J. Microbiol. Biotechnol. (2007), **17**(12), 1965–1969

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

After-Rinsing Hair Growth Promotion of Minoxidil-containing Amino α-Cyclodextrins

KIM, JIN-CHUL* AND MYOUNG-DONG KIM

College of Bioscience & Biotechnology, Kangwon National University and Institute of Bioscience and Biotechnology, Chunchon 200-701, Korea

Received: April 4, 2007 Accepted: July 10, 2007

Abstract Triamino α-cyclodextrin (CD) was synthesized and the inclusion complex with Minoxidil (MXD) was prepared. α-CD was azidated by modifying the 6-hydroxylmethyl CD rim with sodium azide. Then, mono-, di-, tri-, and tetra-azidocyclodextrins were separated by a flash column chromatography and reduced to the corresponding amines by hydrogenation with Pd/C. The substantivities of MXD included in either 2-hydroxypropyl α-CD (HP α-CD) or triamino α-CD were evaluated *in vitro* using hairless mice skins. After applying the preparations onto the skin and rinsing it, the amount of the drug left on the skin was determined using high-performance liquid chromatography (HPLC). It was the highest when the drug was included in triamino α-CD. The electrostatic interaction between the protonated amino CD and the negatively charged skin would be responsible for the relatively high substantivity. The *in vivo* hair growth promotion effect of each preparation was investigated, where the sample application onto the clipped backs of female mice (C57BL6) and the subsequent rinsing of the backs were done once a day for 30 days. Only MXD in triamino α-CD had hair growth promotion effect, possibly due to the significant substantivity.

Keywords: Minoxidil, triamino α-cyclodextrin, inclusion complex, substantivity, hair growth

Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide, MXD) is a potent hypertrichotic agent used topically in treating alopecia androgenetica. Many researches have been done on the percutaneous absorption of MXD into human skin using different vehicles. The drug dissolved in the solvent of ethanol, propylene glycol, and water is the most extensively studied systems. Several factors such as the concentration of the drug, the solvent composition, the application volume, the contact time with skin, and the manner of application (occlusive or nonocclusive), are reported to have an effect on the adsorption of the drug [1, 8, 9, 12]. The lipid vesicular system embodying ethanol, ethosome, was studied to enhance the skin permeation of MXD, and liposomes were employed to enhance transportation of the drug into hair follicles [10, 11]. Hair follicular delivery was also achieved by means of iontophoresis, which transports a cationic derivative of MXD [7]. Besides the skin permeation, the high substantivity of MXD would be a prerequisite for hair growth promotion when hair- and scalp-washing products such as hair shampoo and rinse are used for the treatment of hair growth. One strategy might be to use a cationic carrier, so that an electrostatic interaction between the carrier and skin may lead to the high substantivity of MXD. In a previous study, cationic vesicles composed of *N*-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA) and fatty acids had a significant *in vitro* substantivity and *in vivo* hair growth promotion effect [4].

Much attention has been paid to the development of carriers for drug delivery and tissue engineering [3, 5, 6]. In this study, α-cyclodextrin (α-CD) derivatives were used as carriers for MXD. 2-Hydroxylpropyl α-CD (HP α-CD) was employed as a nonionic carrier, and triamino α-CD was synthesized as a cationic carrier. The 6-hydroxylmethyl (1°) face of α-CD was modified with sodium azide and then the azido CDs were reduced to the amino CDs. The *in vitro* substantivity and the *in vivo* hair growth promotion effects were investigated with the inclusion complexes between MXD and the α-CD derivatives.

