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Preparation of Low-Molecular-Weight 6-Amino-6-Deoxychitosan (LM6A6DC) for Immobilization of Growth Factor

Koo-Yeon Kim, Eun-Hye Kim, Tae-Il Son

***Abstract*—**Epidermal Growth Factor (EGF, Mw=6,045) has been reported to have high efficiency of wound repair and anti-wrinkle effect. However, the half-life of EGF in the body is too short to exert the biological activity effectively when applied in free form. Growth Factors can be stabilized by immobilization with carbohydrates from thermal and proteolytic degradation. Low molecular weight chitosan (LMCS) and its derivate prepared by hydrogen peroxide has high solubility. LM6A6DC was successfully prepared as a reactive carbohydrate for the stabilization of EGF by the reactions of LMCS with alkalization, tosylation, azidation and reduction. The structure of LM6A6DC was confirmed by FT-IR, ^{1}H NMR and elementary analysis. For enhancing the stability of free EGF, EGF was attached with LM6A6DC by using water-soluble carbodiimide. EGF-LM6A6DC conjugates did not show any cytotoxicity on the Normal Human Dermal Fibroblast (NHDF) 3T3 proliferation at least under 100 μg/ml. In the result, it was considered that LM6A6DC is suitable to immobilize of growth factor.

***Keywords*—**Epidermal growth factor (EGF), low-molecular-weight chitosan, immobilization.

I. Introduction

CHITOSAN, natural polymer in nature, is obtained by deacetylation of chitin derived from the shell of crustacean such as crab and crawfish. Generally, chitosan is defined as substances which are deacetylated more than 50% from chitin and soluble in acidic solution [1], [2]. Chitosan is normally present as a form of polymer made by β-(1→4) glucosidic bond of glucosamine and N-acetylglucosamine [3]. Chitosan, differently to cellulose, contains both acetamide group and amino group as representative amino-polysaccharide. Chitosan is noticed because of unique bioactivity such as wound-healing property [4], enhancing secretion of fibroblast growth factor [5] and restoration of bone tissue [6], [7] with chitin and chitin derivates. Also, Chitosan derivates such as Carboxymethylated Chitosan [8], [9], Phosphorylated Chitosan [10] and Sulfonated Chitosan [11] have contributed in making application extent of low solubility and applicable property of Chitosan to be broad.

Growth factors like Epidermal Growth Factor (EGF) have high cell growth activity when Growth factor is applied on a wound. However, the half-life of EGF in body is too short to exert the biological activity effectively because EGF can be easily degraded by proteinases such as trypsin or pepsin secreted from a wound. The carbohydrate part of glycoprotein present in nature plays an important role in preventing amino acid chain to be degraded by proteinase or stabilizing a three dimensional structure of proteins. With this property of carbohydrate, it should be possible that the half-life of growth factors in body will be extended and the three dimensional structure will be stabilized when carbohydrate is attached to EGF.

As chitosan contains amino group, chitosan is suitable for stabilization of proteins by amide bond between carboxyl group of protein and amino group of chitosan. Moreover, chitosan which has positive charge can effectively induce protein to be attached on the cell membrane which has negative charge. LM6A6DC produced through this research was designed for increasing of the degree of coupling by increased amino group which can bind to protein and introducing amino group on 6th carbon of chitosan in order to be attached on the cell membrane more effectively by increasing the density of positive ions. However, there is possibility that macro molecular weight made when chitosan derivate is immobilized with protein can inhibit protein to be attached to a protein receptor of protein membrane. Therefore, low molecular soluble chitosan derivates were produced because 3,000 Da~10,000 Da molecular weight chitosan oligomers show the greatest bioactivity normally [12]. With chitosan oligomers produced, LM6A6DC was produced through Alkalization, Tosylation, Azidation and reduction under the conditions of Fig. 1 [13], [14]. LM6A6DC can be applied for agent of medicinal waters because of its soluble property. LM6A6DC can be also applied for biomaterial field because LM6A6DC is easy to sterilization with the property of heat stability and has no cytotoxicity.

Therefore, the objects of this research are producing LM6A6DC as new carbohydrate for immobilization of protein and stabilized EGF-LM6A6DC conjugates which can make bioactivity of EGF to be maximized by structural stabilization and extending half-life of EGF.

