Smart Polymeric Nanofibers for Topical Delivery of Levothyroxine

Anahita Fathi Azarbayjani¹, Jayarama Reddy Venugopal², Seeram Ramakrishna², Perry Fung Chye Lim¹, Yew Weng Chan³, Sui Yung Chan¹*¹

¹Department of Pharmacy, National University of Singapore, 18, Science Drive 4, 117543, Singapore
²Division of Bioengineering, National University of Singapore, 2 Engineering Drive 3, 119260, Singapore
³Department of Anaesthesiology, Singapore General Hospital, 169608, Singapore

Received, January 1, 2010; Revised, September 2, 2010; Accepted, September 22, 2010; Published, September 24, 2010

ABSTRACT - Topical administration of levothyroxine (T₄) helps to reduces deposits of adipose tissue on skin. The question is whether topical application of T₄ could lead to systemic effects. In the present study a series of nanofibrous membranes were electrospun into blends of poly vinyl alcohol (PVA) and poly-N-isopropylacrylamide (PNIPAM) to develop a sustained topical delivery of T₄. The polymeric nanofiber mats were characterized by field emission scanning electron microscopy (FESEM) and fourier transform infrared (FTIR) spectroscopy. In vitro permeation of the drug from the polymeric nanofibers was studied using excised human skin and the permeation mechanism investigated using confocal microscopy. It was observed that polymeric nanofibers were able to sustain the penetration of T₄ to the skin and help maintain the effective drug concentration in the skin layers for prolonged period. These formulations may have potential uses in topical products and can help to increase the accumulation of the active compound on the skin surface thus minimize the adverse side effects which may be caused by systemic absorption. This may result in consumer compliance, avoid frequent dosing and enhance the therapeutic effectiveness.

INTRODUCTION

Electrospinning technique is a novel method for generating polymeric nanoscale fibers. The nanosized fibers with high surface area-to-volume ratios and porosities are ideal for development of biosensors and biochips, drug delivery systems, wound healing and scaffold for tissue engineering (1-3). Biodegradable polymeric nanofibers are effective carriers for drug delivery and offer advantages such as reduced toxicity and increased therapeutic level and biocompatibility (4-6). Poly vinyl alcohol (PVA) is a hydrophilic polymer with distinct properties such as high degree of swelling, inherent non-toxicity and good biocompatibility (7). Nanofibers of PVA alone or blended with other polymers such as chitosan and gelatin have been studied (4, 5, 7-10). Blends of polymers have been widely used to enhance physical properties and physiological functionality of fiber mats (5, 10, 11).

Poly (N-isopropylacrylamide) (PNIPAM) is a thermally reversible hydrogel with a lower critical solution temperature (LCST) of around 32°C in water. The cross-linked gel of this material swells and shrinks at temperatures below and above the LCST respectively, therefore a PNIPAM delivery system can provide sustained therapeutic levels of a drug by responding to the physiological signals of the body (12, 13). PNIPAM nanoparticles, hydrogels and liposomes coated with PNIPAM have been extensively studied as controlled drug delivery systems (14-21). The potential toxicity of this polymer has been tested subcutaneously and results did not show any toxic effects (22).

Levothyroxine [3, 5, 3’, 5’-tetraiodothyronine (T₄)] is a synthetic hormone administered orally for the treatment of hypothyroidism and goiter (23, 24). T₄ stimulates lipid metabolism and induces lipolysis. Topical administration was reported to reduce deposits of adipose tissue in the skin (25-27). Presence of high concentration of T₄ in cosmetic creams raise questions if topical applications of these creams would lead to systemic absorption effects. Using radioactive marker, radioactivity was found in the plasma after skin application of T₄ (28). However recent in vivo studies using liposomal formulations were not able to detect any systemic effect (26).

Correspondence Author: Sui Yung Chan, Department of Pharmacy, National University of Singapore, Singapore
Email: phacsy@nus.edu.sg
Topical application of dimethyl-β-cyclodextrin (DM β-CD) retained T₄ on the skin without significant transdermal permeation (29). The aim of this work was to develop electrospun nanofiber mats of PVA, PNIPAM and PVA- PNIPAM complex as topical carriers for T₄. We intend to show that polymeric nanofibers can sustain the penetration of T₄ to the skin and help maintain the effective drug concentration in the skin layers for prolonged period while avoiding systemic absorption. The interactive effects of the two polymers on the resultant fiber were studied using FTIR and electron microscopy. Skin permeation of T₄ from these nanofibers was investigated using excised human skin.