Materials and Methods

Materials

Dimethyl sulfoxide (DMSO) was distilled with $CaSO_4$ under reduced pressure (1,000 mtorr) at 45°C. α-CD was dried overnight with P_2O_5 under vacuum. The reactions were done under Ar atmosphere. Thin-layer chromatography

*Corresponding author
Phone: 82-33-250-6561; Fax: 82-33-253-6560;
E-mail: jinkim@kangwon.ac.kr

(TLC) plates were scrutinized by ultraviolet light and developed by 20% H_2SO_4 in H_2O, followed by heating with a heat gun. Normal and flash chromatographies were performed using a silica gel with mesh size of 60–200 and 230–400, respectively. α-CD and 2-hydroxypropyl-α-cyclodextrin (HP-α-CD) were obtained from Aldrich (St. Louis, MO, U.S.A.). Minoxidil (MXD, 98%) was purchased from INFA (Milan, Italy). All other reagents were of analytical grade.

Animals

Female hairless mice (type SKH), 8–14 weeks old, for substantivity test, and female mice (C57BL6), six weeks old, for hair growth promotion study were obtained from LG Chem. (Taejon, Korea). They were housed in suspended wire mesh cages in a room illuminated from 09:00 to 21:00 h and kept at 20–25°C, and given a rodent diet and water *ad libitum*.

Synthesis of Triazido α-CD

Triazido α-CD was synthesized according to the literature [2] except that NaN_3 was used instead of Li N_3. Dry α-CD (11.0 g, 11.31 mmol) was dissolved in dry DMSO (80 ml), and NaN_3 (7.35 g, 10 equiv), triphenyl phosphine (8.90 g, 3 equiv), and carbon terabromide (11.23 g, 3 equiv) were added to the solution. The reaction was stirred under Ar atmosphere for 4 days. The reaction was concentrated to oil under vacuum and 800 ml of acetone was added. The precipitate was filtrated, washed twice with 400 ml of acetone, and air-dried (13.5 g). Half of the crude products (6.75 g) was separated by flash chromatography on a silica gel column (5×50 cm^2) with solvent of acetonitrile: water (9:1, 7 l) and then of acetonitrile:water (4:1, 7 l), and the fractions for each chemical were combined, evaporated, and lyophilized.

Synthesis of Triamino α-CD

Two-hundred mg (0.191 mmol) of triazido α-CD was dissolved in methanol:water (1:1) and 20 mg of Pd/C (10%) was added to the solution. The azido compound was hydrogenated at 40 psi, room temperature, for 48 h. The reaction was filtrated, and then, after removing methanol, the products were lyophilized.

Preparation of MXD/CD Inclusion Complex

MXD, 0.25 g, was added to 10 ml of an aqueous solution of either HP-α-CD or triamino α-CD, of which concentrations were 0, 0.0085, 0.0169, 0.0339, and 0.0678 M. After being whirled at 150 rpm on a shaker (SK-760A, Jeio Tech) for 24 h, the supernatant was filtered through a membrane of pore size 220 μm (Millex-GV, Millipore). The filtrates were diluted 1,000 or 2,000 times with distilled water and the concentrations were determined by measuring the absorbance at 228.8 nm using a UV-spectrophotometer (Unicam 8700). The standard calibration of MXD in water was $A_{228.8}$=0.1585X+0.004 with R^2=0.9998, where $A_{228.8}$ is the absorbance at 228.8 and X is the concentration of MXD in μg/ml. The filtrates obtained from the aqueous solution (8 wt%) of either HP-α-CD or triamino α-CD were diluted with distilled water so that the concentration of MXD was 0.7%, and the final pH was adjusted to 5.0.

In Vitro Substantivity

The detailed process has been described in our previous report [4]. In brief, female hairless mice (type SKH) aged 8–14 weeks were used. After sacrificing with ether, the dorsal skin of each hairless mouse was excised. MXD included either in HP-α-CD or in triamino α-CD was applied on the skin and then the skin was rinsed with distilled water. As a control, MXD solution in ethanol (0.7%) was used. Six pieces of the washed skin were punched out using a biopsy punch (Stiefel, 6 mm in diameter) and put into an eppendorf tube. After 1 ml of ethanol was added to the tube, it was vortexed and left for 24 h to dissolve MXD out of the skin. The concentration of MXD was determined by HPLC. The assay was performed in a Waters liquid chromatograph equipped with a UV detector. A Microsorb-MV column was eluted with acetonitrile (10^{-2} M $NaClO_4$)/H_2O (10^{-2} M $NaClO_4$, pH 3) (9:1, v/v) at a flow rate of 1.0 μl/min, and a sample of 15 μl was injected. The detection wavelength was 283 nm.

