enantiomer from the template was found to be 98% and less than 1% racemisation had been found to occur of either individual enantiomer. The stability of the enantiomer in DMF could be improved by the presence of the polymer mixture since the template molecule might be expected to preferentially interact with the MAA functional monomer rather than the solvent. As a consequence it could position the pendent optically active centre inside the forming polymer network, thereby limiting any racemisation which might otherwise have occurred during the polymerization.

3.4. Enantiomer uptake and imprinting effect

The partition coefficient of propranolol enantiomers from aqueous solutions into S-MIP membranes was compared with that for cellulose membrane, NIP membrane and R-MIP membrane in order to determine the effects of the imprinting procedure (Table 3). In pH 5.5 buffer, the partition coefficients of both *R*- and *S*-isomers decreased with the modification of cellulose membrane, while in pH 7.4 buffer the partition coefficients of propranolol enantiomers were generally increased by the modification of the cellulose membrane. The apparent preferential sorption of *S*-propranolol to the non-modified cellulose membrane (Table 3) confirms the potential role of the parent cellulose membrane in the selective sorption of the propranolol enantiomers, even after the original membranes are transformed to MIP composite cellulose membranes. Thus the R-MIP membrane still demonstrated selectivity for the *S*-isomer, rather than the *R*-isomer, due to the properties of the cellulose membrane itself.

Table 3
Partition coefficient ($K \times 10^3$) of *R*- and *S*-propranolol enantiomers from different donor pH solutions into cellulose and modified cellulose membranes (5 μm thickness), at room temperature (mean ± SE, n=3).

Membrane	pH 5.5			pH 7.4		
	***R*-isomer**	***S*-isomer**	**Ratio *S/R***	***R*-isomer**	***S*-isomer**	**Ratio *S/R***
Cellulose	1.25±0.36	1.38±0.33	1.10±0.14	1.26±0.62	1.53±0.62	1.21±0.01
NIP	0.78±0.28	0.93±0.25	1.19±0.20	1.83±0.95	2.32±0.95	1.27±0.05
R-MIP	1.14±0.25	1.29±0.21	1.14±0.13	1.35±0.34	1.57±0.34	1.16±0.11
S-MIP	0.70±0.20	0.94±0.22	1.35±0.08	1.01±0.22	1.79±0.22	1.74±0.38

Nevertheless the imprinting effect i.e. the difference between the S/R selectivity of the S-MIP membrane and that of the cellulose and NIP membrane was marked, suggesting that the molecular imprinting procedures have produced cavities with a higher affinity for the *S*-enantiomer of propranolol. At the higher pH the enantioselectivity of the membranes as determined by an increased partition coefficient for the *S*-isomer was not significant in the case of the non-modified cellulose membrane but it was for the S-MIP membrane.

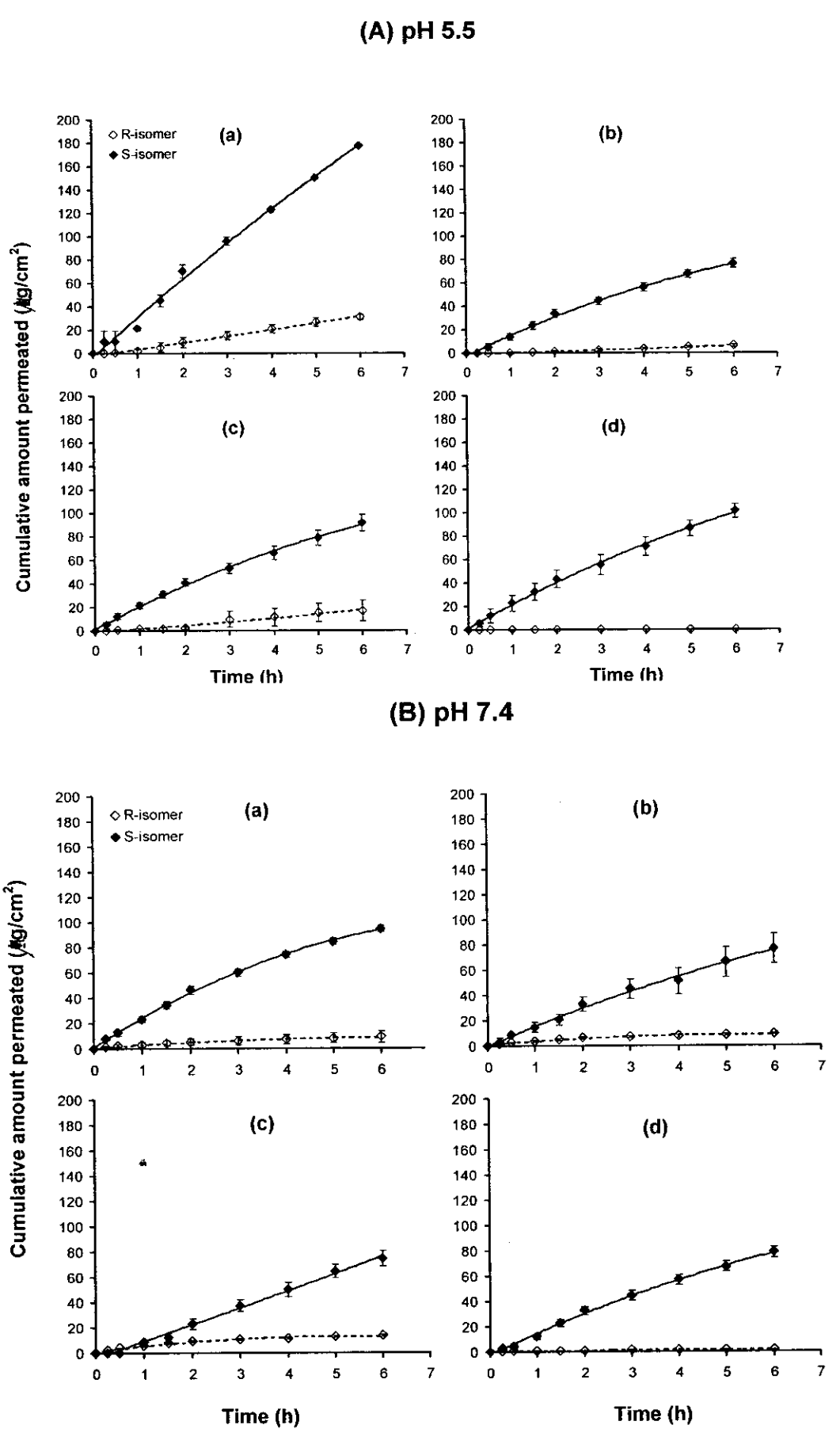


Figure 6: The transport of *R*-and *S*-propranolol enantiomers (HCl) as a function of time (mean ± SE, n=3) from (A) pH 5.5 citrate buffer and (B) pH 7.4 phosphate buffer across (a) cellulose membrane, (b) NIP membrane, (c) R-MIP membrane and (d) S-MIP membrane.

The enhanced enantioselectivity at a higher pH must be due to an increased binding of the favoured *S*-propranolol enantiomer at the binding site. This is most likely as a consequence of the higher degree of ionization of the functional monomer residues (pK_a of MAA < 4) at the higher pH, resulting in an increased electrostatic interaction with the secondary amine of the propranolol molecule [19].

3.5. Selective transport of composite MIP membranes

Preliminary studies using an initial concentration of 40 $\mu g.ml^{-1}$ racemic propranolol established that a steady state flux was achieved during a period of 2-6 h (Figure 6). *R*- and *S*-propranolol enantiomers were transported faster across the unmodified cellulose membranes than those that had been modified, indicating the pore-filling effect of modified cellulose membranes. The faster transport of the *S*-enantiomer in comparison to the *R*-enantiomer confirms the inherent enantioselectivity of the modified cellulose membrane, indicated by the partition data. The S-MIP membrane was found to limit the permeation of *R*-enantiomer, and none was detected in the receptor compartment for at least 6 h, indicating the effects of the imprinting process on the pore structure. However the transport of the *S*-enantiomer through the R-MIP membrane was still faster than that of the *R*-enantiomer. This suggests that the inherent enantioselectivity of the original membrane still dominates the process, despite the modification of some of the pores with polymer synthesised in the presence of the *R*-enantiomer. The *R*-isomer was transported faster through the *R*-MIP membrane than through either the NIP or S-MIP membranes, as a consequence of the *R*-selectivity conferred on some of the membrane pores. These results suggest that the enantiomers are transported *via* a number of routes across the composite membranes. The cellulose fibers and/or the unmodified pores appear to have inherent selectivity for *S*-enantiomer whereas the modified pores will display selectivity for the template enantiomer. The pore filling effects of the polymer does appear to reduce the transport of the *S*-enantiomer across the S-MIP membrane in comparison to the unmodified membrane. However the advantage of employing the S-MIP as opposed to the unmodified membrane is that transport of the *R*-enantiomer over the 6 h of the study was eliminated in the former.

The *S*-isomer/*R*-isomer selectivity of the propranolol enantiomers across the S-MIP membranes from pH 7.4 buffer was ca. 45, while the *S*-isomer/*R*-isomer ratio obtained with cellulose, NIP or R-MIP membranes were below 5. For a donor solution with pH 5.5, the *S*-isomer/*R*-isomer selectivity of S-MIP membrane was ca. 80 (the amount of transported of *R*-isomer was taken assuming the limit of detection was reached after 6 h) whereas the *S*-isomer/*R*-isomer selectivity of cellulose, NIP and R-MIP membranes was less than 20.

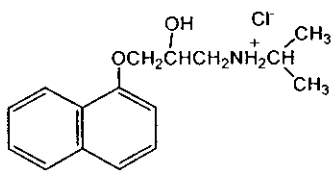
It should also be noted that the faster drug transport rate at the lower pH correlates with the higher degree of swelling of the membrane (Table 2). Such a swelling at pH 5.5 might alter the net pore size of cellulose membrane and partially account for the increased transport of the enantiomers. It is apparent also that the transport of *S*-propranolol and the enantioselectivity was higher for the S-MIP membrane at pH 5.5 as compared to pH 7.4. Two potentially opposing mechanisms are likely to affect the fluxes of the enantiomers at the two different pH values employed in this study. At the acidic pH, the increased swelling of the basic cellulose matrix would be expected to promote the overall transport of propranolol. However when the pH is raised to 7.4 then the improved MIP recognition, and increased electrostatic binding of the *S*-enantiomers to the S-MIP membrane might reduce the overall transport rate, as a consequence of binding and re-binding at sites within the MIP modified pores. Whilst, at pH 5.5 the release of the *S*-propranolol enantiomer absorbed on the cellulose membrane or the MIP/NIP modified cellulose membranes might be expected to increase with swelling of membrane, which leads to change in the mass transfer rate of the enantiomer and the improved efficiency in enantioselectivity at the lower pH. It is apparent that cellulose membranes provided a degree of enantioselective transport for propranolol, but that this is lower than that achieved using the S-MIP membrane. The preferential sorption of *S*-propranolol to cellulose enhances the transport selectivity of S-MIP composite membranes and limits the involvement of the *R*-enantiomers in such a diffusive pathway. Therefore, although it would appear that the selective transport of *S*-propranolol obtained using a composite cellulose membrane is primarily determined by the parent cellulose membrane, a beneficial contribution is derived from the MIP component.

The selective release characteristics of MIP grafted onto the pore surface of the cellulose membrane concurs with the 'fixed-carrier' mechanism that has been proposed for the selective transport achieved *via* ion-exchange carrier membranes. The facilitated transport conferred by a 'fixed-carrier' site membrane involves adsorption and mobility of the target molecule at the 'fixed-carried' site. Previous work has shown that MIPs formed as a membrane [20-22] or prepared into a matrix tablet [23-24] can provide a facilitated and selective transport of the template molecule. The means of controlled release involves reversible complexation and exchange between the template molecule and sites in the MIP membrane (or the MIP tablet). The *S*-isomer of propranolol binds selectively to the MIP binding sites, with its subsequent reactive diffusion taking place by stepwise dissociation and selective binding to neighboring MIP sites. From the results obtained, it shows that the binding of *S*-propranolol enantiomer to MIP is reversible and fast enough to enable the transport of *S*-enantiomer. The *S*-propranolol complexing to and dissociating from the MIP plays an important role in the enantioselectivity of *S*-MIP membrane. Any factor that alters in rate of complexation/decomplexation of *S*-propranolol enantiomer with MIP will change the

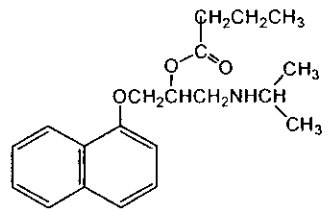
flux and stereoselectivity of the S-MIP composite membrane. In addition as indicated previously, the the basic cellulose membrane in the S-MIP membrane plays a crucial role towards the transport of propranolol enantiomers. Macroscopic changes such as degree of hydration and pH of the medium have a marked effect on transport rate through changes in effective pore size and alteration of enantiomer-membrane partition (Table 3). Indeed any change in the latter will have a direct effect on transport role, since Fick's first law indicates that the rate is dependent upon partition coefficient.

3.6. Selectivity of composite MIP membrane for propranolol prodrugs

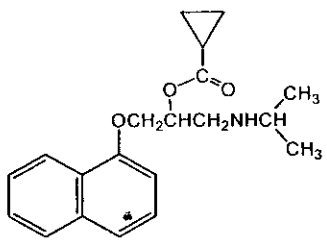
The propranolol prodrugs employed in this study were valeryl propranolol (VL-P) cyclopropanoyl propranolol (CP-P) and succinyl propranolol (SN-P) (see chemical structures below), which were synthesized from racemic propranolol hydrochloride and fatty acid chlorides by substituting different alkyl groups onto the hydroxyl group, according to the method described previously [25]. Solubility, log*P* and pK_a of propranolol and the prodrugs are shown in Table 4.1. In addition, dissociation rate constant data of propranolol prodrugs are presented in Table 4.2



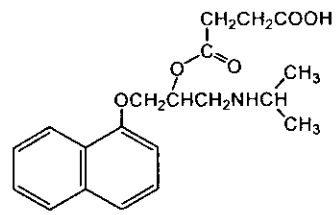
Propranolol HCl (clog*P* = 2.7)



Valeryl propranolol (VL-P) (clog*P* = 5.2)



Cyclopropanoyl propranolol (CP-P) (clog*P* = 3.9)



Succinyl propranolol (SN-P) (clog*P* = 1.1)

The partitioning of racemic prodrugs from saturated solutions was determined at both pH 5.5 and pH 7.4 (Table 4). Generally the *S*-enantiomers were sorbed to both the non-modified and modified cellulose membrane in greater quantities than the *R*-isomer at both pH values. The *S*-enantioselectivity of the unmodified cellulose membrane was apparent for all prodrugs, with the exception of SN-P at the higher pH. As in the case of the parent propranolol molecule, the S-MIP modification of the membrane improved the *S*-enantioselectivity further. Again the enantioselectivity proved to be generally higher at pH 7.4.

Table 4.1: Solubility, Partition coefficient (PC) and pKa of propranolol and its prodrugs

Compound	Solubility (mg/ml)	Log *P*	pK_a
Propranolol	115.9	2.4	9.5
SN-P	598.5	0.68	4.8
VL-P	5.15	89	8.6
CP-P	8.35	14.4	8.7

Table 4.2: Dissociation rate constant of propranolol prodrugs

compound		Rate constant x 10^3							
		Buffer pH 7.4 (min^{-1})		Plasma($min^{-1}.mg^{-1}$)		Liver Homogenate($min^{-1}.mg^{-1}$)		Skin Homogenate($min^{-1}.mg^{-1}$)	
SN-P	(*R*)	6.55±0.95	1.01±0.05	807.59	0.32±0.13	1970.08	0.92±0.07	98.01±16.85	0.68±0.18
	(*S*)	6.55±1.10		2188.32		2103.86		147.02±6.32	
VL-P	(*R*)	5.55±1.05	0.95±0.10	2344.63	1.48±0.69	1495.80	0.89±0.02	149.60±64.43	0.77±0.31
	(*S*)	6.05±1.75		2422.78		1690.38		189.15±60.48	
CP-P	(*R*)	2.65±0.95	1.11±0.05	963.90	1.38±0.38	462.12	1.04±0.27	89.42+28.93	0.38±0.06
	(*S*)	2.35±0.75		703.39		498.60		252.77±80.97	

Table 5
Partition (*K*) and diffusion (*D*) coefficients of propranolol prodrugs into and through non-modified cellulose and modified cellulose membranes at different pH values at room temperature (mean ± SE, n=3).