Koo-Yeon Kim and Eun-Hye Kim are with Department of Systems Biotechnology, Chung-Ang University, Anseong-Si, Gyeonggi-Do 456-756, Republic of Korea (e-mail: kky7792@naver.com, 01032054654@hanmail.net).

Tae-Il Son is with Department of Systems Biotechnology, Chung-Ang University, Anseong-Si, Gyeonggi-Do 456-756, Republic of Korea (corresponding author to provide phone: +82-31-670-3065; e-mail: tisohn@cau.ac.kr).

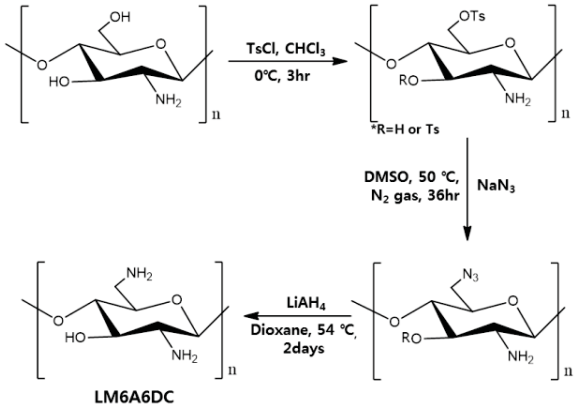

Fig. 1 Overall scheme of modification of low-molecular-weight chitosan

II. Materials & Methods

Insoluble powdered chitosan was purchased from Jakwang Co. (Korea) and the degree of deacetylation was 81%, average molecular weight is more than 3.5x102kDa. Hydrogen peroxide and sodium hydroxide were obtained from Duksan Pure Chem. (Korea). Toluenesulfonyl chloride was obtained from Junsei Chem. (Japan). Sodium azide, dioxane, chloroform and dimethylsulfoxide were obtained from Daesung Chem. (Korea). Lithium aluminium tetrahydride (LiAlH4) was obtained from Shimadzu (Japan). Organic solvents such as Methanol, ethanol, acetone and ether were used without a process of purification. Epidermal Growth Factor (EGF) was obtained from Sigma Chem. Fibroblast 3T3 cell line was obtained as a gift from Korea Cancer Center Hospital (KCCH). DMEM-F12 (Dulbecco's Modified Eagle's Medium-F12), FBS (Fetal Bovine Serum), Trypsin-EDTA (2.5 mg/ml trypsin, 200 μg/ml EDTA) and penicillin/streptomycin were obtained GIBCO BRL (America).

A. Preparation of Low-Molecular-Weight Chitosan (LMCS) by Hydrogen Peroxide

2g of powdered chitosan was dissolved completely in 100 ml of 0.5% HCl solution. Chitosan dissolved in HCl was divided by three fractions. 5 ml of 0.5M, 1.0M, 2M hydrogen peroxide were added to each fractions at 54°C and stirred under less than 254nm wavelength of ultraviolet layer for 2 hours. The samples were cooled to room temperature and neutralized to ph7 with 1N NaOH after dissolved chitosan was removed with decompression filtration. 300 ml of ethanol was added neutralized solutions and participates were obtained by the differences of solubility. The samples were centrifuged at 3000rpm for 5 minutes. From the samples centrifuged, pellets were collected and dried by a vacuum dryer. Dried samples were dissolved in distilled water and ultra-filtered using YM3 (molecular weight cut-off 3,000 Da.) and YM10 (molecular weight cut-off 10,000 Da.) membranes. Ultra-filtered samples were divided depending on molecular weight and dried by a lyophilizer.

B. Alkylation of LMCS

75 ml of 40(w/w)% NaOH was added to 3g of LMCS prepared by hydrogen peroxide and stirred at room temperature for 5 hours under a condition of decompression. After 5 hours, 200 ml of ice water was added and stirred. As ice was melted, colorless and transparent slurry alkali LMCS was prepared.