In Vivo Hair Growth Promotion

Healthy female mice (C57BL6) in telogen phase were used to investigate the hair growth promotion effect. The hairs on the back were removed using an electric clipper, 1 day before applying the samples. Each sample (MXD included either in HP-α-CD or in triamino α-CD, and MXD dissolved in ethanol) of 0.2 ml was applied onto the clipped backs of 6 animals in a separate group. Two min later, the backs of the animals were rinsed with running distilled water (pH 5.0, 37°C) flowing through a perforated water distributor at rate of 35 ml/sec for 20 sec. The sample application and the rinsing were done once a day. The hair-regrowing activities were estimated by taking the photographs 30 days after the first application.

RESULTS AND DISCUSSION

Synthesis of Triazido α-Cyclodextrin

Four kinds of major products were observed on TLC (acetonitrile:water, 4:1) and their R_f values were 0.63, 0.50, 0.3, and 0.16, 0.04, which are tetraazido-, triazido-, diazido-, and monoazido-α-CD. Fig. 1 shows the PDMS spectrum of triazido-α-CD. The observed value of the mass $(M+Na)^+$ was 1,071.7, almost the same as the calculated value, 1,070.9. Thus, it is believed that the

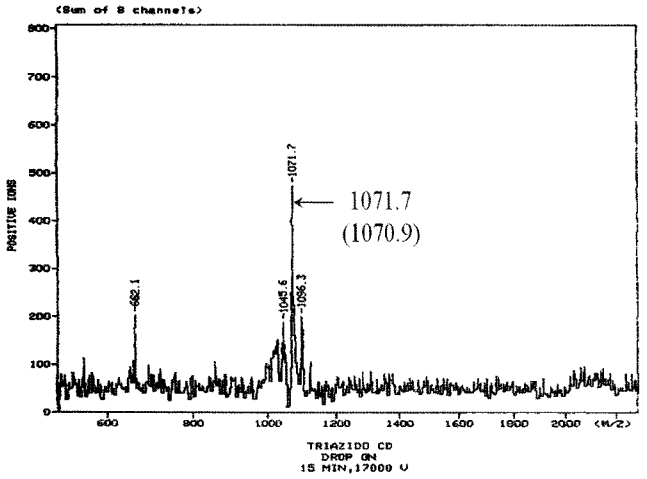

Fig. 1. PDMS spectrum of triazido α-CD.

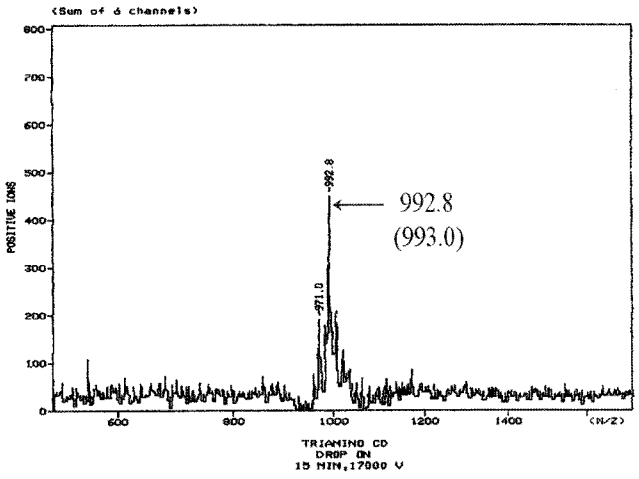

Fig. 3. PDMS spectrum of triamino α-CD.