MATERIALS AND METHODS

Materials
Poly vinylalcohol (Mw 70000-100000 and >98% hydrolyzed), poly (N-isopropylacrylamide) (Mw 20000-25000), levothyroxine, fluorescein and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (Singapore). All other reagents were of analytical grade.

HPLC Analysis
T4 concentration was quantified by HPLC from Agilent HP (USA). The analysis was carried out using an X-bridge column (3.5 μm, 4.6 mm × 100 mm; USA). Mobile phase was a 70:30 volume ratio of acetonitrile and 0.05 M phosphate buffer adjusted to pH 3 using phosphoric acid, flowing at a rate of 0.6 ml/min. UV detection at wavelength 220 nm and injection volume 100 μL gave a retention time of 9 min. Standard solutions of T4 (0.05 - 20 μg/ml) in 40% v/v ethanol were prepared. Coefficients of variation of intra- and inter-day measurements were < 5%.

Electrospinning of PVA/PNIPAM Nanofibers
Polymeric solutions were obtained by dissolving the drug and polymer in water (for PVA and PVA-PNIPAM) or in ethanol (for PNIPAM). Details of each formulation used in the electrospinning process are shown in Table 1. Fluorescein loaded PNIPAM solutions were developed for confocal imaging and studying the depth of the penetration of molecule from these fibers to the lower layers of the skin.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Polymer Concentration (%) w/v</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA</td>
<td>PNIPAM</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>Water</td>
</tr>
<tr>
<td>B</td>
<td>- 10</td>
<td>Ethanol</td>
</tr>
<tr>
<td>C</td>
<td>- 10</td>
<td>Water</td>
</tr>
<tr>
<td>D</td>
<td>10 5</td>
<td>Water</td>
</tr>
<tr>
<td>E</td>
<td>10 10</td>
<td>Water</td>
</tr>
</tbody>
</table>

* All formulations contain 2 mg/ml of T₄.

Nanofibers were fabricated using the electrospinning method. The electrostatic spinner used for the experiments was equipped with an adjustable DC power supply (RP50-1.25 R/230 DDPM, Gamma high voltage Research, USA), a syringe pump (KD-100, KD scientific, Inc., USA) on which a 3-ml syringe was connected to a blunt 27 G stainless steel needle. The applied voltage was set at 25 kV. The distance between the syringe and the fiber collector was kept constant at 13 cm for all experiments. A constant flow rate of 2 ml/h was applied to all the formulations. The depositions were performed at room temperature. The non-woven electrostatically spun fabric was removed from the collector and was dried under vacuum for a week at room temperature to remove residual solvent prior to usage.

Fourier Transform Infrared Measurements (FTIR)
Interaction between polymers and their functional groups in the nanofiber structures were studied using FTIR. Spectra of the fibers were taken with a Perkin Elmer (Spectrum 100; USA) in the wavelength region 500-4000 cm⁻¹ at ambient temperature. Fiber mats were placed on a KBr holder in an enclosed sample chamber for FTIR spectroscopic study.

Field Emission Scanning Electron Microscopy (FESEM)
The surface topography of the electrospun fibers was assessed using a FESEM (Jeol JSM-6701F; Japan). Electrospun fibers were placed at the center of an aluminum stud using carbon tape. A very thin layer of platinum was applied to the fibers by a sputtering unit (Jeol JFC-1600 auto fine coater; Japan). Coated fibers were placed in the microscope chamber at high vacuum. Surface
morphological features were obtained under 5 kV accelerating voltage. The diameter distribution of the electrospun fibers were derived from a random sample of at least 20 fibers.

**In vitro Drug Release**
Total immersion method was used to study the cumulative release profiles of T4 from drug-loaded fiber mats. A known amount of the fibers (5 mg) was suspended in 10 ml of pH 7.4 phosphate buffer and was placed in a shaking incubator at 37°C or 25°C. Samples of 1 ml were taken from the medium periodically and the released drug was determined using HPLC. The volume of the release medium was kept constant by replacement with same volume of fresh medium. Drug content of the fiber mat was calculated by dissolving a certain amount of mat in the solvent and measuring the total amount of drug by HPLC method. All drug release data were averaged from three measurements.