C. Tosylation of Alkali LMCS

61g of tosyl chloride (20 times excess tosyl chloride/pyranose unit) was dissolved in 120 ml of chloroform. The dissolved sample was added alkali LMCS solution cooled at 0°C with strong stirred. The sample was stirred at 0°C for 1 hour and at room temperature for 4 hours. Distilled water was added in order to induce precipitation. Precipitates were filtered with using decompression filtration and washed with distilled until neutralized completely. Neutralized sample was washed with 500 ml of ethanol and 300 ml of ether. The sample was dried by a vacuum dryer for 1 day and 7.32g of tosyl LMCS was prepared.

D. Azidation of Tosyl LMCS

4g of tosyl LMCS was added to 500 ml of dimethylsulfoxide (DMSO) and stirred for 3 hours. After 3 hours, 7.35g of NaN3 (10 times excess sodium azide/tosylated pyranose unit) was added and stirred at 50°C for 36 hours under an air stream of nitrogen gas. 300 ml of ice water added and after 3 hours at room temperature, the sample filtered with using decompression drier. The sample filtered was washed with distilled water, ethanol, acetone and ether in order and dried by a vacuum dryer for a day. 2.46g of 6-azido-6-deoxy was prepared.

E. Reduction of 6-azido-6-deoxy LMCS

2.4g of 6-azido-6-deoxy LMCS was swelled in 360 ml of dioxane for 3 hours. 4.3g of LiAlH4 (10 times excess sodium azide/azido pyranose unit) was added to the swelled sample and reacted at 75°C for 72 hours. The mixed solution, 32 ml of dioxane and 8 ml of distilled water, was added gradually and slowly. 300 ml of distilled water added in order to neutralize reducing agent, LiAlH4, completely and stirred at room temperature for 24 hours. The sample was filtered with using a decompression drier and washed with distilled water, ethanol, acetone and ether in order. After vacuum drying for one day, 11.07g of LM6A6DC was prepared.

F. Purification of LM6A6DC

LM6A6DC was dispersed 300 ml of 2N HCl added for 3 hours and the dispersed sample was dialyzed for 72 hours against distilled water (membrane cut-off 12,000~14,000 Da, Cellu•Sep T4). Purified LM6A6DC was prepared after evaporation and lyophilization.

G. Characterization of LM6A6DC

IR spectra were obtained in SHIMADZU FT-IR 8400S (JAPAN) using KBr pellets. The substitution rates of each intermediates produce on the process of tosylation, azidation and reduction were obtained by elementary analysis (C, H, N, and S) in Flash-1112 (Thermo electron, U.S.A.). 1H NMR spectra of LMCS and LM6A6DC were recorded at 400 MHz in JNN ECP 400 spectrometer (U.S.A.) after dissolved in D2O

solvent. However, 1H NMR spectra of tosyl LMCS and 6-azido-6-deoxy LMCS could not be recorded because of insoluble property in polarity organic solvent and acid due to tosyl and azido groups introduced to LMCS newly.

H. Preparation of EGF-LM6A6DC Conjugates

To find the optimized condition for preparation of EGF-LM6A6DC conjugates, research was carried out with some of various conditions. Conjugates were prepared using EDC as a cross-linker reported by Anderson (Fig. 2). The concentration ratio of EGF, EDC and LM6A6DC was 1:200:1,000. EGF was dissolved in PBS, pH 6. 5, to prepare 0.338nmol/ml, 3.38nmol/ml and 33.8nmol/ml of EGF solutions. The cross-linker, EDC, was dissolved in PBS, pH 7.4, to prepare 6.68nmol/ml, 66.8nmol/ml and 668nmol/ml of EDC solutions. LM6A6DC was dissolved in PBS, pH 8.5, to prepare 0.338μmol/ml, 3.38μmol/ml and 33.8μmol/ml of LM6A6DC solutions. 250μl of EDC solution was added to 500μl of EGF solution and gentle shaking was carried out for pre-activation of carboxyl group of EGF at 4°C for 2 minutes. The activated EGF was added to 250μl of LM6A6DC and stirred at room temperature for 4 hours. As the final step, 100μl of glycine solution (0.138M in pH 8.5 PBS) was added and left at room temperature for 90 minutes. To remove EGF, EDC, LM6A6DC, glycine and a by-product, urea, remained in prepared EGF-LM6A6DC conjugates solution, the sample was dialyzed for 72 hours (membrane cut-off 12,000~14,000 Da, Cellu•Sep T4). Finally, EGF-LM6A6DC conjugates were prepared.