triazido-α-CD was successfully synthesized. α-CD was azidated according to the method of Hanessian *et al.* [2]. They reported that treatment of α-CD with triphenylphosphine and carbon tetrabromide (3 equiv) and lithium azide (10 equiv) in DMSO for 6 h gave monoazido, diazido, and triazido α-CD in yields of 20%, 26%, and 5%–10%, respectively. We used the same reaction condition, except that sodium azide was used instead of lithium azide and the reaction period was 4 days. Since our goal was to synthesize triamino α-CD, we tried to produce triazido-α-CD in a higher yield by prolonging the reaction period and increasing the amount of sodium azide. However, the higher yield of triazido α-CD resulted in the higher yield of tetraazido α-CD, which made separation of triazido α-CD from teraazido α-CD more difficult. With sodium azide, a 4-day reaction led to tetra, tri, di, mono α-CD in the yield of 16.2% (0.19 g), 21.5% (1.24 g), 17.5% (1.04 g), and 8.3% (0.503 g), respectively. With our experimental conditions, a workable yield of triazido α-CD was obtained with a low yield of tetraazido α-CD. Fig. 2 shows the 500 MHz proton NMR spectrum of the triazido α-CD in CD_3SOCD_3. The assignments are as follows: 3.24–3.46 (m, H2, H4, H6*, H_2O), 3.52–3.86 (m, 24H, H3, H5, H6), 4.49–4.70 (m, 3H, OH6), 4.76–4.82 (br, 3H, H1), 4.82–4.88 (m, 3H, H1*), 5.38–5.76 (m, 12H, OH2, OH3). The * symbol indicates the glucose units containing the azido group. The degree of azidation was determined by the ratio of the number of OH2 and OH3 to that of OH6. For triazido α-CD, the calculated ratio is 4.0, and it was almost the same as the observed ratio, 4.03. It means that the triazido α-CD was successfully synthesized.

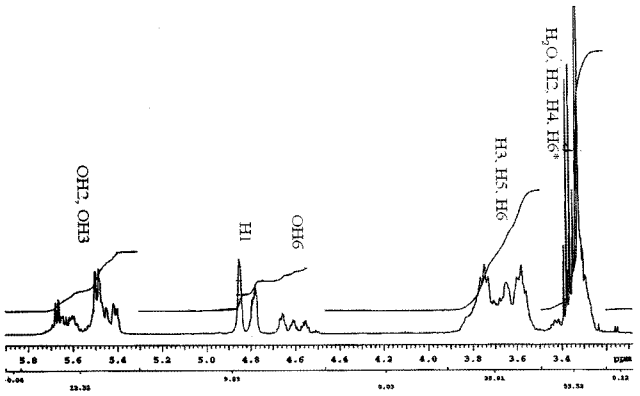

Fig. 2. Five-hundred MHz proton NMR spectrum of the triazido α-CD in CD_3SOCD_3.

Synthesis of Triamino α-Cyclodextrin

The product was observed on TLC (n-PrOH:H_2O:NH_4OH, 6:2:1) and its R_f value was 0.06. The yield was 93% (172 mg). Fig. 3 shows the PDMS spectrum of triamino-α-CD. The observed value of the mass $(M+H)^+$ was 971.0 and the calculated value was the same. The observed value of $(M+Na)^+$ was 992.8 and the calculated value was 993.0. Fig. 4 shows the 300 MHz proton NMR spectrum of the triamino α-CD in D_2O. The assignments are as follows. 2.59–2.83 (m, 3H, H6*), 2.83–3.1 (m, 3H, H6*), 3.1–3.53 (m, 12H, H2, H4), 3.53–3.97 (m, 18H, H3, H5, H6), 4.78–

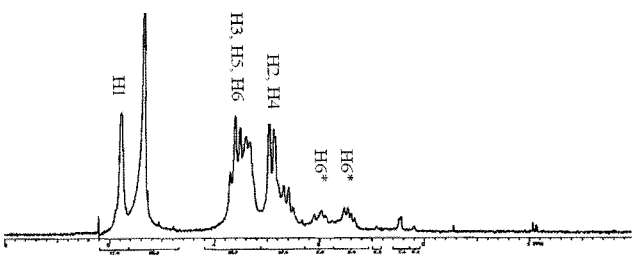

Fig. 4. Three-hundred MHz proton NMR spectrum of the triamino α-CD in D_2O.