**Preparation of Human Epidermis**
Skin samples of adult Chinese females were obtained with patient consent and ethics approval, after abdominal reduction surgery. This study was approved by the Institutional Review Board (IRB) of Singapore General Hospital, Republic of Singapore (IRB Reference Number 196/2006). This IRB operates in accordance with the International Conference on Harmonization/Singapore Guideline for Good Clinical Practices, and with the applicable regulatory requirements. Subcutaneous fat and dermis were carefully separated from the epidermis after immersing the whole skin in 60 ± 5°C distilled water for 2 min. Samples were stored at -80°C until use. Prior to permeation studies, skin samples with stratum corneum side facing upwards, were equilibrated in 0.9% w/v sodium chloride solutions containing 1% v/v antibacterial antimycotic solution (30).

**In vitro Skin Permeation Studies**
Permeation studies of drug-loaded 10% PNIPAM nanofibers were performed using a flow-through diffusion cell apparatus at 37 ± 0.5 °C. The donor compartment was filled with 25 mg of fiber mat (by spreading the mat in the donor chamber). A volume of 250 μl of water was added to all samples to initiate wetting and drug release from the fiber mat. The control sample was 250 μl of aqueous solution, having an equal amount of drug as the test sample (n=4). The exposed surface area of the skin for the permeation of the drug was 0.785 cm². Samples from the receptor compartment were collected at predetermined time points over a 24-h period, and the amount of T4 permeated was analyzed by HPLC. The steady state flux (J) was estimated from the slope of the straight line portion of the cumulative haloperidol absorbed against time profile.

The permeation of drug across the epidermis can be described by Fick’s 2nd law of diffusion (31):

\[
Q = AKLC_o \left[ \frac{D}{L^2} t - \frac{1}{6} \sum_{n=1}^{\infty} \left( \frac{(-1)^n}{n^2} \right) e^{-\left(\frac{D}{L^2} \pi^2 n^2 \right) t} \right]
\]

(1)

Permeation parameters are interpreted from the cumulative amount of released drug per unit skin area (Q/A) versus time (t) plot. The gradient and x-intercept of the linear portion of the plot yield steady-state flux (Jss) and lag time (tL) respectively.

The drug permeability (KD/L) is derived as shown in Eq 2:

\[
\frac{KD}{L} = \frac{J_{ss}}{C_o}
\]

(2)

where C_o is the initial drug concentration in the donor cell. The diffusion parameter, D/L² reflecting the mobility of the drug solute in the skin was calculated using the following equation:

\[
\frac{D}{L^2} = \frac{1}{6t_L}
\]

(3)

The partition parameter, KL, reflecting the distribution of the drug between the skin and the donor solution is derived from:

\[
KL = \frac{KD/L}{D/L^2}
\]

(4)

All permeation experiments were carried out using skin samples from the same donor.
Confocal Laser Scanning Microscopy (CLSM)

Skin penetration of fluorescein loaded 10% w/w PNIPAM-10% w/w PVA nanofibers were viewed using a CLSM. Control sample was fluorescein dissolved in water. The CLSM was a Nikon A1R laser scanning spectral confocal and digital camera. All samples were excited at 488 nm and X-Z sectioning was used to determine the depth of permeation using an objective of ×60. After 24-h exposure to fluorescein-loaded PNIPAM-PVA nanofibers, the skin samples were washed three times, placed on a glass slide and covered with a glass cover-slip. The slides were inverted and images were captured through the cover-slip side of the prepared samples. The full epidermis was scanned at different increments through the z-axis of the microscope.

STATISTICS

Skin permeation of T4 was analyzed using one-way analysis of variance (ANOVA; Graph Pad Prism, Version 2). The differences were considered statistically significant if p < 0.05.

RESULTS AND DISCUSSION

FTIR Measurements of the Drug-loaded Nanofibers

The interactions between the polymers and T4 were analyzed by FTIR (Fig. 1). For pure PVA (Fig. 1a), a broad band around 3336 cm⁻¹ is attributed to the O–H stretching vibration of the hydroxyl group. The vibrational bands at 2942 and 1438 cm⁻¹ represent the –CH stretching. The sharp peak band at 1095 cm⁻¹ corresponds to C–O stretching present in the PVA backbone (Fig. 1a). There was a decline in the intensity of the –OH band when PVA was mixed with T4 (Fig. 1b). It is clear that hydrogen abstraction occurred from PVA molecule in the presence of T4 suggesting the formation of hydrogen bond between PVA and T4 molecules (32-34).