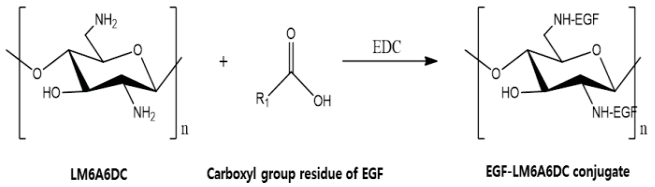

Fig. 2 Condensation reaction between EGF and LM6A6DC by EDC

I. Cell Culture and Cytotoxicity Assay

Using Normal Human Dermal Fibroblast 3T3 (NHDF 3T3) cell line obtained as a gift from Korea Cancer Center Hospital, cytotoxicity tests of LM6A6DC and EGF-LM6A6DC conjugates was carried out. The growth rate of cell when EGF and EGF-LM6A6DC was applied to NHDF 3T3 cell line was also researched. The cell was cultured on DMEM-F12, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (10,000 U/ml penicillin, 10,000μl/ml streptomycin) medium at 37°C, under condition of 5% CO_2. The cell was divided to 3 x 103 a well of 96-well plate and cultured for 12 hours in order to attachment on plate. Various concentrations of LM6A6DC and EGF-LM6A6DC conjugates were added to each well and cytotoxicity test carried out after 24 hours. Pure EGF and EGF-LM6A6DC conjugates were added and the growth rate was researched after 24 hours and 48 hours by MTT assay method using ELISA microplate reader in 595nm.

III. Results and Discussion

A. FT-IR and ^{1}H-NMR

There were specific peaks at around 1,200 cm^{-1} which are derived from SO_2 of tosyl group and 820 cm^{-1} comes from *para*-phenyl group of tosyl group (Fig. 3). On the other hand, 6-azido-6-deoxy LMCS had a peak at around 2,100 cm^{-1}. This peak comes from azido group of 6-azido-6-deoxy LMCS (Fig. 4). For analysis of successful reduction from 6-azido-6-deoxy LMCS to LM6A6DC, we compared each FT-IR spectra. Specific peaks at 1,200 cm^{-1} and 820 cm^{-1} were disappeared at FT-IR spectrum of LM6A6DC; because, tosyl group of 6-azido-6-deoxy LMCS was reduced into primary amine by $LiAlH_4$ (Fig. 5).

^{1}H-NMR was conducted to confirm the introducing of amino group to LMCS. In ^{1}H-NMR data of LM6A6DC, specific peak around 2.8ppm which is derived from amino group was observed. As a result, amino group was introduced to LMCS successfully.