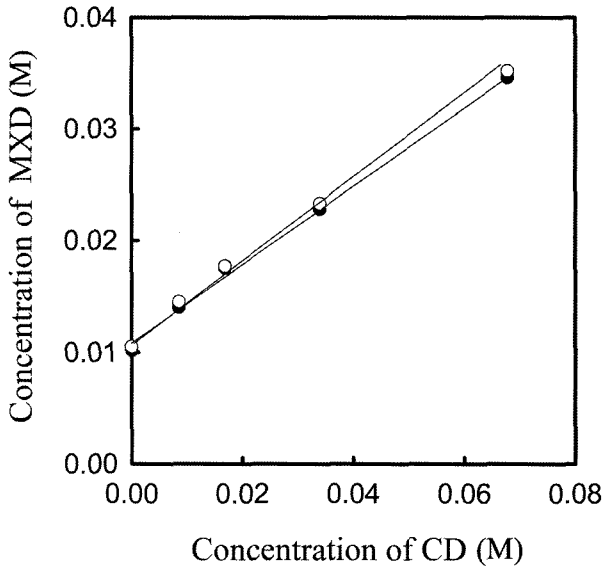

Fig. 5. Solubility of MXD in HP α-CD solution (●) and triamino α-CD solution (○).

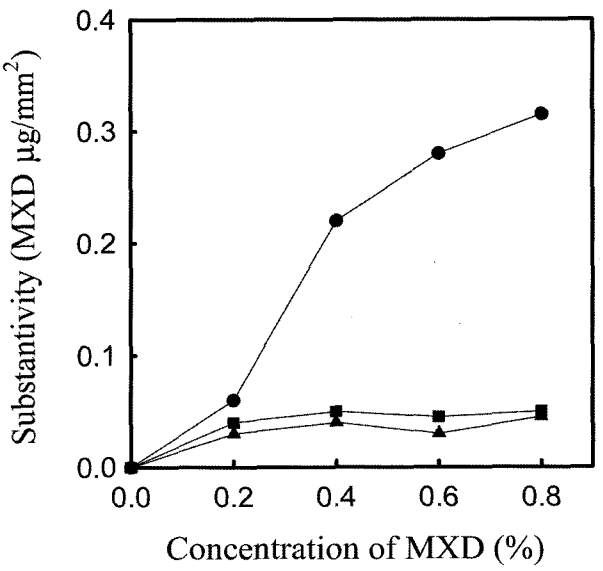

Fig. 6. Substantivity of MXD in HP α-CD solution (■), triamino α-CD solution (●), and ethanol (▲).

5.0 (m, 6H, H1). The * symbol indicates the glucose units containing the amino group. The ratio of number of protons of H6* to that of H1 was found to be 1.02 and the calculated value is 1.0.

Preparation of MXD/CD Inclusion Complex

Fig. 5 shows the solubility of MXD in either HP α-CD or triamino α-CD solution. The solubilities of MXD increased linearly with the concentration of CDs. They are expressed as S_M=0.3531C+0.0108 (R^2=0.9974) for HP α-CD and S_M=0.3572C+0.0112 (R^2=0.9978) for triamino α-CD, where S_M is the solubility of MXD in *M* and C is the concentration of the CD derivatives in *M*. The inclusion complex between the CD derivatives and MXD would account for the increased solubility. The hydrophobic part of MXD, a pyridine moiety, is likely to be included in the hydrophobic cavity of the CD derivatives. When either CD derivatives were used, no significant difference in the solubility of MXD was observed. It means that the solubility of MXD mainly depends on the interaction with the cavity of CD but is not affected by the chemical moiety on the primary surface of CD.