Compound	Membrane	*K* (x 10^3)						*D* (x 10^{-6} cm.h^{-1})					
		pH 5.5			pH 7.4			pH 5.5			pH 7.4		
		R	*S*	*S/R*	*R*	*S*	*S/R*	*R*	*S*	*S/R*	*R*	*S*	*S/R*
VL-P	Cellulose	1.11±0.51	1.38±0.93	1.20±0.39	1.26±0.14	1.63±0.68	1.43±0.69	62.3±4.52	77.9±3.84	1.26±0.07	64.3±9.74	70.7±13.7	1.08±0.06
	NIP	1.88±0.30	2.10±0.45	1.09±0.07	1.26±0.38	1.67±0.73	1.32+0.12	77.8±4.94	86.8±5.57	1.12±0.09	69.8±5.03	64.4±7.48	0.94±0.15
	S-MIP	0.74±0.21	1.38±0.22	1.71±0.17	0.74±0.29	1.53±0.19	2.11±0.17	57.6±2.57	95.9±10.5	1.68±0.22	32.8±8.61	66.7±5.81	2.33±0.56
SN-P	Cellulose	1.30±0.38	1.82±0.53	1.42±0.10	1.03±0.25	0.94±0.30	0.91±0.11	38.8±8.00	51.3±8.53	1.32±0.93	28.7±4.77	34.1±9.48	1.25±0.38
	NIP	1.61±0.12	1.94±0.38	1.16±0.16	0.26±0.17	0.22±0.18	0.85±0.24	29.3+6.91	42.9±11.5	1.46±0.31	23.4±2.61	26.6±3.60	1.13±0.04
	S-MIP	0.62±0.17	0.74±0.09	1.36±0.17	0.44±0.23	0.60±0.33	1.31±0.11	32.8±3.51	50.5±7.01	1.57+0.24	27.1±4.35	29.5±5.99	1.23±0.37
CP-P	Cellulose	0.68±0.12	0.82±0.21	1.20±0.52	0.83±0.02	1.44±0.11	1.73±0.17	69.8±7.48	73.2±9.64	1.08±0.15	38.2±0.23	44.5±7.82	1.17±0.32
	NIP	1.28±0.23	1.44±0.34	1.12±0.13	0.85±0.07	0.82±0.34	0.98±0.30	52.8±2.64	64.0±10.4	1.21±0.17	49.3±1.53	46.3±12.2	0.93±0.23
	S-MIP	1.02±0.53	1.58±0.23	1.55±0.59	1.59±0.04	2.62±0.20	1.65±0.09	59.5±3.79	68.2±7.58	1.18±0.22	49.4±6.90	61.5±18.1	1.22±0.30

There are many factors affect to sorption of propranolol prodrugs on the membranes. Since the ester modification of hydroxyl group increases the hydrophobicity of the propranolol molecule, i.e., in case of VL-P and CP-P, interaction between propranolol enantiomers and cellulose might be expected to be reduced. The introduction of different bulkier side chains would be expected to reduce the interaction with any recognition site and the diffusion coefficients of VL-P and CP-P *S*-isomers through the S-MIP membrane (Table 5) were markedly higher than that of *S*-propranolol HCl (Table 1). However the diffusion of the *S*-isomer of the most hydrophilic prodrug, SN-P was comparable to that of the parent compound. Since enantioselectivity is preserved when the prodrugs are employed as the diffusing species, this demonstrates that the hydroxyl group, present in propranolol is not crucial. It is apparent that it is the position of the charged nitrogen group within the partitioning and diffusing species, relative to the carboxylic acid residues of MAA that is the dominant mechanism involved in the interaction between the enantiomers and substrate.

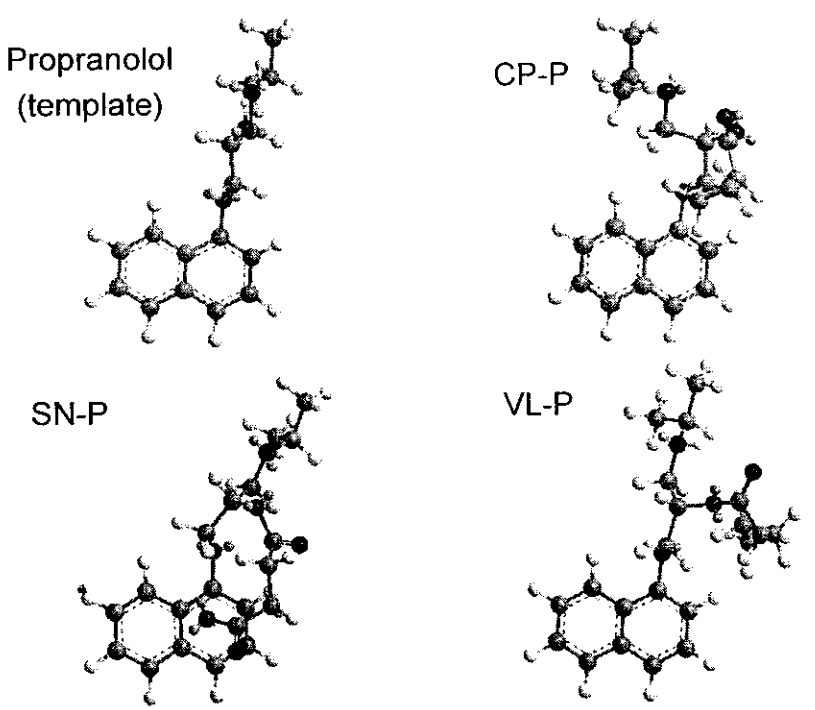


Figure 7: The energy-minimised Chem3D structures of *S*-propranolol and *S*-propranolol prodrugs as calculated by molecular mechanics using MM2 force field (Chem3D Ultra 8.0, CambridgeSoft, MA, USA).

A preliminary examination of the energy-minimised structures of *S*-propranolol and its prodrugs (Figure 7) suggests that VL-P might be expected to adopt a conformation most similar to the parent compound. The principal interaction, as indicated previously, is likely to

be between the secondary amine group within the molecule(s) and the carboxylic acid group of the functional monomer. However the pendant groups within VL-P would appear to be most similarly positioned to the parent molecule in comparison to the other prodrug species, such that interaction with active sites in the MIP might occur more readily.

3.7. Transdermal enantioselective-controlled release of composite MIP membranes

The *in vitro* percutaneous permeation study of propranolol enantiomers was investigated by applying drug to cellulose, NIP or S-MIP membranes in direct contact with isolated excised rat skin contained in a Franz cell. The initial concentration of racemic propranolol (HCl) in the donor phase was varied from 100 - 300 $\mu g.ml^{-1}$. Control experiments were also carried out to determine the percutaneous transport of drug across skin alone.

The cumulative amounts of propranolol transported across rat skin as a function of time, when drug was applied to the membrane positioned in place on the skin surface are shown in Figure 8. The resulting curves indicate that the diffusion of *R*-propranolol across the S-MIP membrane-skin layer is delayed whereas a relatively facilitated transport of *S*-propranolol across S-MIP membrane through the rat skin occurred over the initial 18 h of transport for every donor concentration. The percutaneous transfer of the *S*-enantiomer across cellulose membrane, was less than if the S-MIP membrane was *in situ* and was higher than the *R*-form across both membrane types. The percutaneous permeation of both *R*- and *S*-enantiomers of propranolol across NIP membranes applied to the skin was initially delayed, which this is possibly due to the MAA-EDMA steric hindrances which might occur in the pores of the modified basic cellulose membrane. NIP membranes allowed slightly faster *S*-propranolol enantiomer transport than for the *R*-propranolol enantiomer. There is little facilitated *S*-propranolol enantiomer transport across NIP membranes and this does not increase markedly as the initially applied concentration of drug is increased. In addition, *S/R* selectivity in terms of the permeability coefficient through NIP membranes is not significantly changed with donor concentration (Table 6).

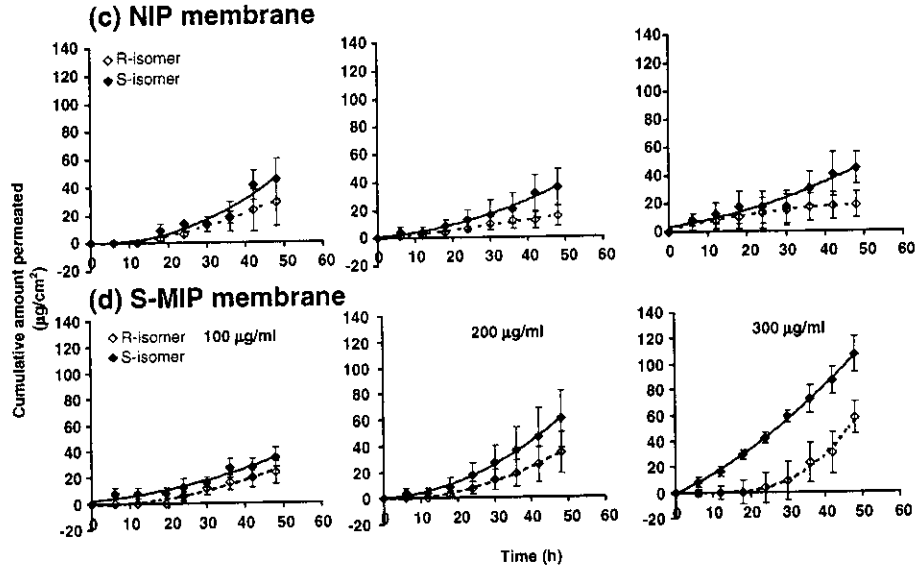


Figure 8: The permeation of propranolol enantiomers (HCl) from pH 7.4 buffer solution (a) across full-thickness rat skin at 37°C in the absence of membrane or under the same conditions but with a (b) cellulose membrane, (c) NIP membrane or (d) S-MIP membrane placed on the surface of the skin (mean ± SE, n=6). The initially applied donor concentrations of racemic propranolol were 100, 200 and 300 $\mu g.ml^{-1}$.

Table 6
Steady-state flux (J_{ss}), Permeability coefficient (K_p), and Lag time (τ) of *R*- and S-propranolol from pH 7.4 buffer solution containing racemic propranolol at different concentrations through full-thickness rat skin alone or for transport through cellulose, NIP or S-MIP membrane applied to the skin surface (mean ± SE, n=6).

Donor concentration (μg/ml)	Membrane	J_{ss} (μg.cm^{-2}h^{-1})		K_p x 10^{-3} (cm.h^{-1})			τ (h)		
		R-isomer	*S*-isomer	*R*-isomer	*S*-isomer	*S*/R ratio	*R*-isomer	*S*-isomer	*S*/R ratio
100	Control	0.30±0.04	0.28±0.01	1.01±0.13	2.10±0.15	1.41±0.38	18.67±0.67	16.76±0.38	0.90±0.05
	Cellulose	0.24±0.04	0.54±0.05	2.43±0.45	5.39±0.62	2.38±0.48	12.07±0.76	1.64±1.40	0.13±0.11
	NIP	0.45±0.05	0.73±0.07	4.46±0.49	7.28±0.65	1.69±0.29	14.07±0.67	11.33±1.33	0.81±0.02
	S-MIP	0.36±0.07	0.66±0.02	3.59±0.72	6.60±0.04	2.04±0.52	18.85±0.85	5.69±1.69	0.30±0.10
200	Control	0.91±0.21	0.52±0.03	4.55±1.03	2.59±0.19	0.57±0.90	10.31±0.61	8.87±0.90	0.87±0.10
	Cellulose	0.24±0.03	0.48±0.01	1.20±0.13	2.39±0.06	2.07±0.04	15.72±3.00	5.90±0.02	0.44±0.11
	NIP	0.32±0.09	0.50±0.01	1.61±0.43	2.49±0.07	1.87±0.62	8.88±0.85	10.57±0.57	1.46±0.18
	S-MIP	0.49±0.05	1.10±0.02	2.46±0.22	5.50±0.10	2.27±0.23	16.23±1.44	4.65±0.60	0.30±0.60
300	Control	1.01±0.14	0.33±0.16	3.33±0.45	1.09±0.54	0.33±0.18	9.54±1.87	6.00±0.01	0.63±0.13
	Cellulose	0.55±0.04	0.72±0.16	1.84±0.86	2.39±0.52	1.71±0.35	12.45±0.90	9.48±2.90	0.75±0.20
	NIP	0.44±0.12	0.68±0.02	1.48±0.38	2.28±0.07	1.74±0.38	1.33±0.94	1.72±1.30	1.04±0.24
	S-MIP	0.52±0.06	1.73±0.36	1.73±0.36	5.76±1.20	3.50±1.01	17.58±4.46	2.45±1.77	0.11±0.06

At a donor concentration of 100 $\mu g.ml^{-1}$, the transport of propranolol enantiomers through skin alone was very low and the flux rates of the two enantiomers were found to be similar. Increasing the donor compartment concentrations (to 200 and 300 $\mu g.ml^{-1}$) resulted in a greater diffusion rate through the skin layer in the absence of membrane, but no enantiomeric differences in transport was observed initially. However, after 12 h the diffusion of *R*-enantiomer across the skin into receptor phase was found to be significantly higher than that of the *S*-enantiomer. The stereoselective penetration of propranolol through excised rat skin has been reported by Miyazaki et al [26] who showed that the percutaneous permeation of *S*-propranolol was 4 times higher than that of *R*-propranolol. These latter results were, however, contrary to the results reported by Heard et al. [27] when the transfer of propranolol from sub-saturated solutions across rat skin was not found to be an enantioselective process. The enantioselective transfer of propranolol across rat skin therefore remains unclear. However, the results obtained in this study indicate that although an enantiomeric difference in the permeation of *R*- and *S*-propranolol through rat skin was shown, particularly after 18 h application, an enantioselective-controlled transport was obtained when the S-MIP membrane was placed *in situ* on the skin. The flux of *S*-isomer was increased in comparison to the control when the S-MIP membrane was *in situ* for all test concentrations, with enhancement ratios of ca. 2-5 being obtained for the different drug concentrations employed (Table 5). The flux of *R*-isomer was reduced with the S-MIP membrane *in situ* compared with controls at donor concentrations of 200 and 300 $\mu g.ml^{-1}$. Due to higher permeability and greater partitioning of *S*-propranolol from S-MIP membrane, the lag time of *S*-isomer was reduced when the membrane was applied as compared to control. However the lag times of the *R*-isomer were found generally to increase in the presence of the S-MIP membrane compared to those obtained in control experiments.

Furthermore, the permeability coefficients were greater for the *S*-isomer through the S-MIP membrane and skin than skin alone, whatever the initial donor drug concentration ($P<0.05$). The permeability coefficients of *R*-enantiomer through S-MIP membrane were generally lower when the S-MIP membrane was *in situ* than through skin alone. The higher permeability coefficients observed for the *S*-enantiomer would appear to confirm the enantioselective delivery properties of S-MIP membrane. When the concentration of racemic propranolol was increased, the lag time for the permeability of *R*-isomer through the S-MIP membrane and skin appeared to be relatively constant. This was in contrast, to the change in lag times observed when the *S*-isomer was applied since these were reduced, as the initial drug concentration was increased (Table 5). This caused the *S/R* ratio of lag time for the transport of propranolol through S-MIP membrane to remain constant or even be reduced as the initial applied concentration was increased. However the results indicated

that increasing the donor concentration promoted the stereoselective transport of S-MIP membrane.