The bands at 2971, 2932 and 2875 cm⁻¹ are associated with the –CH stretching vibration of PNIPAM fiber in 100% v/v ethanol (Fig. 1f). The positions of these three peaks are sensitive to changes in the conformation of the hydrocarbon chain and they shift towards lower frequencies when placed in aqueous solutions. This could be related to the interaction of the alkyl chain with water causing a decrease in the degree of freedom of the PNIPAM molecules (35-37). FTIR spectra of the polymer blends (Fig. 1c, d) show the presence of both PVA and PNIPAM in the nanofibrous networks. The interactions between the two polymers could be due to hydrogen bonding between hydroxyl group in PVA and amide groups in PNIPAM.

FESEM Analysis of Nanofibers

Morphological structures of electrospun PVA, PNIPAM and polymer mixtures are shown in Fig. 2. In our study, the electrospinning parameters such as voltage, flow rate and distance between the injector and collector were kept constant, therefore any difference in the morphology or structure of the fibers is related to the intrinsic properties of the polymeric solution. PVA fibers loaded with 1 mg/ml of T4 were obtained using drug/polymer solutions at a concentration of 10% w/w polymer in water. Formulation A exhibits uniform fibers with diameter ranging from ~ 100 to 200 nm (Fig. 2a). Continuous fibers of formulation B without beads or sputtering of the solution were obtained with fiber diameter ranging from ~ 30 to 300 nm. High viscosity and lower surface tension of the ethanolic solution favored the formation of continuous nanofibers as observed with formulation B (38-40).

Formulation C resulted in spindle-like defects therefore it was not used in the other experiments (Fig. 2c). Nevertheless continuous nanofibers were obtained upon electrospinning formulation D and E, however some elongated and semi-spherical defects were formed along these fibers (Fig. 2d, e).

The mean diameter of the fibers correlated with viscosity of the solution. Fiber diameter increased (from about 100 to 1000 nm) when blends of PVA and PNIPAM were used. This could be due to the hydrogel formed when PNIPAM was added into aqueous solutions resulting in higher surface tension values. FESEM detected no levothyroxine crystals on the surface of the fibers, which indicated that levothyroxine was completely embedded in the fibers.

In vitro Drug Release

The release of T4 from formulation A, B, D and E were investigated in phosphate buffer solution of pH 7.4 both at body temperature, 37°C, (Fig. 3) and room temperature, 25°C (Fig. 4).
Figure 1. FTIR spectra of formulations (a) A, without the drug (b) A, (c) D, (d) E, (e) B and (f) B without the drug. All formulations contain drug unless otherwise mentioned.

T₄ content of the nanofiber mat was calculated, it was found that 25 mg of the polymer fiber mat contained 20 μg of the drug. It can be seen that PVA and PNIPAM polymer released around 48% and 73% of their total drug content respectively, within the first 15 min in contact with PBS in 37°C, where as the rest of the drug was released gradually over the time period. T₄ is speculated to be released from polymer mats by drug diffusion and polymer erosion (degradation) or both mechanisms.

At body temperature of 37°C, formulation B released approximately 97% of its drug content whereas formulation A released only 65% of its drug content. The release of T₄ from mixed polymer mat was found to be a function of PNIPAM concentration used, therefore formulation E released more drug compared to formulation D. This could be explained by the high water solubility of PNIPAM which dissolved almost immediately leading to a rapid release of T₄. PVA dissolves in water at temperatures above 90°C. At body temperature, electrospun fibers of PVA have a disintegration time of a few weeks (7). Drug release rates from formulations containing PVA were lower due to the slow degradation of PVA nanofibers compared to PNIPAM nanofibers. Thus, PNIPAM and PVA blends could prolong drug release with less risk of toxicity compared to pure PNIPAM fibers. The drug release at 25°C from formulation B was relatively lower than at 37°C (>LCST). Considering that PNIPAM is a thermosensitive polymer, it can be used for regulating drug release via response to temperature change. At temperatures below LCST, the polymer is stable and the drug release is slower, however at higher temperatures the polymer collapses thereby enhancing drug release (41).