***In Vitro* Substantivity**

Fig. 6 shows the substantivity of MXD, the amount left on the skin after rinsing formulation-applied skin with running water. The substantivity of MXD included in triamino α-CD was appreciable when the concentration was greater than 0.4%. However, no significant amount of MXD was detected in the case where MXD was either included in HP α-CD or dissolved in ethanol. Triamino α-CD might electrostatistically interact with negatively charged skins since the amine groups could be protonated. This may explain the higher substantivity. In a previous study, among three kinds of topical dosage forms of MXD, namely vesicles, double emulsions and inclusion complex with hydoxypropyl-β-cyclodextrin (HP-β-CD), cationic vesicles composed of *N*-[3-(dimethylamino)propyl]-octadecanamide (DMAPODA) and fatty acids had a significant *in vitro* substantivity [4]. The substantivity of MXD encapsulated in the cationic vesicles was approximately 2.8 μg/mm^2 when the concentration of MXD in the preparations was 1%. This value is about 9 times higher than in the case where triamino α-CD was employed as a vehicle for MXD. In the vesicle preparation, the molar ratio of lipid comprising the cationic vesicle to MXD was around 12:1. It means that the positive charge density per one molecule of MXD is much higher than that of the triamino α-CD/MXD complex. Since the molar ratio of triamino α-CD to MXD in the complex is approximately 1:1 [4], the positive charge density per one molecule of MXD would be relatively low. This may account for the difference in the substantivity between the cationic vesicle formulation and the inclusion complex.

***In Vivo* Hair Growth Promotion**

Fig. 7 shows the photographs of mice, treated with MXD included either in HP α-CD or in triamino α-CD, or MXD dissolved in ethanol. MXD in triamino α-CD exhibited a significant hair growth promoting effect. However, when MXD in either HP α-CD or ethanol was applied, no significant hair growth was observed (a photograph is not shown here when ethanol was used as a vehicle). These results are in accordance with the substantivity of MXD,

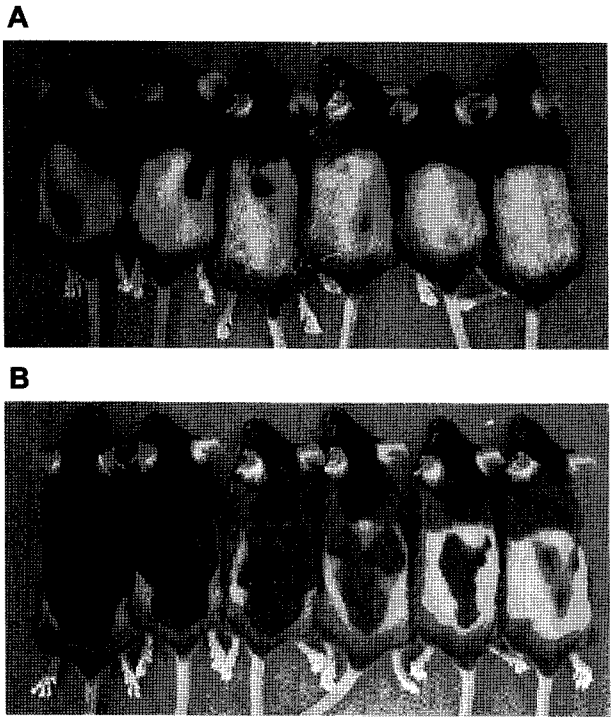

Fig. 7. *In vivo* hair-growth promotion of MXD in HP α-CD solution (**A**) and triamino α-CD solution (**B**).
The sample application onto the clipped backs of mice and the subsequent rinsing of the backs were done once a day for 30 days.

because only MXD in triamino α-CD showed an appreciable amount of MXD on the skin after rinsing it. The drug should release to the site of action to exhibit its therapeutic effect. It was reported that the inclusion complex is stable and the release of the drug hardly occurs [4]. In this circumstance, how does the drug exhibit its therapeutic action, hair growth promotion? The lipidic components comprising sebum, secreted from hair follicles, could replace MXD in the cavity of the α-CD derivative, leading to the release of MXD, but it is not clear yet.