The results obtained in this study indicate that the enantioselectivity shown with S-MIP membrane placed on rat skin is related to the high combined affinity of cellulose and S-MIP for the *S*-propranolol along with the lesser affinity for *R*-enantiomer inherent in the cellulose and induced in the S-MIP by the imprinting process. The enantioselective-controlled release of S-MIP membrane was clearly apparent when racemic propranolol placed in the donor compartment at concentrations up to 300 $\mu g.ml^{-1}$. Beyond this concentration, *i.e.,* 400 $\mu g.ml^{-1}$, the stereoselectivity was reduced (data not shown). The reason for this might involved the saturation of binding sites within the excised rat skin.

3.8. The effect of the gelling agents on enantioselectivity

In this part, two gel formulations, containing either poloxamer or chitosan were selected as drug reservoirs for the formulation of racemic propranolol within the MIP thin-layer composite cellulose membrane. The ability of the fabricated devices to deliver the *S*-enantiomer of propranolol was tested by conducting release studies *in vitro* using modified Franz diffusion cells. The enantiomer release profiles showed that the *S*-enantiomer delivery is completely controlled by the composite MIP membrane. The rate of drug release was demonstrated to be dependent on the type of gel used. The gel type was found to affect the enantioselective transport of propranolol, with the more rheologically structured poloxamer gel formulation providing no selective release of *S*-propranolol. The greater gel structure may have interfered with the enantioselective controlling process at the pore surface of the composite MIP membrane. In contrast the chitosan gel was found to allow excellent selectivity for delivery of the *S*-propranolol enantiomer (*S*/*R* ratio = 4), as shown in Figure 8.

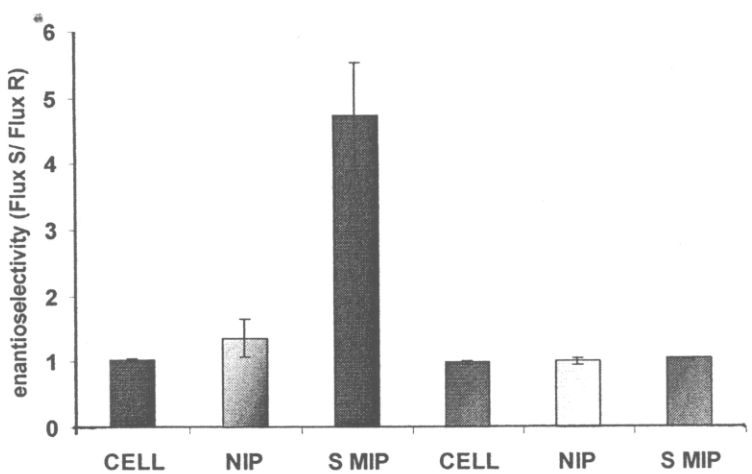


Figure 8: The effect of gelling agent on Flux *S*/Flux *R* of the membranes

3.9 *In vivo* evaluation

The chitosan gel formulation was selected for *in vivo* studies by fabricating in the S-MIP patch with the addition of 0.75 mg racemic propranolol and compared with control (gel alone). The plasma drug level profile in rats following transdermal application of control and the patch is shown in Figure 9a and 9b, respectively. The plasma levels of both *R*- and *S*-propranolol after application of control were lower than the plasma levels after transdermal application with the patch. The plasma levels of *R*-propranolol from the patch and control were not different. The plasma levels after application of the patch gradually increased and reached a peak plasma drug level of 800 ng ml^{-1} for the *S*-propranolol and 390 ng $ml^{-1}$for the *R*-propranolol. Besides, the plasma levels of both *R*- and *S*-propranolol after application of control were not statistically different ($p>0.5$). There was enantioselectivity for the plasma drug level from the transdermal application of the patch. The plasma drug level of *S*-propranolol from the patch than control must have been contributed by absorbed drug that enantioselectively released by the S-MIP membrane. Thus the enantioselective delivery of the patch comprising with S-MIP membrane is clearly demonstrated *in vivo*.

The skin irritation following application of either the gel or the S-MIP patch at 24 h was examined. The edema was absent in the both cases, as shown in Figure 10a,b.

Figure 9a Plasma level profile following transdermal application of gel formulation.

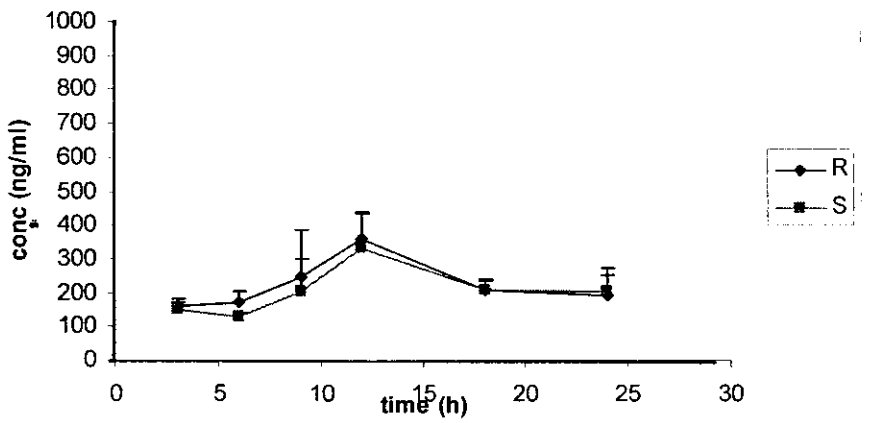


Figure 9a Plasma level profile following transdermal application of the S-MIP patch.

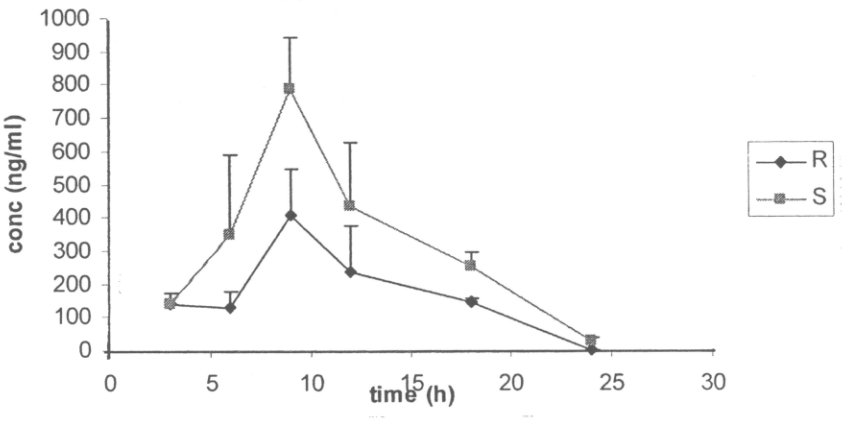


Figure 10a: The illustration showing skin before and after application racemic propranolol gel

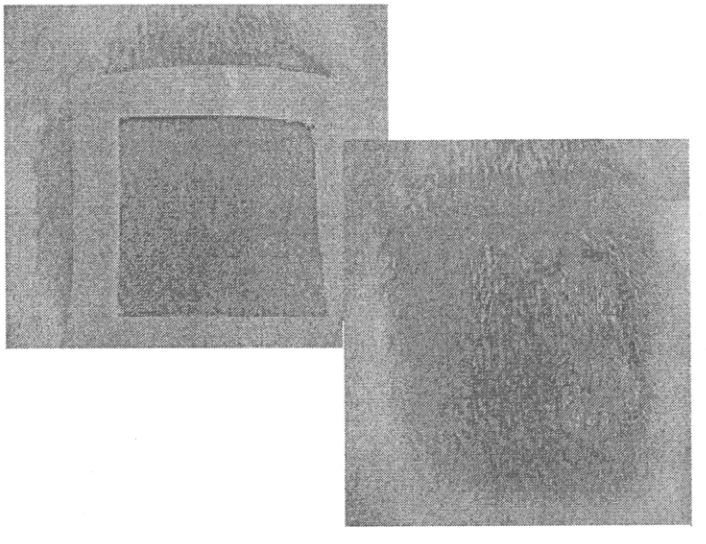
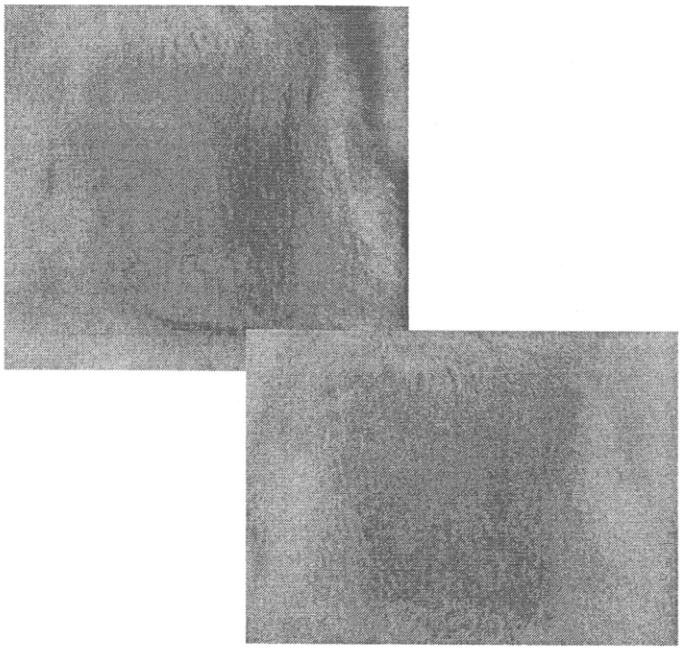


Figure 10b: The illustration showing skin before and after application the racemic propranolol gel



4. Conclusions

The current study demonstrated the potential of MIP molecularly imprinted polymer composite membranes based on cellulose in controlling the release of *S*-propranolol into the skin. The degree of stereoselectivity demonstrated would result in considerably higher therapeutic advantage when considering the differential pharmacological activities of the two enantiomers of propranolol. The cellulose membranes containing modified pores and a surface with imprinted polymer selective for *S*-propranolol were capable of limiting release of the distomer. Also the enantioselective delivery by S-MIP membrane is demonstrated *in vivo*

References

[1] J.G. Riddel, D.W.G. Harron, R.G. Shanks, Clinical pharmacokinetics of β-adrenoceptor antagonists, Clin. Pharmacokin. 12 (1987) 305-320.

[2] A. Parkinson, Biotransformation of xenobiotics, in: C.P. Klaassen (Ed.), Casarett and Doulls Toxicology, The Basic Science of Poisons, International Edition, 1st Edition, McGraw-Hill, New York, 1996, pp. 152-153.

[3] M.J. Quigley, G.M.C. Jordan, F.R. Timoney, The synthesis, hydrolysis kinetics and lipophilicity of *O*-acyl esters of propranolol, Int. J. Pharm.101 (1994) 145-163.

[4] B.D. Anderson, W.W. Chu, R.E. Galinsky, Reduction of first-pass metabolism of propranolol after oral administration of ester prodrugs, Int. J. Pharm. 43 (1988) 261-265.

[5] A. Buur, H. Bundgaard, V.H.L. Lee, Prodrugs of propranolol: hydrolysis and intramolecular aminolysis of various propranolol esters and oxazolidin-2-one derivative, Int. J. Pharm. 42 (1988) 51-60.

[6] A.M. Barrett, V.A. Cullum, The biological properties of the optical isomers of propranolol and their effects on cardiac arrythemias, Br. J. Pharmacol. 34 (1968) 43-55.

[7] S. Ahmed, T. Imai, Y. Yoshigae, M. Otagiri, Stereoselective activity and nature of metabolizing esterases for propranolol prodrug in hairless mouse skin, liver and plasma, Life Science 61 (1997) 1879-1887.

[8] S. Ahmed, T. Imai, M. Otagiri, Stereoselective hydrolysis and penetration of propranolol prodrugs: *In vitro* evaluation using hairless mouse skin, J. Pharm. Sci. 84 (1995) 877-883.

[9] M. Ulbricht, Review, Membrane separations using molecularly imprinted polymers, J. Chromatogr. B. 804 (2004) 113-125.

[10] S.A. Piletsky, T.L. Panasyuk, E.V. Piletskaya, I.A. Nicholls, M. Ulbricht. Receptor and transport properties of imprinted polymer membranes-a review, J. Memb. Sci. 157 (1999) 263-278.

[11] M. Ulbricht, M. Belter, U. Langenhangen, F. Schneider, W. Weigel, Novel molecularly imprinted polymer (MIP) composite membranes via controlled surface and pore functionalizations, Desalination 149 (2002) 293-295.

[12] K. Hattori, K. Yoshimi, K. Sakai, Gate effect of cellulosic dialysis membrane grafted with molecularly imprinted polymer, J. Chem. Eng. Jpn 34 (2001) 1466-1468.

[13] T.A. Sergeyeva, H. Matuschewski, S.A. Piletsky, J. Bendig, U. Schedler, M. Ulbricht, Molecular imprinted polymer membranes for substance-selective solid-phase extraction from water by surface photo-grafting polymerization, J. Chromatogr. A 907 (2001) 89-99.

[14] D. Klemm, D. Schumann, U. Udhardt, S. Marsch, Bacterial synthesised cellulose-artificial blood vessels for microsurgery, Prog. Polm. Sci. 26 (2001) 1561-1603.

[15] J.D. Fontana, A.M. De-Souza, C.K. Fontana, I.L. Torriani, J.C. Moreschi, B.J. Gallotti, S.J. De-Souza, G.P. Narcisco, J.A. Bichara, L.F.X. Farah, Acetobacter cellulose pellicle as a temporary skin substitute, Appli. Biochem. Biotechnol. 25 (1990) 253-264.

[16] C.M. Heard, R. Suedee, Stereoselective adsorption and trans-membrane transfer of propranolol enantiomer using cellulose derivatives, Int. J. Pharm. 139 (1996) 15-23.

[17] Y. Okamoto, M. Kawashima, K. Yamamoto, K. Hatada, Useful chiral packing materials for high-performance liquid chromatographic resolution. Cellulose triacetate and tribenzoate coated on macroporous silica gel, Chem. Lett. (1984) 739-742.

[18] R. Suedee, C.M. Heard, Direct resolution of propranolol and bupranolol by thin-layer chromatography using cellulose derivatives as stationary phase, Chirality 9 (1997) 139-144.

[19] J. Haginaka, Y. Sakai, Uniform-sized molecularly imprinted polymer material for (*S*)-propranolol, J. Pharm. Biomed. Anal. 22 (2000) 899-907.

[20] R.D. Noble, Generalized microscopic mechanism of facilitated transport in fixed site carrier membranes, J. Membr. Sci. 75 (1992) 121-129.

[21] T. Yamaguchi, C. Baertsch, C.A. Koval, R.D. Noble, C.N. Bow, Olefin separation using silver impregnated ion-exchange membranes and silver salt/polymer blend membranes, J. Memb. Sci. 117 (1996) 151-161.

[22] J. Mathew-Krots, K.J. Shea, Imprinted polymer membranes for the selective transport of targeted neutral molecules, J. Am. Chem. Soc. 118 (1996) 8154-8155.

[23] R. Suedee, T. Srichana, G.P. Martin, Evaluation of matrices containing molecularly imprinted polymers in the enantioselective-controlled delivery of β-blockers, J. Contr. Rel. 66 (2000) 135-147.

[24] R. Suedee, T. Srichana, R. Chotivatesin, G.P. Martin, Stereoselective release behaviors of imprinted bead matrices. Drug Dev. Ind. Pharm. 28 (2002) 547-556.

[25] J.M. Quigley, C.G.M. Jordan, R.F. Timoney, The synthesis, hydrolysis kinetics and lipophilicity of *O*-acyl esters of propranolol, Int. J. Pharm. 101 (1994) 145-163.