In vitro Skin Permeation

Fig. 5 represents cumulative T₄ from formulation A, B, E and control as a function of time. It can be seen that T₄ permeation across the skin was gradual (p>0.05) and there is evidence of drug accumulation in the skin as shown by the low amounts of drug permeation across the skin (Fig. 5) and the high amounts of T₄ deposition detected on the skin surface (results not shown). Skin retention of T₄ was improved when polymeric carriers were used. T₄ skin retention from formulation A was markedly higher than that of formulation B, probably due to the low degradation rate of PVA nanofibers. Blending PVA and PNIPAM (formulation E) managed to increase the skin retention of T₄ when compared to pure PNIPAM nanofiber mat containing T₄. Table 2 summarizes skin permeation parameters of T₄ from different nanofiber formulations across the skin.
Figure 2. FESEM images of formulations (a) A (b) B (c) C (d) D (e) E.
Figure 3. In vitro release profile of T₄ from electrospun mat in phosphate buffer (pH 7.4) at body temperature (37°C).

Figure 4. In vitro release profile of T₄ from electrospun mat in phosphate buffer (pH 7.4) at room temperature (25°C).
It can be seen that flux rate of the control samples ($J = 0.846 \pm 0.073 \, \mu g/cm^2/h$) is higher than the nanofiber mat formulations ($p<0.05$). Nanofibers containing pure PVA had lower flux when compared to samples containing pure PNIPAM or a mixture of the two polymers. This shows that a combination of PVA and PNIPAM polymers effectively retarded the flux rate of $T_4$, and PVA polymer alone was the most effective in retarding the flux rate.

The recommended therapeutic dose of $T_4$ is 50-100 $\mu g$/day. Therefore the cumulative $T_4$ skin penetration obtained in this study would not be sufficient to produce a systemic effect in vivo. Topical efficacy was improved as the drug accumulation on the skin prolonged release (29).

To support the above hypothesis CLS microscopic studies were conducted. Skin samples were treated with formulation E loaded with fluorescein and the control samples were treated with an aqueous solution containing same concentration of fluorescein. The binary images (Fig. 6b) indicate the localization of green fluorescein on the outer layer of the skin samples treated with this formulation as compared with the control. It can be seen that the fluorescein dye could not penetrate the skin samples treated with formulation E and the dye concentrated at the outer layer of the skin.

**Table 2.** Permeation profile of different nanofiber formulation (n=4).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Mean flux ± SD ($\mu g/cm^2/h$)</th>
<th>Lag time (h)</th>
<th>Drug permeability (cm/h)</th>
<th>Diffusion parameter ($h^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.314 ± 0.045***</td>
<td>2.978±0.691*</td>
<td>6.335±0.376***</td>
<td>0.496±0.213*</td>
</tr>
<tr>
<td>B</td>
<td>0.598 ± 0.025**</td>
<td>2.372±0.145</td>
<td>5.047±0.240***</td>
<td>0.395±0.015</td>
</tr>
<tr>
<td>E</td>
<td>0.399 ± 0.031***</td>
<td>3.333±1.114*</td>
<td>7.092±0.325***</td>
<td>0.556±0.027*</td>
</tr>
<tr>
<td>Control</td>
<td>0.846 ± 0.073</td>
<td>1.152±0.205</td>
<td>2.451±0.157</td>
<td>0.192±0.011</td>
</tr>
</tbody>
</table>

* Statistically significant at $P < 0.05$
** Statistically significant at $P<0.01$
*** Statistically significant at $P<0.001$
This may be due to the impermeable barrier of the polymer above its LCST temperature which prevents the penetration of the fluorescein. However, fluorescein was detected in the lower layers of the epidermis treated with aqueous solution that did not contain any polymer. For better visualization, skin cell nuclei were counterstained with DAPI.

These polymeric nanofiber delivery systems may have potential use in skin formulations containing active ingredients that are meant to be concentrated on the skin surface and can help to increase the retention of the drug on the skin layers. Due to the lower dosing frequency and simpler dosage regimes, patient compliance may also be improved.
CONCLUSION

Effective sustained delivery of T₄ has been achieved by using polymeric nanofibers which could relatively improve therapy by reducing number of applications. These nanofiber mats may be promising carriers for sustained topical delivery of drugs while keeping the drugs concentrated on the skin over a prolonged period with reduced systemic uptake.

ACKNOWLEDGMENT

The authors would like to thanks Dr Clement Khaw from Singapore Bioimaging Consortium (SBIC) for his guidance and assistance in using the confocal laser microscopy.

REFERENCES