In summary, the inclusion complexes of MXD either in HP α-CD or in triamino α-CD were prepared, and the substantivity and after-rinsing hair growth promotion effects were evaluated. Compared with MXD in HP α-CD, the one in triamino α-CD exhibited relatively high substantivity. Furthermore, only MXD in triamino α-CD had after-rinsing hair growth promotion effect. Therefore, the ionic interaction between the vehicles and the skins is likely to be one of the dominant factors in the substantivity and, in turn, the hair growth promotion.

Acknowledgments

This work is supported by the Chuncheon Center of Korea Basic Science Institute

References

1. Ferry, J. J., J. H. Shepard, and G. J. Szpunar. 1990. Relationship between contact time of applied dose and percutaneous absorption of minoxidil from a topical solution. *J. Pharm. Sci.* **79:** 483–486.
2. Hanessian, S., A. Benalil, and C. Laferriere. 1995. The synthesis of functionalized cyclodextrins as scaffolds and templates for molecular diversity, catalysis, and inclusion phenomena. *J. Org. Chem.* **60:** 4786–4797.
3. Kim, C.-J., S.-A. Jun, N.-K. Lee, K.-T. Kim, S. K. Lee, C.-H. Kim, and H.-D. Paik. 2006. Encapsulation of *Bacillus polyfermenticus* SCD with alginate-methylcellulose and evaluation of survival in artificial conditions of large intestine. *J. Microbiol. Biotechnol.* **16:** 443–449.
4. Kim, J.-C., M.-H. Lee, and M.-J. Rang. 2002. Minoxidil-containing dosage forms: Substantivity and after-rinsing hair growth promotion. *Drug Deliv.* **10:** 119–123.
5. Lee, J.-H., D.-H. Lee, J. H. Son, J.-K. Park, and S.-K. Kim. 2005. Optimization of chitosan-alginate encapsulation process using pig hepatocytes for development of bioartificial liver. *J. Microbiol. Biotechnol.* **15:** 7–13.
6. Park, K.-H., D. M. Kang, and K. Na. 2006. Physicochemical characterization and carcinoma cell interaction of self-organized nanogels prepared from polysaccharide/biotin conjugates for development of anticancer drug carrier. *J. Microbiol. Biotechnol.* **16:** 1369–1376.
7. Poulos, C. W., G. M. Brenner, L. V. Allen Jr., V. A. Prabhu, and P. L. Huerta Jr. 1994. Method for simulating hair growth with cationic derivative of minoxidil using therapeutic iontophoresis. *USP.* 5,466,695.
8. Tata, S., G. L. Flynn, and N. D. Weiner. 1995. Penetration of minoxidil from ethanol/propylene glycol solution: Effect of application volume and occlusion. *J. Pharm. Sci.* **84:** 688–691.
9. Tata, S., N. Weiner, and G. Flynn. 1994. Relative influence of ethanol and propylene glycol cosolvents on deposition of minoxidil into the skin. *J. Pharm. Sci.* **83:** 1508–1510.
10. Touitou, E., N. Dayan, L. Bergelson, B. Godin, and M. Eliaz. 2000. Ethosomes - novel vesicular carriers for enhanced delivery: Characterization and skin penetration properties. *J. Control. Release* **65:** 403–418.
11. Tsai, J.-C., G. L. Flynn, N. Weiner, and J. J. Ferry. 1993. Effect of minoxidil concentration on the deposition of drug and vehicle into the skin. *Int. J. Pharm.* **96:** 111–117.
12. Tsai, J.-C., M. J. Cappel, G. L. Flynn, N. D. Weiner, J. Kreuter, and J. J. Ferry. 1992. Drug and vehicle deposition from topical applications: Use of *in vitro* mass balance technique with minoxidil solutions. *J. Pharm. Sci.* **81:** 736–743.