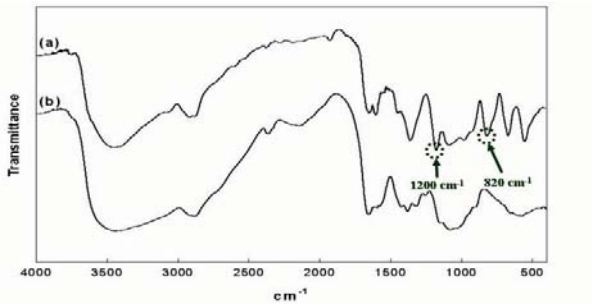

Fig. 3 FT-IR spectra of (a) Tosyl LMCS and (b) LMCS

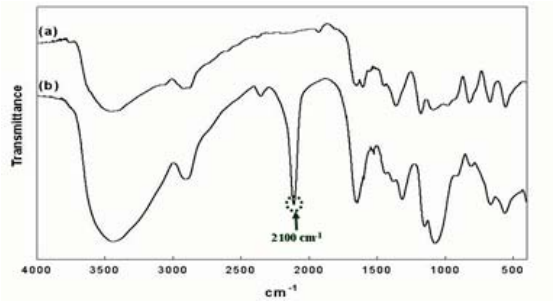

Fig. 4 FT-IR spectra of (a) Tosyl LMCS and (b) 6-Azido-6-Deoxy LMCS

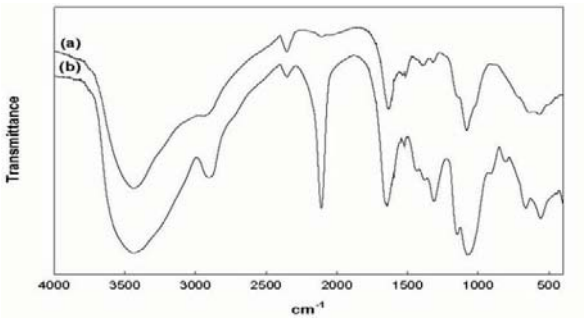

Fig. 5 FT-IR spectra of (a) LM6A DC and (b) 6-Azido-6-Deoxy LMCS

B. Elementary Analysis

From the results of elementary analysis, the substitution ratios were calculated. The substitution ratio of tosylation was 0.92, that of azidation was 0.74, and that of reduction was 0.75 (Table I).

TABLE I
RESULTS OF ELEMENTARY ANALYSIS

Sample	C (%)	H (%)	N (%)	S (%)
Chitosan	42.64	8.25	7.68	-
LMCS	37.36	6.47	6.92	-
Tosyl LMCS	38.54	5.00	3.78	7.95
6-Azido-6-Deoxy LMCS	44.54	5.03	16.84	2.06
LM6A6DC	34.87	6.24	11.85	2.74

C. Cytotoxicity Assay

Cytotoxicity was determined by MTT assay using the NHDF 3T3 cell line. There was no cytotoxicity under 100 μg/mL concentrations (Fig. 6).

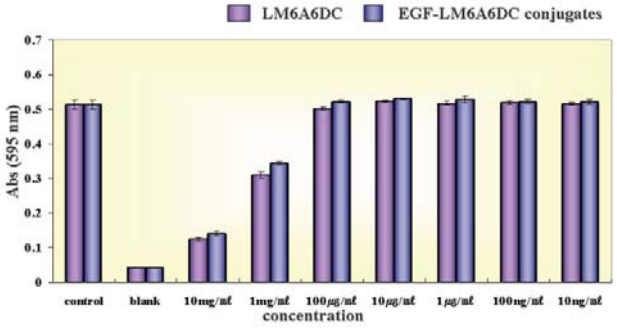

Fig. 6 Cytotoxicity assay of LM6A6DC and EGF-LM6A6DC conjugates

D. Cell Proliferation of EGF and EGF-LM6A6DC Conjugates

Cell proliferation test was carried out to confirm the proliferation effect of EGF-LM6A6DC conjugates compared to free EGF. Each sample were added as 40ng/mL concentrations and cultured during 24h and 48h. After the culturing, the group with EFG or EGF-LM6A6DC conjugates showed the better cell proliferation than control. Also, the result showed that EGF activity was lasted for over 48hours.

In the result of 48h culturing, EGF-LM6A6DC conjugates showed the better cell proliferation effect than free EGF. It is consider that EGF showed activity slowly than free EGF because EGF was encapsulated by LM6A6DC so that EGF was released gradually by biodegradation of LM6A6DC (Fig. 7).

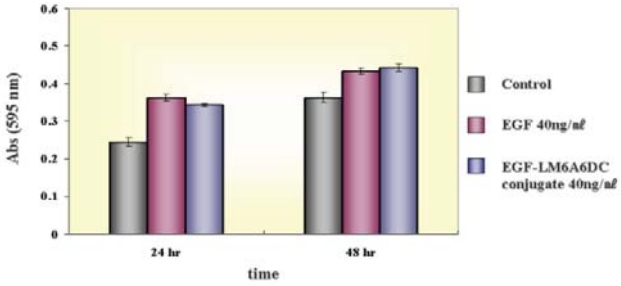

Fig. 7 NHDF 3T3 proliferation of free EGF and EGF-LM6A6DC conjugate

IV. CONCLUSION

The modification like tosylation, azidation, and reduction for the immobilization of EGF was carried out. As a result, LM6A6DC was prepared successfully. In the result of cytotoxicity assay and cell proliferation test, there was no cytotoxicity under 100 μg/mL concentration and EGF-LM6A6DC conjugates had good immobilization ability. LM6A6DC should overcome drawback like an insoluble property of high-molecular-weight chitosan so that LM6A6DC could immobilize various growth factors like an EGF and help the growth factors to active consistently.

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