[26] K. Miyazaki, F. Kaiho, A. Inagaki, M. Dohi, N. Hazemoto, M. Haga, H. Hara, Y. Kato, Enantiomeric difference in percutaneous penetration of propranolol through rat excised skin, Chem. Pharm. Bull. 40 (1992) 1075-1076.

[27] C.M. Heard, A.C. Watkinson, K.R. Brain, J. Hadgraft, In vitro skin penetration of propranolol enantiomers, J. Int. J. Pharm. 90 (1993) R5-R8.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Controlled Release 113 (2006) 43–56
journal of controlled release
www.elsevier.com/locate/jconrel

Composite membrane of bacterially-derived cellulose and molecularly imprinted polymer for use as a transdermal enantioselective controlled-release system of racemic propranolol

Chatchada Bodhibukkana [a], Teerapol Srichana [a], Sanae Kaewnopparat [a], Naruedom Tangthong [a], Pisit Bouking [a], Gary P. Martin [b], Roongnapa Suedee [a,*]

[a] *Molecular Recognition Material Research Unit, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hatyai, Songkla 90112, Thailand*

[b] *King's College London, Pharmaceutical Sciences Research Division, Franklin-Wilkins Building, London SE1 9NH, UK*

Received 11 May 2005; accepted 7 March 2006
Available online 20 March 2006

Abstract

A composite membrane for transdermal delivery of *S*-propranolol enantiomer was developed based on the controlled pore functionalization of bacterial cellulose membranes using a molecularly imprinted polymer (MIP) layer synthesis. The reactive pore-filling of an asymmetric porous cellulose membrane with a MIP thin-layer was effected using a silanised coupler as an additional anchor for the MIP. MIP thin-layers with specific binding sites for *S*-propranolol were synthesized by copolymerisation of methacrylic acid with a cross-linker, ethylene glycol dimethacrylate in the presence of *S*-propranolol as the template molecule and the latter was subsequently extracted. Selective transport of *S*-propranolol through the MIP composite membrane was obtained, although this was determined mostly by the parent cellulose membrane with some ancillary contributory effect from the MIP layer. In addition, an enantioselectivity in the transport of propranolol prodrug enantiomers was found, suggesting that the shape and functional groups orientation, which are similar to that of the print molecule were essential for enantiomeric recognition of the MIP composite membrane. The enantioselectivity of S-MIP membranes was also shown when the release of propranolol enantiomers was studied in vitro using rat skin, with racemic propranolol contained in the donor compartment. The composite membrane of bacterially-derived cellulose and molecularly imprinted polymer may have great potential for use as a transdermal enantioselective controlled-release system for racemic propranolol.

Keywords: Molecularly imprinted polymer; Cellulose membrane; Propranolol; Transdermal delivery; Enantioselectivity

1. Introduction

The efficiency of delivery of propranolol, a non-selective β-adrenergic receptor antagonist, via the oral route is affected by the low absorption [1] and extensive first-pass metabolism of the drug [2] whereas the transdermal delivery of propranolol enables the hepatic first-pass metabolism to be avoided, potentially improving bioavailability. The absorption of propranolol hydrochloride, which is the commercially available form of propranolol, through skin is very poor. A prodrug strategy has been used with some success where the drug after absorption through the stratum corneum is subsequently metabolized to the parent compound [3–5]. Propranolol possesses one chiral center and the *S*-enantiomer is 100–130 times more pharmacologically active than the *R*-enantiomer [6]. Propranolol is marketed as racemate and no stereoselective permeation of propranolol and propranolol ester prodrug was observed through skin in vitro [7,8]. Thus, if the *S*-enantiomer were to be selectively transported across the skin, a better therapeutic response might be expected relative to that obtained using a racemic mixture of the drug.

A molecularly imprinted polymer (MIP) is a synthetic polymer possessing selective molecular recognition properties because of recognition sites within the polymer matrix that are complementary to the analyte molecule in the shape and positioning of functional groups. The use of MIPs as new sorbents for affinity membrane extraction is becoming more widespread,

* Corresponding author. Tel.: +66 74 428239; fax: +66 74 288862.
E-mail address: roongnapa.s@psu.ac.th (R. Suedee).

0168-3659/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2006.03.007

due to their potentially high selectivity [9,10]. MIP membranes utilising such an approach may comprise either a simple MIP membrane or alternatively the MIP could be integrated with other materials within a composite. A composite MIP membrane is an optimised porous support membrane which has been functionalized with a suitable thin selective layer to yield a membrane with appropriate transport properties for the target compound [9]. A number of composite membranes functionalized with thin MIP layers have been previously developed to attain adsorption specificity [11–13]. The preparation of MIP composite membranes can allow the transport of drugs to be controlled by both membrane pore structure and MIP recognition. The structure of the base membrane in MIP composite membranes may be used as a means to adapt pore size, permeability, internal surface area and binding capacity to the desired application with synthetic membranes often being used.

Cellulose is a naturally occurring linear β-1,4-linked glucose polymer synthesized by plants and some species of bacteria. In contrast to some synthetic membranes, cellulose membrane exhibits a high resistance to corrosive chemicals but is biodegradable and hence eco-friendly. The unique properties of the bacterially-derived cellulose synthesized by *Acetobacter xylinum* have inspired a number of attempts to use it in a number of commercial products. Proposed applications include its use as a temporary skin substitute, a dressing material, a material for surgery or microsurgery and as membranes [14,15]. For example Biofill® is a specially prepared membrane which can be used as a temporary skin substitute for patients with burns and ulcers. Bacterially-derived cellulose can be easily processed into a porous membrane possessing good mechanical strength, unlike the cellulose derived from plant biomass. These unique features make such bacterially-derived membranes potentially suitable for use as a component of transdermal delivery system although to date no studies have reported the use of such cellulose membranes in controlled drug delivery systems.

In a previous study, cellulose triphenylcarbamates were shown to have a good potential for controlling transport of propranolol enantiomers through a silastic membrane [16]. Cellulose and its derivatives have proven stereoselectivity to propranolol in adsorption chromatography both in column chromatography [17] and thin layer chromatography [18], where they demonstrate a high affinity to the *S*-enantiomer of propranolol.

In this study, MIPs and bacterially-derived membranes were integrated to form composite membranes which could have a potential as a transdermal delivery system of the model drug *S*-propranolol. Through optimisation of MIP affinity and ensuring a uniform distribution of the polymer within cellulose membrane pores as well as minimizing non-selective diffusion by pore-filling, a selective absorption and subsequent release of the eutomer of propranolol through skin might be achieved. Accordingly MIP membranes selective for *S*-propranolol were developed and their enantioselective-controlled release was examined in vitro. Microporous cellulose membranes were generated by bacterial-cultivation and were used as a matrix for a two-step grafting procedure to yield a MIP by in situ copolymerisation within the thin barrier layer of the base material. Using a silanisation procedure, supramolecular complexes of template molecules and suitable functional isopropenylates were fixed onto pore-containing surface of cellulose membranes. MIP synthesis mixtures comprising methacrylic acid as a functional monomer and ethylene glycol dimethacrylate as a cross-linker, in the presence of the template enantiomer were successfully polymerized. This procedure allowed the generation of MIP composite membranes which included a thin MIP layer that could act as a selective barrier.

2. Experimental

2.1. Chemicals and reagents

Ethylene glycol dimethacrylate (EDMA), methacrylic acid (MAA) and propranolol hydrochloride, *R*-propranolol HCl, *S*-propranolol HCl and 3-methacryloxypropyltrimethoxysilane (3-MPS) and valeryl chloride were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2′-Azobis-(isobutyronitrile) (AIBN) was obtained from Janssen (Geel, Belgium). Yeast extract and peptone were obtained from Difco Laboratories (MO, USA). Succinic anhydride and cyclopropanecarbonyl chloride were supplied by Fluka Chemie AG (Buchs, Switzerland). EDMA was purified by extraction with 10% (w/v) $CaCO_3$, washing with water, drying over anhydrous sodium sulfate and subsequent distillation under reduced pressure. MAA was purified by distillation under reduced pressure prior to use. All solvents used were of analytical grade and were dried with molecular sieves before use. Working standard solutions were prepared daily.

2.2. Preparation of bacterial cellulose membranes

Cellulose membrane was obtained by incubating *A. xylinum* TISTR 975, sub-cultured five times, in coconut juice supplemented with 4% sucrose (w/v) at pH 5.0. A stainless steel round shallow tray of 39 cm diameter was used to grow the cellulose-producing bacteria at 30 °C at the surface of culture medium under static conditions. The culture volume was 500 ml and the effective area for membrane growth was 20 cm^2. Buffered Schamm and Hestrin's medium (BSH medium) was employed as the pre-culture medium, composed of 2.0% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.033% (w/v) $Na_2HPO_4{\cdot}2H_2O$ and 0.011% (w/v) citric acid·H_2O. *A. xylinum* TISTR 975 was grown in 50 ml of BSH medium for 3 days to use as a pre-culture. The pellicles of bacterial cellulose (0.01 mm thickness) formed on the surface of this medium surface were harvested aseptically.

Two 'thin' cellulose membranes were prepared using two different bacterial cultures, comprising suspensions containing either 1×10^8 or 3×10^8 cfu ml^{-1}. One 'thick' cellulose membrane was prepared by placing 4 pieces of cut pellicle (1 × 1 cm) in the tray, approximately 10 cm from the edge of the tray and an equal distance from each other. For cellulose membrane production, the pre-culture of bacteria was inoculated into 500 ml of the culture media and the tray was covered with a linen cloth, followed by incubation for 1 day. The cellulose pellicle formed on the surface of the media was removed and

discarded. The remaining culture was further incubated under the same conditions for one more day. The formed cellulose membrane was harvested and transferred to 1% (w/v) NaOH at 80 °C for 24 h and then thoroughly washed with distilled water to remove any remaining associated microorganisms and proteins. The pure cellulose sheets were dried at 37 °C overnight and kept in a dust free atmosphere until required for use.

2.3. Preparation of MIP composite membrane

The grafting procedure of ethylene glycol dimethacrylate and methacrylic acid copolymer onto the pore-containing surface of the bacterially-derived membrane is illustrated in Fig. 1. The grafting was carried out in the presence of the propranolol enantiomer template. A cellulose membrane was reacted with 3-MPS (10% w/w in toluene) at 80 °C for 5 h. The resulting membrane was then thoroughly washed in methanol and dried. The reacted cellulose membrane was then placed in crystallizing dish, 18 cm in diameter. A solution containing 12 mmol of MAA as a functional monomer, 0.05 mol of EDMA as a cross-linking monomer, 2 mmol of *R*- or *S*-propranolol (HCl) as template and 0.7 mmol of AIBN as a radical polymerization initiator in DMF (2 ml) was poured onto the surface of cellulose membranes. The dish was then purged with nitrogen for 2–3 min to remove oxygen (which acts as a radical scavenger) before closure and the temperature maintained at 60 °C for 18 h. After polymerisation, membranes were transferred to a Soxhlet extractor and extracted with 10% (w/v) acetic acid in methanol for at least 72 h before further extraction with methanol for 72 h to remove any non-grafted polymer, monomer, residual initiator and template molecule. The prepared membranes were dried in vacuo overnight. The complete removal of the template molecule from the polymer was confirmed by its absence from the methanol extract of the polymer, as verified by assay (see Section 2.8) and the absence of nitrogen content in elemental analysis results obtained by using a JSM-5800 LV electron microscope (Jeol, MA, USA) equipped with an Oxford Instruments LINK-ISIS 30 X-ray detector and microanalysis system. The membrane prepared with an imprint of *R*-propranolol was referred to as R-MIP membrane and the membrane having the imprint of *S*-propranolol was referred to as S-MIP membrane. Blank composite membranes (non-imprinted polymer (NIP) cellulose membranes), for control experiments were prepared in the same manner as MIP modified cellulose membrane but with the template molecule omitted (these membranes were referred to as NIP membranes). The change in weight of the membrane induced by the grafting procedure was measured using a microbalance (Precisa 300 A, USA: capacity 305 g, readability 0.1 mg). The degree of modification (DM) was calculated from the difference in weight between the sample modified with a deposited MIP layer and the initial unmodified sample.

2.4. Characterisation methods

2.4.1. Surface morphology and cross-section study

The membrane morphology of the initial and modified cellulose was studied by scanning electron microscope observation of the cross-section and inner and outer surfaces at an accelerating voltage of 15 kV with the samples being sputter-coated with gold

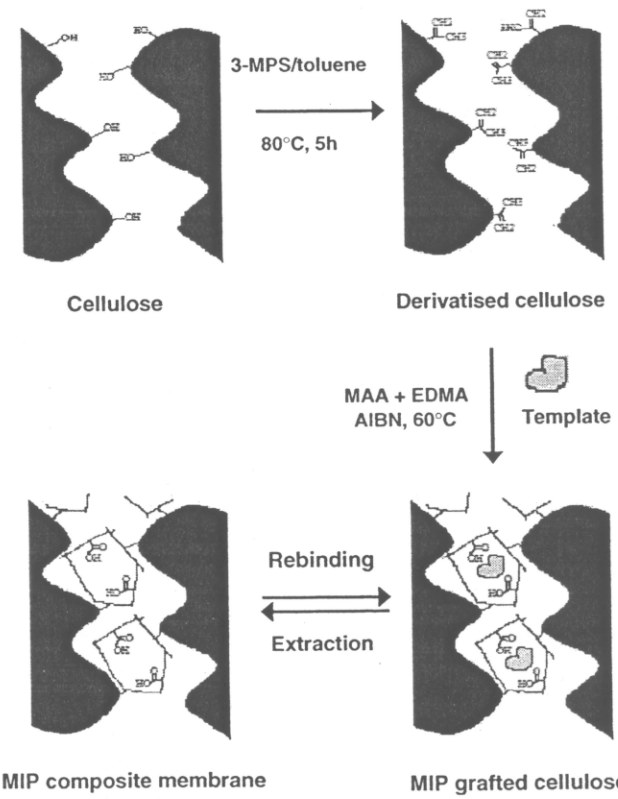


Fig. 1. Surface pore modification of cellulose membrane with a molecularly imprinted polymer against propranolol enantiomer.

before imaging (Jeol series JSM 5800LV, CA, USA). The morphology was further examined with an atomic force microscope (AFM) using a Digital Instruments NanoScope IIIa scanning probe microscope (Veeco Instruments GmbH, Germany). AFM observations were carried out at room temperature without any previous treatment, using rectangular silicon nitride cantilevers with pyramidal tips.

2.4.2. Pore size measurements

The pore size of membranes was estimated from surface pictures of the membrane obtained by SEM with the aid of a Carnoy computer imaging program (Lab of Plant Systemics, Belgium).

2.4.3. Electrical resistance measurements

The degree of membrane fibrillation was assessed from the value obtained for the resistance of membrane. Also, impedance measurement was used to confirm the deposition of a MIP layer onto the surface of the cellulose membranes. Electrochemical resistance measurements of membranes were carried out by short-circuit current technique using a Revision G Voltage-Current Clamp, Model VCC 600 (Harvard Apparatus, CA, USA). Chambers having an effective area of 1 cm^2 were used. The test membrane was mounted in the measuring cell and a current established across it using a potentiostat via an amplifier with high-resistance inputs. The four electrode potentiostat assured a passage of current between the two calomel electrodes in such a manner as to hold constant amplitude of voltage between the two identical reversible silver–silver chloride electrodes and the intensity and phase of current in the circuit. A 60 μA current was applied and the membrane potential difference, PD (mV) and the short circuit current, I_{sc} (A) were recorded simultaneously. The membrane resistance, R_m (Ω cm^2) was calculated from PD/I_{sc}, based on the Ohm's law. These were corrected by eliminating the offset voltage between the electrodes and solution resistance, which was determined prior to each experiment using identical bathing solutions. All experiments were carried out at 25±1 °C.

2.4.4. Mechanical properties measurements

The mechanical strength of the membranes was measured using a Universal testing machine (Lloyd, UK) with an operating head load of 100 N. The membranes were placed between the grips (2.5 cm in length) of the testing machine. The grip length was 2.5 cm and the speed of testing was set at the rate of 30 mm/min. Tensile strength was calculated according to the equation: tensile strength (kN/m^2)=max load (kN)/cross-sectional area (m^2).

2.4.5. Degree of swelling of membranes

The degree of swelling of cellulose and modified cellulose membranes was evaluated in pH 5.5 and pH 7.4 buffers, since for some applications membranes were used in these conditions. All polymer membranes were vacuum-dried at room temperature for at least 3 days before testing. The membrane samples were weighed and then soaked in individual tubes containing phosphate buffer pH 7.4 (μ=0.55) or citrate buffer pH 5.5 (μ=0.35) at room temperature (~30 °C). The membranes were incubated in the medium until the weight of wet membranes remained stable, which usually occurred after approximately 7 h incubation. Before measuring the weight of the wet membrane, surface water was gently removed with a tissue. The wet films were dried under vacuum at room temperature to a constant weight (over a minimum of 2 days) and stored in a desiccator at room temperature before measurements. The degree of swelling of the membrane (%) was calculated from the equation: $(W_{wet}-W_{dry})/W_{dry}\times 100\%$ where W_{dry} and W_{wet} are the weights of dried and wet samples, respectively. Each test was carried out in sets of three.

2.5. Stability studies

Samples of *R*- and *S*-propranolol (50 mg) were incubated separately with pH 5.5 citrate or pH 7.4 phosphate buffer solution (1 ml) at both room temperature (30 °C) and 37 °C for 7 days. The same quantities of enantiomer (50 mg) were incubated also at 60 °C in 1 ml of DMF (or chloroform) for 24 h. Samples were removed at the end of the incubation times and assayed for enantiomer content according to the method described in Section 2.9.

2.6. Measurement of partition coefficient

The membrane-pH 5.5 (μ=0.35) or pH 7.4 (μ=0.55) buffer partition coefficient was evaluated by equilibrating racemic propranolol solutions with a membrane. The difference between the initial and equilibrium concentrations of each enantiomer in the aqueous phase was determined (Section 2.9) and hence the amount of propranolol enantiomer sorbed to the membrane was calculated. In a typical binding assay, the membrane (1 cm^2) was added to 5 ml of an aqueous solution containing 100 μg ml^{-1} of racemic propranolol, and stirred overnight at room temperature for equilibrium to be established. The partition coefficient (K) was calculated from the equation: $K=C_p/C_s$, where C_p is the concentration of the analyte associated with the membrane, and C_s is the concentration of the analyte in the solution. The selectivity factor representing the effect of the imprinting process was the ratio of K of the *S*-isomer to K of the *R*-isomer.

2.7. Permeation determination

The enantioselective transport of the cellulose and modified cellulose membranes (NIP, R-, and S-MIP membranes) was evaluated by a dialysis method using a vertical Franz-type diffusion cell. A membrane (exposed area 0.78 cm^2) was mounted between the two chambers of the diffusion cells, the volumes of the donor and receptor chambers being 1 and 2.5 ml, respectively. The required amount of racemic propranolol (40 μg ml^{-1}) was dissolved in either pH 5.5 citrate buffer (μ=0.35) or 7.4 phosphate buffer (μ=0.55) solutions to obtain the donor solutions (0.5 ml). Buffer (pH 7.4) was introduced into the receiver compartments of the diffusion cells. The solubility of propranolol enantiomers in the receiver medium was 0.75 mg ml^{-1}, thus assuring the maintenance of sink conditions

for the duration of diffusion experiments. Drug release was measured by the removal of samples (250 μl) from the receiving chamber at appropriate time intervals over 6 h. The volume of the sample withdrawn was replaced by the same volume of the medium. Each test was carried out in sets of six. MIP membranes were reusable at least twice after regeneration by incubating with water to release sorbed propranolol.

The diffusion of each propranolol enantiomer was determined using the chiral-HPLC analytical method outlined in Section 2.9. The cumulatively permeated amounts (μg) were calculated and plotted as a function of time. The flux J (μg cm^{-2} h^{-1}) is defined by:

$$J = Q^{*}A^{-1}{*}t^{-1}$$

where Q (μg) is the amount of analyte permeated, A (cm^2) is the effective membrane area and t (h) is the time. The selectivity of the membrane was defined as the ratio of the permeation flux of *S*-isomer to that of the *R*-isomer.

2.8. In vitro percutaneous penetration study

Propranolol was used as the hydrochloride salt. Racemic propranolol hydrochloride was dissolved in phosphate buffer saline (PBS pH 7.4) to produce a range of drug concentrations; 100, 200, 300, 400 μg ml^{-1}.

The in vitro percutaneous penetration study of S-MIP membrane was performed with a Franz-type diffusion cell (see Section 2.7). Adult male, Wistar rats weighing 230–250 g were sacrificed by snapping the spinal cord at the neck. Rectangular sections of dorsal skin were shaved and excised from the animal using surgical scissors. Adhering fat and other visceral debris were removed from the under surface with tweezers. The excised skin was immediately mounted between the half-cells, with and without a coupled test membrane, such that the dermal surface was in contact with the receptor fluid and the epidermal side in contact with the test membrane (if present). Drug solution (0.5 ml) was applied to the membrane surface and the cell was maintained at 37 °C by an external circulating water-bath. The receptor phase was stirred constantly at 250 rpm with a magnetic bar. An aliquot (250 μl) of receptor fluid was collected at set time intervals over 2 days and replaced with the same volume of fresh PBS. The concentration of propranolol in the collected sample was determined by HPLC (Section 2.9).

To 'clean-up' the receptor phase samples, a liquid–liquid extraction method was employed as follows. The receptor phase samples (250 μl) were added to 1 ml of 0.1 M phosphate buffer pH 4 that was saturated with NaCl. Propranolol enantiomers were extracted into 2 ml diethyl ether by shaking for 10 min, using mutually pre-saturated phases. Organic phase (2 ml) was evaporated to dryness under reduced pressure and dissolved in 125 μL of HPLC mobile phase, in readiness for assay.

The in vitro permeation data were obtained from the equation: $J_{ss}=K_pC_s$ where J_{ss} is the steady state flux measured as the slope of the profile after regression analysis. K_p is the apparent permeability coefficient through the skin, calculated by dividing J_{ss} by the starting concentration C_s. The x-intercept of the extrapolated linear region of the curve was employed to obtain a lag time (τ).

2.9. Stereospecific HPLC method

The analysis of the propranolol enantiomer content of the samples was performed directly using a chiral-HPLC method. The HPLC system (Shimadzu Corporation, Kyoto, Japan) comprised a Shimadzu LC-10AD pump, FCL-10AL gradient valve, DGU-14A in-line solvent degasser, SCL-10A system controller, SIL-10AD auto injector (20 μl injection loop), equipped with a Shimadzu SPD-10A UV–Vis detector set at 290 nm. Data were collected and analysed on a personal computer using Class VP software (version 4.2, Shimadzu). The column employed was a 250×4.6 mm I.D., particle size 10 μm Chiralcel OD-R column (Daicel, Chemical Industries Ltd., Japan). The mobile phase was 40:60 (v/v) 1 M sodium perchlorate:acetonitrile and a flow rate of 1.0 ml/min was employed. Typical retention times were 10.0 min for *R*-propranolol and 14.7 min for *S*-propranolol. Correlation coefficients for the calibration curves in the range 2–25 μg ml^{-1} for *R*- and *S*-propranolol enantiomers were greater than 0.999. The sensitivity of detection was 1.3 μg ml^{-1} and the reproducibility of the peak areas of both enantiomers was more than 95%.

Cumulative corrections were made for the previously removed samples in determining the total amount permeated. The cumulative percentages of each enantiomer which permeated into the receptor compartment were calculated and plotted as a function of time.

The results of the controlled membrane and skin permeation experiments were expressed as a mean ± SE. Paired two sided t-tests were performed on the data and a significance limit of 5% level was applied, where appropriate.

2.10. Molecular modelling

The 3-D chemical structures of *S*-propranolol and *S*-prodrugs have been modelled with molecular mechanics (Chem3D Ultra 8.0, CambridgeSoft, MA, USA) followed by energy minimisation using Truncated Newton (MM2 force field).

3. Results and discussion

3.1. Preparation of composite MIP membranes

Composite MIP membranes were prepared by reactive filling of the pores of the bacterial cellulose membranes with MIPs having recognition sites for the *S*-propranolol enantiomer. In order to achieve an additional anchor for the MIP, asymmetric porous cellulose membranes were treated with 3-MPS. Thin-layer MIPs were synthesised by a free radical copolymerization of MAA functional monomer with EDMA as a cross-linker in the presence of propranolol enantiomer (as a template molecule) in DMF, and this was followed by subsequent template molecule extraction. MAA was chosen as the functional monomer since the acid group of the monomer had the facility to interact with the amine groups of the print molecule. The cross-linker,

EDMA was selected due to the anticipated rigidity and compactness it might confer to the imprinted polymer. Porous asymmetric cellulose membranes were obtained as a product of *A. xylinum* fermentation. The properties of prepared cellulose membrane such as thickness, degree of fibrillation, porosity and pore size can be adjusted by controlling the loading concentration of bacteria during culture as well as the time of cultivation. Three bacterial cellulose membranes having different thickness and electrical membrane resistance were used in this work for screening experiments to optimise the MIP affinity and distribution of the MIP within the membrane. The three lots of cellulose membrane displayed variation in membrane thicknesses and resistance respectively as follows: (1) 5 μm/1 Ω cm^2; (2) 10 μm/2 Ω cm^2; (3) 15 μm /4 Ω cm^2. Cellulose membranes were imprinted with *S*-propranolol enantiomer during pore-filling and their stereoselective sorption and transport properties determined. The same polymer mixtures were used in the pore-filling process of all three lots of cellulose membranes.

The membrane thickness and pore diameter of cellulose membrane appeared to have no significant effect on degree of modification (DM). The DMs of the three modified membranes were similar with an average DM of 0.80 mg/cm^2. The variation in the DM of individual membranes was found to be less than 10%. The increase of membrane resistance (R_m) after the modification was found to be 0.7 Ω cm^2 for the 5 μm membrane, 1.3 Ω cm^2 for the 10 μm membrane and 2.2 Ω cm^2 for the 15 μm membrane. The tensile strengths of the cellulose membranes were found to increase when each was modified with MIP to form composite membranes. An increase from 3.16 to 7.20 kN/m^2 occurred for the 5 μm membranes, from 6.60 to 8.78 kN/m^2 for the 10 μm membranes and from 8.83 to 21.45 kN/m^2 for the 15 μm membrane. SEM cross-sectional images of initial and modified cellulose membranes show the different membrane thicknesses (Fig. 2). The cross-sectional view shows an array of closely packed MIP domains in the cellulose membranes. The thickness of the deposited MIP layer tends to increase as the thickness of cellulose membrane increases. Even so the relatively small thickness of the MIP layer might be expected to allow relatively high fluxes through the MIP composite cellulose membranes. The cellulose membranes varied in the degree of porosity and fibrillation (Fig. 3). Fibers were still apparent after modification of the thinnest membrane (5 μm) with MIP but when the same amount of MIP was applied to the 15 μm membrane, it did not penetrate as effectively into the tighter meshed polymer chains (see Fig. 3), presenting a smoother, but thicker layer with a fibrous surface. The SEM micrographs would suggest that a greater amount of MIP is coupled to the outer surface of the 15 μm membrane compared to the 5 μm membrane.

The AFM images of the membrane (Fig. 4) show the vertical profile of the sample with the light regions representing the highest points and the darkest regions showing the pores. The pore size obtained from the AFM images of the MIP membrane indicated that this was smaller than that of the parent non-modified membrane.

Table 1 shows that the partitioning of the propranolol enantiomers between S-MIP membranes and pH 7.4 buffer decreased with increase in the membrane thickness. The differences in the partition coefficients of *S*-enantiomer and *R*-enantiomer were greatest when the thickness of the composite membrane was smallest. Differential transport of the template enantiomer significantly increased with a decrease in the thickness of the membrane. The increase in the thickness of

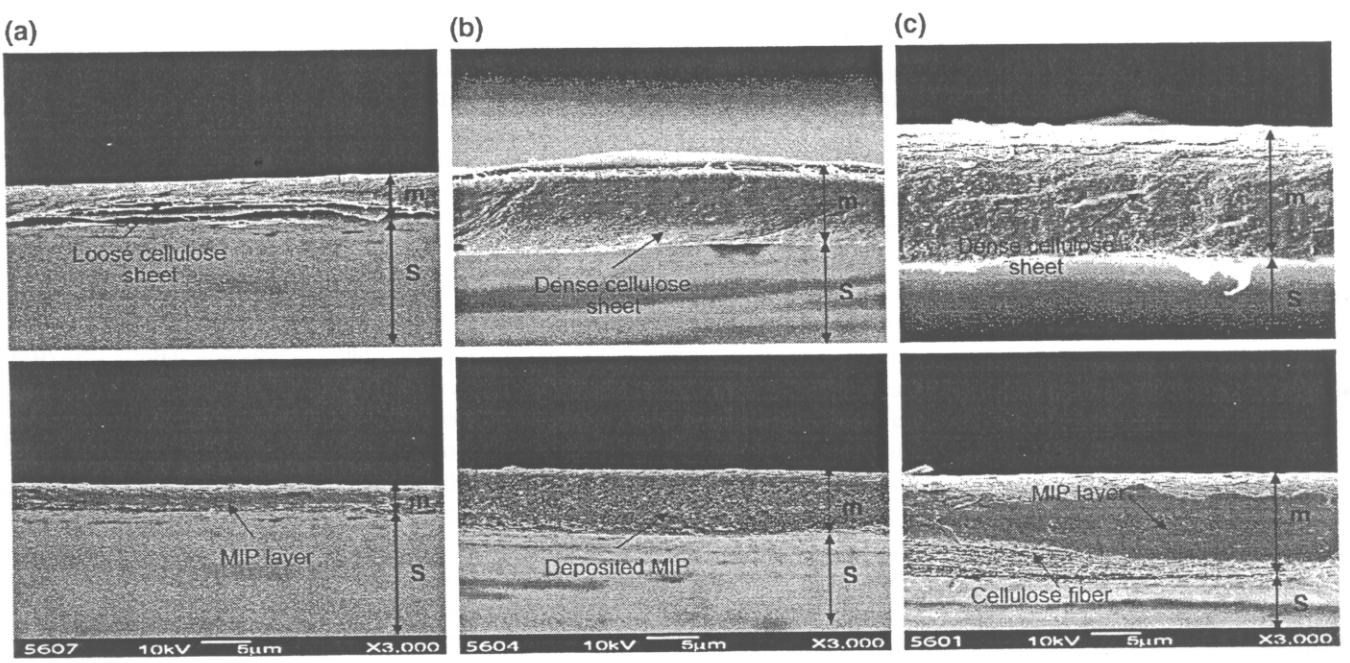


Fig. 2. SEM cross-section images of the initial cellulose membranes (top) and the MIP modified membranes (bottom), showing the different membrane thicknesses with differed membrane resistances. (a) 5 μm/1 Ω cm^2 membrane (b) 10 μm/2 Ω cm^2 membrane and (c) 15 μm/4 Ω cm^2 membrane. Picture designation: s=stub, m=membrane.

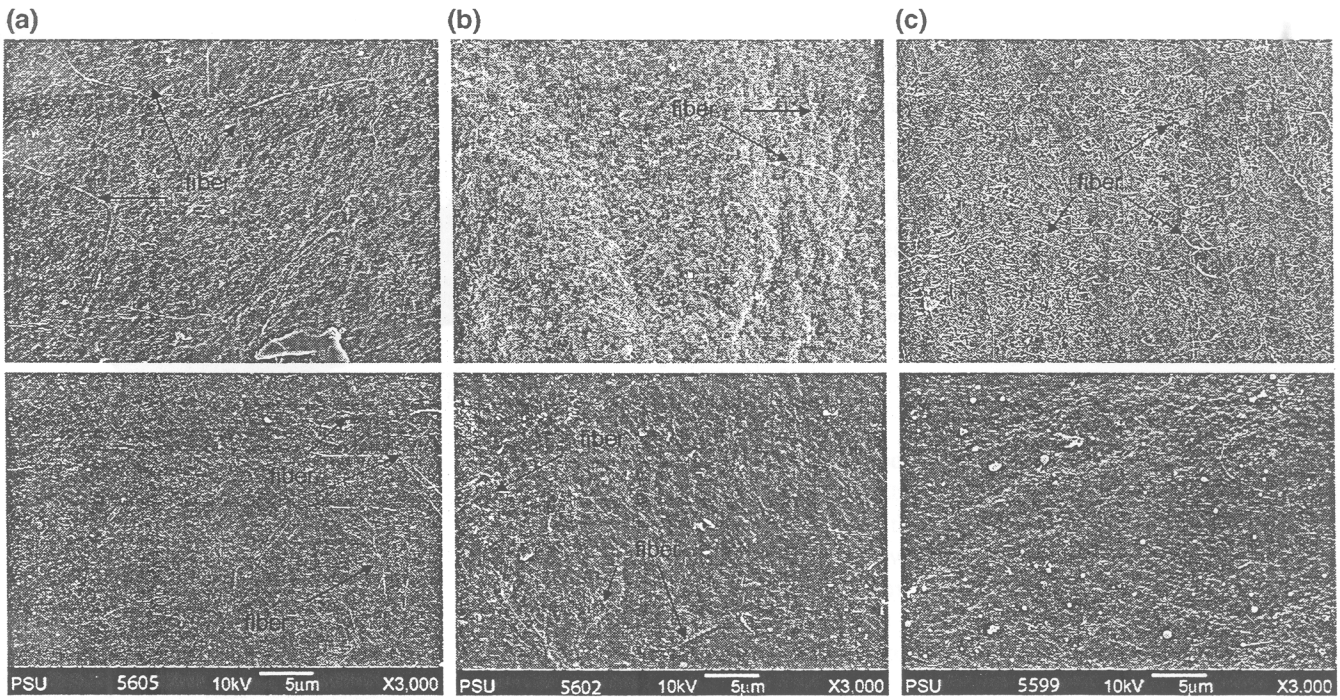


Fig. 3. Surface morphology of the initial cellulose membranes (top) and the MIP modified membranes (bottom). (a) 5 μm/1 Ω cm^2 membrane (b) 10 μm/2 Ω cm^2 membrane and (c) 15 μm/4 Ω cm^2 membrane.

membrane resulted in a reduction in the specificity of S-MIP membrane. This phenomenon may be attributed to the effects of a gradient in DMF concentration being established in the monomer mixture near the surface of the membrane during the imprinting process. Such a gradient would be expected to lead to an alteration in the density of the created polymer network dependent upon depth of the grafted MIP layer. Supporting evidence for this can be seen from the SEM images (Fig. 2) with much of the MIP not fully penetrating into the membrane pores of the thickest (15 μm) membrane but accumulating at the surface. Since the 5 μm MIP membrane enabled the highest diffusion rates of the enantiomers to be obtained with the greatest imprinting effect, this membrane was selected for further study as a basic membrane for the development of a composite MIP membrane, which might be applied to transdermal delivery.

It was necessary to confirm that the basic membrane prepared with the method described in Section 2.2 each time provided similar appearances and release properties. Therefore the thinnest cellulose membranes were prepared on a number of occasions and their morphology examined and the mechanical strength determined. The percentage coefficient of variation (CV) for the thickness of the non-modified cellulose membranes was found to be 5% ($n=6$). Membrane porosity was found to vary from 1.4% to 2.4%, with the average pore size 170 nm. The %CV of the partition coefficients and enantioselectivity of the modified cellulose membranes obtained for three different batches of the initial membrane were found to be 5.0% and 10.0%, respectively. The mechanical stability of the cellulose membranes was not damaged during grafting process. The stability of cellulose membrane after modification was good, since the membrane could be reused after washing and similar release characteristics and selectivity were obtained.

3.2. Characterisation of composite MIP membranes

The general properties of 5 μm S-MIP membranes were compared with those of NIP membrane and R-MIP membranes, which were all produced from the same basic cellulose membrane (Table 2). The values obtained for the electrical resistance of membranes provided an indication of the leakage of membrane and it was found that the electrical resistance of the cellulose membrane increased upon modification. This was presumably a consequence of the potential occlusion of the pores due to modification with the copolymer. An increase in the tensile strength of the membranes after modification was also found, possibly as a consequence of the introduction of a rigid copolymer of MAA/EDMA within the pores of cellulose membrane. The average pore diameters obtained by SEM, however, proved to be very similar, within the range 150–200 nm. The results presented in Table 2 indicated that cellulose membranes swelled greatly in aqueous solvent confirming the hydrophilic and highly porous nature, which promotes water absorption capacity. Swelling of the non-modified cellulose membranes in citrate buffer (pH 5.5) is clearly inherently greater than when they are placed in phosphate buffer (pH 7.4). When EDMA-MAA based polymers are bound at the pore surface, this causes a decrease in the degree of swelling. It might also have been expected that the introduction of ionizable carboxylic acid groups would have resulted in an increased swelling of the composite membrane at the higher pH. However the (modified) cellulose membrane still shows a greater swelling at

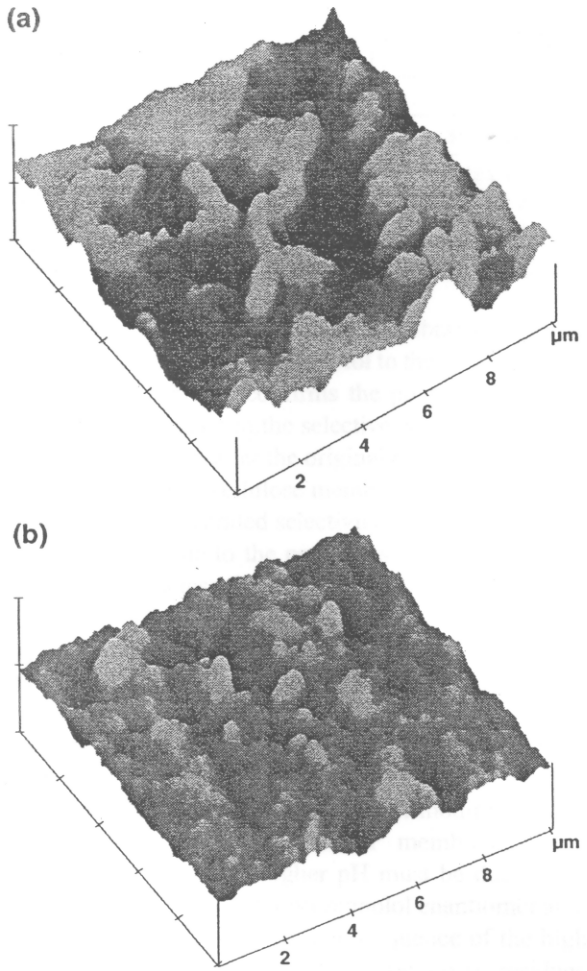


Fig. 4. Three-dimensional AFM image of (a) 5 μm cellulose membrane and (b) 5 μm S-MIP membrane.

pH 5.5 than at pH 7.4. This suggesting that the pH-dependency in the swelling of the MIP composite cellulose membranes is governed predominantly by the cellulose membrane.

3.3. The stability issues of propranolol enantiomers

The stability of *R*- and *S*-propranolol in pH 5.5 citrate and pH 7.4 phosphate buffer solutions was investigated at room temperature (30 °C), 37 °C and in DMF solvent at 60 °C since the propranolol enantiomers were used under these conditions. The results showed that more than 99% content of both *R*- and *S*-propranolol was found after 7 days incubation in buffer solution at room temperature and 37 °C, indicating good

Table 2
Characteristics of cellulose and modified cellulose membranes (5 μm thickness)

Membrane	Membrane resistance (Ω cm^2)	Tensile strength (kN/m^2)	Pore size (nm)[a]	Degree of swelling (%)[b]	
				pH 5.5	pH 7.4
Cellulose	1.11	3.16	177.9±35.4	101.25±9.21	55.57±5.72
NIP	1.71	8.23	146.3±36.5	74.30±9.94	34.01±3.30
R-MIP	1.71	7.20	176.9±47.8	77.61±8.62	33.41±3.82
S-MIP	1.71	7.20	198.3±4.2	72.36±3.81	44.44±9.84

[a] Refer to mean pore size estimated from surface pictures of membrane obtained by SEM (n=10).
[b] Mean±SE, n=3.

stability of the enantiomers under these conditions. At 60 °C, where the polymerization procedure of the imprinting was effected, it was found that 14.7±3.0% (n=3) racemised when the pure *R*-enantiomer was incubated alone in DMF for 24 h. In contrast, under the same conditions only 1.5±0.3% (n=3) of the pure *S*-isomer was found to racemise. However when the individual *R*- and *S*-propranolol enantiomer was incubated in chloroform at temperature 60 °C, both remained stable with no racemisation of enantiomer after 24 h.

This indicates that the type of solvent in which the enantiomers are dissolved affects the stability of the propranolol enantiomers. Therefore it is possible that if racemisation occurs during imprinting then the selectivity of the final MIP might be decreased. However the total recovery of enantiomer from the template was found to be 98% and less than 1% racemisation had been found to occur in either individual enantiomer. The stability of the enantiomer in DMF could be improved by the presence of the polymer mixture since the template molecule might be expected to preferentially interact with the MAA functional monomer rather than the solvent. As a consequence it could position the pendent optically active center inside the forming polymer network, thereby limiting any racemisation which might otherwise have occurred during the polymerization.

3.4. Enantiomer uptake and imprinting effect

The partition coefficient of propranolol enantiomers from aqueous solutions into S-MIP membranes was compared with that for cellulose membrane, NIP membrane and R-MIP membrane in order to determine the effects of the imprinting procedure (Table 3). In pH 5.5 buffer, the partition coefficients of both *R*- and *S*-isomers decreased with the modification of cellulose membrane, while in pH 7.4 buffer the partition coefficients of propranolol enantiomers were generally increased by

Table 1
Partition (K) and diffusion coefficients (D, cm h^{-1}) of propranolol into/through MIP modified cellulose membranes of different thicknesses and membrane resistances at room temperature (mean±SE, n=3)

Solute[a]	5 μm/1 Ω cm^2 membrane		10 μm/2 Ω cm^2 membrane		15 μm/4 Ω cm^2 membrane	
	$K\times10^3$	D ($\times10^{-6}$ cm h^{-1})	$K\times10^3$	D ($\times10^{-6}$ cm h^{-1})	$K\times10^3$	D ($\times10^{-6}$ cm h^{-1})
R	1.01±0.22	0.70±0.26	1.59±0.18	3.46±0.41	0.37±0.20	6.11±1.23
S	1.79±0.22	30.84±2.51	1.56±0.38	8.10±1.82	0.29±0.16	6.62±0.73
S/*R*	1.77±0.58	44.06±2.96	0.98±0.25	2.34±0.42	0.78±0.12	1.08±0.09

[a] Refer to *R*- or *S*-isomer of propranolol.

Table 3
Partition coefficient ($K \times 10^3$) of *R*- and *S*-propranolol enantiomers from different donor pH solutions into cellulose and modified cellulose membranes (5 μm thickness), at room temperature (mean ± SE, $n=3$)

Membrane	pH 5.5			pH 7.4		
	R-isomer	*S*-isomer	Ratio *S/R*	*R*-isomer	*S*-isomer	Ratio *S/R*
Cellulose	1.25±0.36	1.38±0.33	1.10±0.14	1.26±0.62	1.53±0.62	1.21±0.01
NIP	0.78±0.28	0.93±0.25	1.19±0.20	1.83±0.95	2.32±0.95	1.27±0.05
R-MIP	1.14±0.25	1.29±0.21	1.14±0.13	1.35±0.34	1.57±0.34	1.16±0.11
S-MIP	0.70±0.20	0.94±0.22	1.35±0.08	1.01±0.22	1.79±0.22	1.74±0.38

the modification of the cellulose membrane. The apparent preferential sorption of *S*-propranolol to the non-modified cellulose membrane (Table 3) confirms the potential role of the parent cellulose membrane in the selective sorption of the propranolol enantiomers, even after the original membranes are transformed to MIP composite cellulose membranes. Thus the R-MIP membrane still demonstrated selectivity for the *S*-isomer, rather than the *R*-isomer, due to the properties of the cellulose membrane itself. Nevertheless the imprinting effect i.e. the difference between the *S/R* selectivity of the S-MIP membrane and that of the cellulose and NIP membrane was marked, suggesting that the molecular imprinting procedures have produced cavities with a higher affinity for the *S*-enantiomer of propranolol. At the higher pH the enantioselectivity of the membranes as determined by an increased partition coefficient for the *S*-isomer was not significant in the case of the non-modified cellulose membrane but it was for the S-MIP membrane. The enhanced enantioselectivity at a higher pH must be due to an increased binding of the favoured *S*-propranolol enantiomer at the binding site. This is most likely as a consequence of the higher degree of ionization of the functional monomer residues (pK_a of MAA < 4) at the higher pH, resulting in an increased electrostatic interaction with the secondary amine of the propranolol molecule [19].

3.5. Selective transport of composite MIP membranes

Preliminary studies using an initial concentration of 40 μg ml^{-1} racemic propranolol established that a steady state flux was achieved during a period of 2–6 h (Fig. 5). *R*- and *S*-propranolol enantiomers were transported faster across the unmodified cellulose membranes than those that had been modified, indicating the pore-filling effect of modified cellulose membranes. The faster transport of the *S*-enantiomer in comparison to the *R*-enantiomer confirms the inherent enantioselectivity of the modified cellulose membrane, indicated by the partition data. The S-MIP membrane was found to limit the permeation of *R*-enantiomer, and none was detected in the receptor compartment for at least 6 h, indicating the effects of the imprinting process on the pore structure. However the transport of the *S*-enantiomer through the R-MIP membrane was still faster than that of the *R*-enantiomer. This suggests that the inherent enantioselectivity of the original membrane still dominates the process, despite the modification of some of the pores with polymer synthesised in the presence of the *R*-enantiomer. The *R*-isomer was transported faster through the R-MIP membrane than through either the NIP or S-MIP membranes, as a consequence of the *R*-selectivity conferred on some of the membrane pores. These results suggest that the enantiomers are transported via a number of routes across the composite membranes. The cellulose fibers and/or the unmodified pores appear to have inherent selectivity for *S*-enantiomer whereas the modified pores will display selectivity for the template enantiomer. The pore-filling effects of the polymer do appear to reduce the transport of the *S*-enantiomer across the S-MIP membrane in comparison to the unmodified membrane. However the advantage of employing the

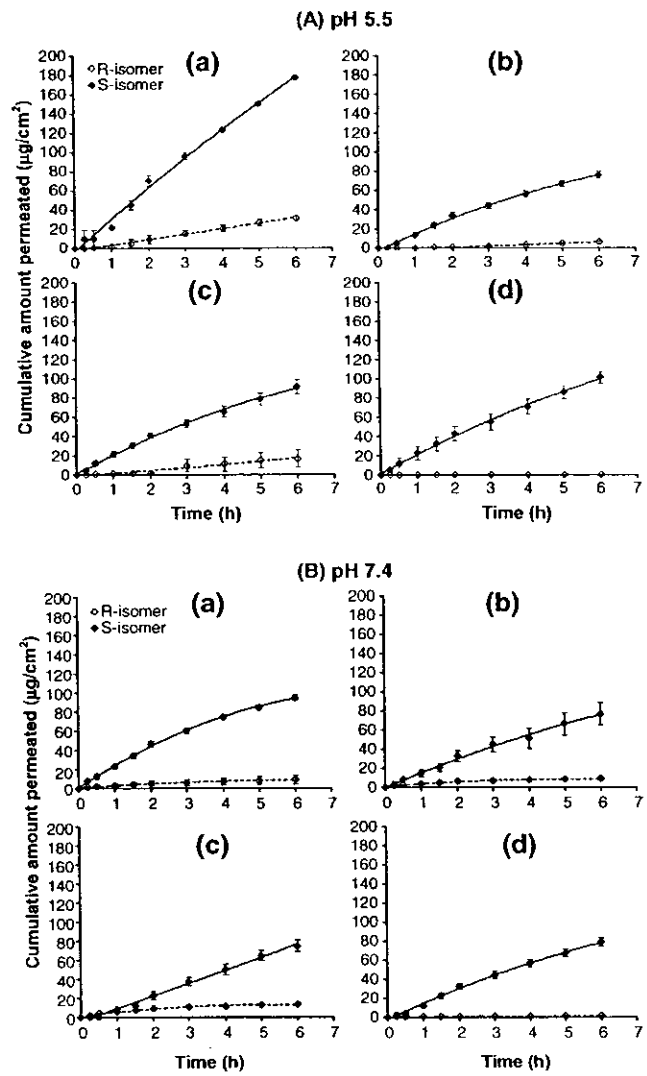


Fig. 5. The transport of *R*- and *S*-propranolol enantiomers (HCl) as a function of time (mean ± SE, $n=3$) from (A) pH 5.5 citrate buffer and (B) pH 7.4 phosphate buffer across (a) cellulose membrane, (b) NIP membrane, (c) R-MIP membrane and (d) S-MIP membrane.

S-MIP as opposed to the unmodified membrane is that transport of the *R*-enantiomer over the 6 h of the study was eliminated in the former.

The *S*-isomer/*R*-isomer selectivity of the propranolol enantiomers across the S-MIP membranes from pH 7.4 buffer was ca. 45, while the *S*-isomer/*R*-isomer ratio obtained with cellulose, NIP or R-MIP membranes was below 5. For a donor solution with pH 5.5, the *S*-isomer/*R*-isomer selectivity of S-MIP membrane was ca. 80 (the amount of transported of *R*-isomer was taken assuming the limit of detection was reached after 6 h) whereas the *S*-isomer/*R*-isomer selectivity of cellulose, NIP and R-MIP membranes was less than 20.

It should also be noted that the faster drug transport rate at the lower pH correlates with the higher degree of swelling of the membrane (Table 2). Such a swelling at pH 5.5 might alter the net pore size of cellulose membrane and partially account for the increased transport of the enantiomers. It is apparent also that the transport of *S*-propranolol and the enantioselectivity was higher for the S-MIP membrane at pH 5.5 as compared to pH 7.4. Two potentially opposing mechanisms are likely to affect the fluxes of the enantiomers at the two different pH values employed in this study. At the acidic pH, the increased swelling of the basic cellulose matrix would be expected to promote the overall transport of propranolol. However when the pH is raised to 7.4 then the improved MIP recognition, and increased electrostatic binding of the *S*-enantiomers to the S-MIP membrane might reduce the overall transport rate, as a consequence of binding and re-binding at sites within the MIP modified pores. Whilst, at pH 5.5 the release of the *S*-propranolol enantiomer absorbed on the cellulose membrane or the MIP/NIP modified cellulose membranes might be expected to increase with swelling of membrane, which leads to change in the mass transfer rate of the enantiomer and the improved efficiency in enantioselectivity at the lower pH. It is apparent that cellulose membranes provided a degree of enantioselective transport for propranolol, but that this is lower than that achieved using the S-MIP membrane. The preferential sorption of *S*-propranolol to cellulose enhances the transport selectivity of S-MIP composite membranes and limits the involvement of the *R*-enantiomers in such a diffusive pathway. Therefore, although it would appear that the selective transport of *S*-propranolol obtained using a composite cellulose membrane is primarily determined by the parent cellulose membrane, a beneficial contribution is derived from the MIP component.

The selective release characteristics of MIP grafted onto the pore surface of the cellulose membrane concurs with the 'fixed-carrier' mechanism that has been proposed for the selective transport achieved via ion-exchange carrier membranes. The facilitated transport conferred by a 'fixed-carrier' site membrane involves adsorption and mobility of the target molecule at the 'fixed-carried' site. Previous work has shown that MIPs formed as a membrane [20–22] or prepared into a matrix tablet [23,24] can provide a facilitated and selective transport of the template molecule. The means of controlled release involves reversible complexation and exchange between the template molecule and sites in the MIP membrane (or the MIP tablet). The *S*-isomer of propranolol binds selectively to the MIP binding sites, with its

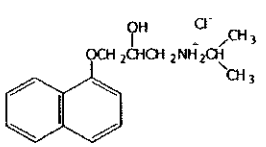
Table 4
Partition (K) and diffusion (D) coefficients of propranolol prodrugs into and through non-modified cellulose and modified cellulose membranes at different pH values at room temperature (mean±SE, $n=3$)

Compound	Membrane	K ($\times 10^3$)			D ($\times 10^{-6}$ cm h^{-1})					
		pH 5.5			pH 7.4			pH 5.5			pH 7.4		
		R	*S*	*S/R*	R	S	*S/R*	*R*	*S*	*S/R*	R	*S*	*S/R*
VL-P	Cellulose	1.11±0.51	1.38±0.93	1.20±0.39	1.26±0.14	1.63±0.68	1.43±0.69	62.3±4.52	77.9±3.84	1.26±0.07	64.3±9.74	70.7±13.7	1.08±0.06
	NIP	1.88±0.30	2.10±0.45	1.09±0.07	1.26±0.38	1.67±0.73	1.32±0.12	77.8±4.94	86.8±5.57	1.12±0.09	69.8±5.03	64.4±7.48	0.94±0.15
	S-MIP	0.74±0.21	1.38±0.22	1.71±0.17	0.74±0.29	1.53±0.19	2.11±0.17	57.6±2.57	95.9±10.5	1.68±0.22	32.8±8.61	66.7±5.81	2.33±0.56
SN-P	Cellulose	1.30±0.38	1.82±0.53	1.42±0.10	1.03±0.25	0.94±0.30	0.91±0.11	38.8±8.00	51.3±8.53	1.32±0.93	28.7±4.77	34.1±9.48	1.25±0.38
	NIP	1.61±0.12	1.94±0.38	1.16±0.16	0.26±0.17	0.22±0.18	0.85±0.24	29.3±6.91	42.9±11.5	1.46±0.31	23.4±2.61	26.6±3.60	1.13±0.04
	S-MIP	0.62±0.17	0.74±0.09	1.36±0.17	0.44±0.23	0.60±0.33	1.31±0.11	32.8±3.51	50.5±7.01	1.57±0.24	27.1±4.35	29.5±5.99	1.23±0.37
CP-P	Cellulose	0.68±0.12	0.82±0.21	1.20±0.52	0.83±0.02	1.44±0.11	1.73±0.17	69.8±7.48	73.2±9.64	1.08±0.15	38.2±0.23	44.5±7.82	1.17±0.32
	NIP	1.28±0.23	1.44±0.34	1.12±0.13	0.85±0.07	0.82±0.34	0.98±0.30	52.8±2.64	64.0±10.4	1.21±0.17	49.3±1.53	46.3±12.2	0.93±0.23
	S-MIP	1.02±0.53	1.58±0.23	1.55±0.59	1.59±0.04	2.62±0.20	1.65±0.09	59.5±3.79	68.2±7.58	1.18±0.22	49.4±6.90	61.5±18.1	1.22±0 .30

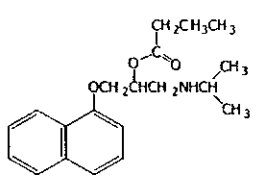
subsequent reactive diffusion taking place by stepwise dissociation and selective binding to neighboring MIP sites. From the results obtained, it shows that the binding of *S*-propranolol enantiomer to MIP is reversible and fast enough to enable the transport of *S*-enantiomer. The *S*-propranolol complexing to and dissociating from the MIP plays an important role in the enantioselectivity of S-MIP membrane. Any factor that alters in rate of complexation/decomplexation of *S*-propranolol enantiomer with MIP will change the flux and stereoselectivity of the S-MIP composite membrane. In addition as indicated previously, the basic cellulose membrane in the S-MIP membrane plays a crucial role towards the transport of propranolol enantiomers. Macroscopic changes such as degree of hydration and pH of the medium have a marked effect on transport rate through changes in effective pore size and alteration of enantiomer-membrane partition (Table 3). Indeed any change in the latter will have a direct effect on transport role, since Fick's first law indicates that the rate is dependent upon partition coefficient.

3.6. Selectivity of composite MIP membrane for propranolol prodrugs

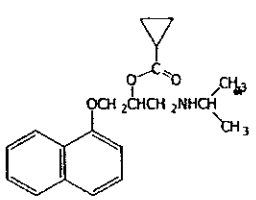
The propranolol prodrugs employed in this study were valeryl propranolol (VL-P) cyclopropanoyl propranolol (CP-P) and succinyl propranolol (SN-P) (see chemical structures below), which were synthesized from racemic propranolol hydrochloride and fatty acid chlorides by substituting different alkyl groups onto the hydroxyl group, according to the method described previously [25].



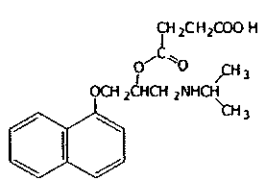
Propranolol HCl (clog*P* = 2.7)



Valeryl propranolol (VL-P) (clog*P* = 5.2)



Cyclopropanoyl propranolol (CP-P) (clog*P* = 3.9)



Succinyl propranolol (SN-P) (clog*P* = 1.1)

The partitioning of racemic prodrugs from saturated solutions was determined at both pH 5.5 and pH 7.4 (Table 4). Generally the *S*-enantiomers were sorbed to both the non-modified and modified cellulose membrane in greater quantities than the *R*-isomer at both pH values. The *S*-enantioselectivity of the unmodified cellulose membrane was apparent for all prodrugs, with the exception of SN-P at the higher pH. As in the case of the parent propranolol molecule, the S-MIP modification of the membrane improved the *S*-enantioselectivity further. Again the enantioselectivity proved to be generally higher at pH 7.4. There are many factors that affect the sorption of propranolol prodrugs on the membranes. Since the ester modification of hydroxyl group increases the hydrophobicity of the propranolol molecule, i.e., in case of VL-P and CP-P, interaction between propranolol enantiomers and cellulose might be expected to be reduced. The introduction of different bulkier side chains would be expected to reduce the interaction with any recognition site and the diffusion coefficients of VL-P and CP-P *S*-isomers through the S-MIP membrane (Table 4) were markedly higher than that of *S*-propranolol HCl (Table 1). However the diffusion of the *S*-isomer of the most hydrophilic prodrug, SN-P was comparable to that of the parent compound. Since enantioselectivity is preserved when the prodrugs are employed as the diffusing species, this demonstrates that the hydroxyl group, present in propranolol is not crucial. It is apparent that it is the position of the charged nitrogen group within the partitioning and diffusing species, relative to the carboxylic acid residues of MAA that is the dominant mechanism involved in the interaction between the enantiomers and substrate.

A preliminary examination of the energy-minimised structures of *S*-propranolol and its prodrugs (Fig. 6) suggests that VL-P might be expected to adopt a conformation most similar to the parent compound. The principal interaction, as indicated previously, is likely to be between the secondary amine group within the molecule(s) and the carboxylic acid group of the functional monomer. However the pendant groups within VL-P would appear to be most similarly positioned to the parent molecule in comparison to the other prodrug species, such that interaction with active sites in the MIP might occur more readily.

3.7. Transdermal enantioselective-controlled release of composite MIP membranes

The in vitro percutaneous permeation study of propranolol enantiomers was investigated by applying drug to cellulose, NIP or S-MIP membranes in direct contact with isolated excised

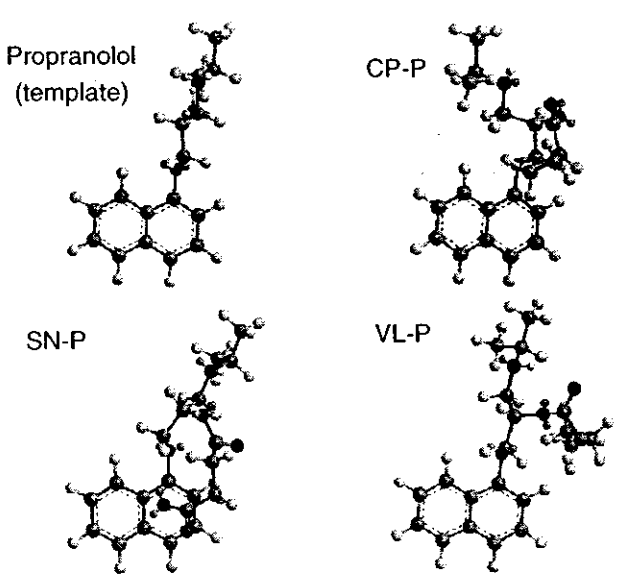


Fig. 6. The energy-minimised Chem3D structures of *S*-propranolol and *S*-propranolol prodrugs as calculated by molecular mechanics using MM2 force field (Chem3D Ultra 8.0, CambridgeSoft, MA, USA).

rat skin contained in a Franz cell. The initial concentration of racemic propranolol (HCl) in the donor phase was varied from 100 to 300 μg ml^{-1}. Control experiments were also carried out to determine the percutaneous transport of drug across skin alone.

The cumulative amounts of propranolol transported across rat skin as a function of time, when drug was applied to the membrane positioned in place on the skin surface are shown in Fig. 7. The resulting curves indicate that the diffusion of *R*-propranolol across the S-MIP membrane-skin layer is delayed whereas a relatively facilitated transport of *S*-propranolol across S-MIP membrane through the rat skin occurred over the initial 18 h of transport for every donor concentration. The percutaneous transfer of the *S*-enantiomer across cellulose membrane, was less than if the S-MIP membrane was in situ and was higher than the *R*-form across both membrane types. The percutaneous permeation of both *R*- and *S*-enantiomers of propranolol across NIP membranes applied to the skin was initially delayed, which is possibly due to the MAA-EDMA steric hindrances which might occur in the pores of the modified basic cellulose membrane. NIP membranes allowed slightly faster *S*-propranolol enantiomer transport than for the *R*-propranolol enantiomer. There is little facilitated *S*-propranolol enantiomer transport across NIP membranes and this does not increase markedly as the initially applied concentration of drug is increased. In addition, *S*/*R* selectivity in terms of the permeability coefficient through NIP membranes is not significantly changed with donor concentration (Table 5).

At a donor concentration of 100 μg ml^{-1}, the transport of propranolol enantiomers through skin alone was very low and the flux rates of the two enantiomers were found to be similar. Increasing the donor compartment concentrations (to 200 and 300 μg ml^{-1}) resulted in a greater diffusion rate through the skin layer in the absence of membrane, but no enantiomeric differences in transport were observed initially. However, after

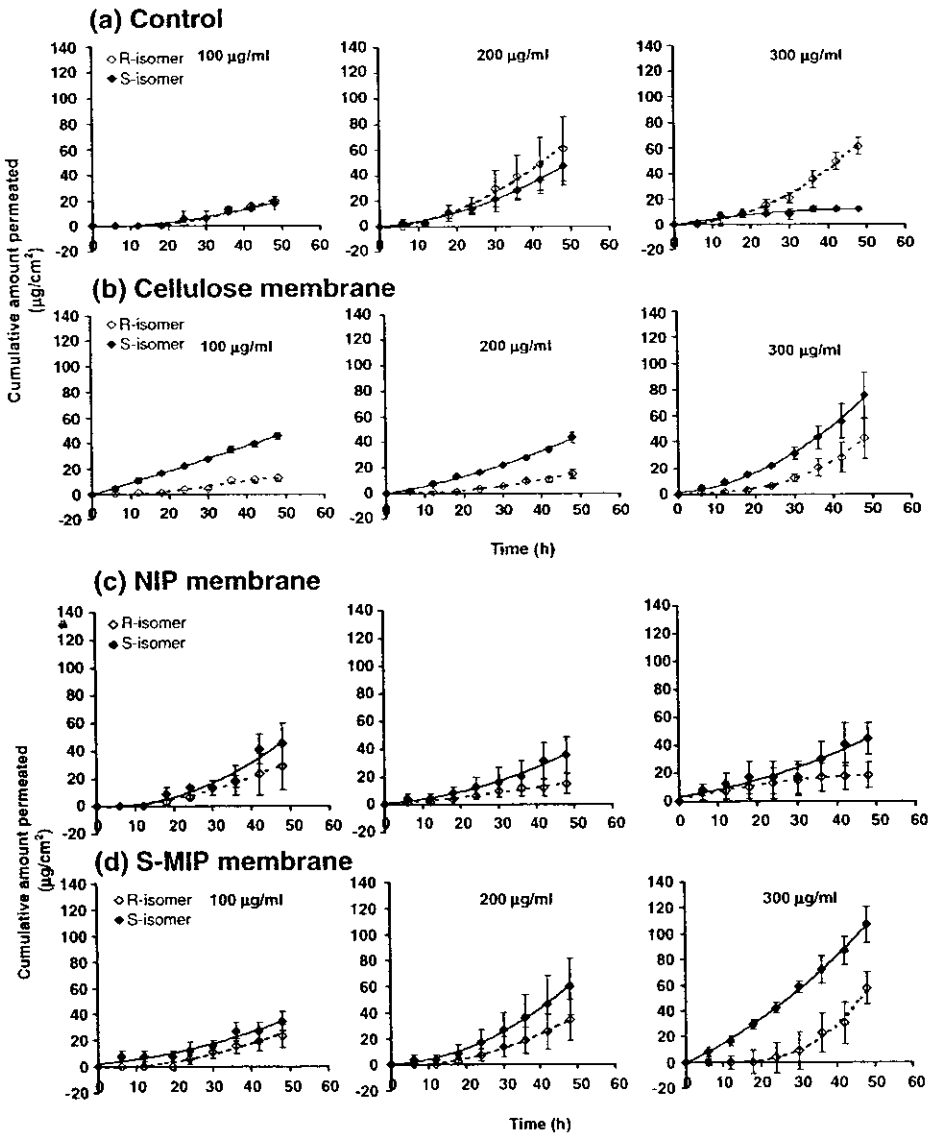


Fig. 7. The permeation of propranolol enantiomers (HCl) from pH 7.4 buffer solution (a) across full-thickness rat skin at 37 °C in the absence of membrane or under the same conditions but with a (b) cellulose membrane, (c) NIP membrane or (d) S-MIP membrane placed on the surface of the skin (mean±SE, n=6). The initially applied donor concentrations of racemic propranolol were 100, 200 and 300 μg ml^{-1}.

Table 5
Steady state flux (J_{ss}), permeability coefficient (K_p), and lag time (τ) of *R*- and *S*-propranolol from pH 7.4 buffer solution containing racemic propranolol at different concentrations through full-thickness rat skin alone or for transport through cellulose, NIP or S-MIP membrane applied to the skin surface (mean+SE, $n=6$)

Donor concentration (μg/ml)	Membrane	J_{ss} (μg cm^{-2} h^{-1})		$K_p \times 10^{-3}$ (cm h^{-1})			τ (h)		
		R-isomer	*S*-isomer	*R*-isomer	*S*-isomer	*S/R* ratio	*R*-isomer	*S*-isomer	*S/R* ratio
100	Control	0.30±0.04	0.28±0.01	1.01±0.13	2.10±0.15	1.41±0.38	18.67±0.67	16.76±0.38	0.90±0.05
	Cellulose	0.24±0.04	0.54±0.05	2.43±0.45	5.39±0.62	2.38±0.48	12.07±0.76	1.64±1.40	0.13±0.11
	NIP	0.45±0.05	0.73±0.07	4.46±0.49	7.28±0.65	1.69±0.29	14.07±0.67	11.33±1.33	0.81±0.02
	S-MIP	0.36±0.07	0.66±0.02	3.59±0.72	6.60±0.04	2.04±0.52	18.85±0.85	5.69±1.69	0.30±0.10
200	Control	0.91±0.21	0.52±0.03	4.55±1.03	2.59±0.19	0.57±0.90	10.31±0.61	8.87±0.90	0.87±0.10
	Cellulose	0.24±0.03	0.48±0.01	1.20±0.13	2.39±0.06	2.07±0.04	15.72±3.00	5.90±0.02	0.44±0.11
	NIP	0.32±0.09	0.50±0.01	1.61±0.43	2.49±0.07	1.87±0.62	8.88±0.85	10.57±0.57	1.46±0.18
	S-MIP	0.49±0.05	1.10±0.02	2.46±0.22	5.50±0.10	2.27±0.23	16.23±1.44	4.65±0.60	0.30±0.60
300	Control	1.01±0.14	0.33±0.16	3.33±0.45	1.09±0.54	0.33±0.18	9.54±1.87	6.00±0.01	0.63±0.13
	Cellulose	0.55±0.04	0.72±0.16	1.84±0.86	2.39±0.52	1.71±0.35	12.45±0.90	9.48±2.90	0.75±0.20
	NIP	0.44±0.12	0.68±0.02	1.48±0.38	2.28±0.07	1.74±0.38	1.33±0.94	1.72±1.30	1.04±0.24
	S-MIP	0.52±0.06	1.73±0.36	1.73±0.36	5.76±1.20	3.50±1.01	17.58±4.46	2.45±1.77	0.11±0.06

12 h the diffusion of *R*-enantiomer across the skin into receptor phase was found to be significantly higher than that of the *S*-enantiomer. The stereoselective penetration of propranolol through excised rat skin has been reported by Miyazaki et al. [26] who showed that the percutaneous permeation of *S*-propranolol was 4 times higher than that of *R*-propranolol. These latter results were, however, contrary to the results reported by Heard et al. [27] when the transfer of propranolol from sub-saturated solutions across rat skin was not found to be an enantioselective process. The enantioselective transfer of propranolol across rat skin therefore remains unclear. However, the results obtained in this study indicate that although an enantiomeric difference in the permeation of *R*- and *S*-propranolol through rat skin was shown, particularly after 18 h application, an enantioselective-controlled transport was obtained when the S-MIP membrane was placed in situ on the skin. The flux of *S*-isomer was increased in comparison to the control when the S-MIP membrane was in situ for all test concentrations, with enhancement ratios of ca. 2–5 being obtained for the different drug concentrations employed (Table 5). The flux of *R*-isomer was reduced with the S-MIP membrane in situ compared with controls at donor concentrations of 200 and 300 μg ml^{-1}. Due to higher permeability and greater partitioning of *S*-propranolol from S-MIP membrane, the lag time of *S*-isomer was reduced when the membrane was applied as compared to control. However the lag times of the *R*-isomer were found generally to increase in the presence of the S-MIP membrane compared to those obtained in control experiments.

Furthermore, the permeability coefficients were greater for the *S*-isomer through the S-MIP membrane and skin than skin alone, whatever the initial donor drug concentration ($P<0.05$). The permeability coefficients of *R*-enantiomer through S-MIP membrane were generally lower when the S-MIP membrane was in situ than through skin alone. The higher permeability coefficients observed for the *S*-enantiomer would appear to confirm the enantioselective delivery properties of S-MIP membrane. When the concentration of racemic propranolol was increased, the lag time for the permeability of *R*-isomer through the S-MIP membrane and skin appeared to be relatively constant. This was in contrast, to the change in lag times observed when the *S*-isomer was applied since these were reduced, as the initial drug concentration was increased (Table 5). This caused the *S/R* ratio of lag time for the transport of propranolol through S-MIP membrane to remain constant or even be reduced as the initial applied concentration was increased. However the results indicated that increasing the donor concentration promoted the stereoselective transport of S-MIP membrane.

The results obtained in this study indicate that the enantioselectivity shown with S-MIP membrane placed on rat skin is related to the high combined affinity of cellulose and S-MIP for the *S*-propranolol along with the lesser affinity for *R*-enantiomer inherent in the cellulose and induced in the S-MIP by the imprinting process. The enantioselective-controlled release of S-MIP membrane was clearly apparent when racemic propranolol was placed in the donor compartment at concentrations up to 300 μg ml^{-1}. Beyond this concentration, i.e., 400 μg ml^{-1}, the stereoselectivity was reduced (data not shown). The reason for this might involved the saturation of binding sites within the excised rat skin.

4. Conclusions

The current study demonstrated the potential of MIP molecularly imprinted polymer composite membranes based on cellulose in controlling the release of *S*-propranolol into the skin. The degree of stereoselectivity demonstrated would result in considerably higher therapeutic advantage when considering the differential pharmacological activities of the two enantiomers of propranolol. The cellulose membranes containing modified pores and a surface with imprinted polymer selective for *S*-propranolol were capable of limiting release of the distomer. However, it remains to be determined if the results observed can be reproduced in vivo.

Acknowledgements

Financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0160/2545) is acknowledged. The authors would like to thank Miss Kemmarut Bouking for assistance in cultivation processes.

References

[1] J.G. Riddel, D.W.G. Harron, R.G. Shanks, Clinical pharmacokinetics of β-adrenoceptor antagonists, Clin. Pharmacokinet. 12 (1987) 305–320.

[2] A. Parkinson, Biotransformation of xenobiotics, in: C.P. Klaassen (Ed.), Casarett and Doulls Toxicology, the Basic Science of Poisons, International Edition, 1st edition, McGraw-Hill, New York, 1996, pp. 152–153.

[3] M.J. Quigley, G.M.C. Jordan, F.R. Timoney, The synthesis, hydrolysis kinetics and lipophilicity of *O*-acyl esters of propranolol, Int. J. Pharm. 101 (1994) 145–163.

[4] B.D. Anderson, W.W. Chu, R.E. Galinsky, Reduction of first-pass metabolism of propranolol after oral administration of ester prodrugs, Int. J. Pharm. 43 (1988) 261–265.

[5] A. Buur, H. Bundgaard, V.H.L. Lee, Prodrugs of propranolol: hydrolysis and intramolecular aminolysis of various propranolol esters and oxazolidin-2-one derivative, Int. J. Pharm. 42 (1988) 51–60.

[6] A.M. Barrett, V.A. Cullum, The biological properties of the optical isomers of propranolol and their effects on cardiac arrythemias, Br. J. Pharmacol. 34 (1968) 43–55.

[7] S. Ahmed, T. Imai, Y. Yoshigae, M. Otagiri, Stereoselective activity and nature of metabolizing esterases for propranolol prodrug in hairless mouse skin, liver and plasma, Life Sci. 61 (1997) 1879–1887.

[8] S. Ahmed, T. Imai, M. Otagiri, Stereoselective hydrolysis and penetration of propranolol prodrugs: in vitro evaluation using hairless mouse skin, J. Pharm. Sci. 84 (1995) 877–883.

[9] M. Ulbricht, Review, Membrane separations using molecularly imprinted polymers, J. Chromatogr. B, Biomed. Sci. Appl. 804 (2004) 113–125.

[10] S. Piletsky, T.L. Panasyuk, E.V. Piletskaya, I.A. Nicholls, Receptor and transport properties of imprinted polymer membranes—a review, J. Membr. Sci. 157 (1999) 263–278.

[11] M. Ulbricht, M. Belter, U. Langenhangen, F. Schneider, W. Weigel, Novel molecularly imprinted polymer (MIP) composite membranes via controlled surface and pore functionalizations, Desalination 149 (2002) 293–295.

[12] K. Hattori, K. Yoshimi, K. Sakai, Gate effect of cellulosic dialysis membrane grafted with molecularly imprinted polymer, J. Chem. Eng. Jpn. 34 (2001) 1466–1468.

[13] T.A. Sergeyeva, H. Matuschewski, S.A. Piletsky, J. Bendig, U. Schedler, M. Ulbricht, Molecular imprinted polymer membranes for substance-selective solid-phase extraction from water by surface photo-grafting polymerization, J. Chromatogr. A 907 (2001) 89–99.

[14] D. Klemm, D. Schumann, U. Udhardt, S. Marsch, Bacterial synthesised cellulose-artificial blood vessels for microsurgery, Prog. Polym. Sci. 26 (2001) 1561–1603.

[15] J.D. Fontana, A.M. De-Souza, C.K. Fontana, I.L. Torriani, J.C. Moreschi, B.J. Gallotti, S.J. De-Souza, G.P. Narcisco, J.A. Bichara, L.F.X. Farah, Acetobacter cellulose pellicle as a temporary skin substitute, Appl. Biochem. Biotechnol. 25 (1990) 253–264.

[16] C.M. Heard, R. Suedee, Stereoselective adsorption and trans-membrane transfer of propranolol enantiomer using cellulose derivatives, Int. J. Pharm. 139 (1996) 15–23.

[17] Y. Okamoto, M. Kawashima, K. Yamamoto, K. Hatada, Useful chiral packing materials for high-performance liquid chromatographic resolution. Cellulose triacetate and tribenzoate coated on macroporous silica gel, Chem. Lett. (1984) 739–742.

[18] R. Suedee, C.M. Heard, Direct resolution of propranolol and bupranolol by thin-layer chromatography using cellulose derivatives as stationary phase, Chirality 9 (1997) 139–144.

[19] J. Haginaka, Y. Sakai, Uniform-sized molecularly imprinted polymer material for (*S*)-propranolol, J. Pharm. Biomed. Anal. 22 (2000) 899–907.

[20] R.D. Noble, Generalized microscopic mechanism of facilitated transport in fixed site carrier membranes, J. Membr. Sci. 75 (1992) 121–129.

[21] T. Yamaguchi, C. Baertsch, C.A. Koval, R.D. Noble, C.N. Bow, Olefin separation using silver impregnated ion-exchange membranes and silver salt/polymer blend membranes, J. Membr. Sci. 117 (1996) 151–161.

[22] J. Mathew-Krots, K.J. Shea, Imprinted polymer membranes for the selective transport of targeted neutral molecules, J. Am. Chem. Soc. 118 (1996) 8154–8155.

[23] R. Suedee, T. Srichana, G.P. Martin, Evaluation of matrices containing molecularly imprinted polymers in the enantioselective-controlled delivery of β-blockers, J. Control. Release 66 (2000) 135–147.

[24] R. Suedee, T. Srichana, R. Chotivatesin, G.P. Martin, Stereoselective release behaviors of imprinted bead matrices, Drug Dev. Ind. Pharm. 28 (2002) 547–556.

[25] J.M. Quigley, C.G.M. Jordan, R.F. Timoney, The synthesis, hydrolysis kinetics and lipophilicity of *O*-acyl esters of propranolol, Int. J. Pharm. 101 (1994) 145–163.

[26] K. Miyazaki, F. Kaiho, A. Inagaki, M. Dohi, N. Hazemoto, M. Haga, H. Hara, Y. Kato, Enantiomeric difference in percutaneous penetration of propranolol through rat excised skin, Chem. Pharm. Bull. 40 (1992) 1075–1076.

[27] C.M. Heard, A.C. Watkinson, K.R. Brain, J. Hadgraft, In vitro skin penetration of propranolol enantiomers, Int. J. Pharm. 90 (1993) R5–R8.