Original Article

Synthesis, Characterization, and Activity Determination of Dendrimer-streptokinase Conjugated and Finding Protein Corona and Evaluation of Cytotoxicity

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Abstract

Nowadays, dendrimer conjugation with low molecular weight drugs has been considered for pharmacokinetics improvement, targeting drugs to particular sites, and helping cellular uptake. Increasing the performance of relatively great therapeutic proteins like streptokinase (SK) using dendrimers is being explored. In this study, G2 dendrimer and dendrimer-streptokinase-conjugates were synthesized. After synthesis of dendrimer-SK conjugates, characterization of these conjugates by Fourier-transform infrared radiation (FT-IR), nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC) showed that the conjugation reaction was a success, which resulted in relatively pure SK-dendrimer conjugates and checked the cytotoxicity effect. Here, we reveal that the dendrimer-streptokinase conjugates formed protein corona in whole blood cells. These results make emphasis on the need for testing the formation and biological importance of the protein corona in the blood as the nanoparticles are released into. Moreover, the potency of dendrimer-SK conjugates was greater than streptokinase. It seems that high enzymatic activity of dendrimer-SK conjugates can be a favorable way for changing bioactive macromolecules with dendrimer. Furthermore, injection of dendrimer-streptokinase conjugates may be promising for the treatment of many conditions.

Keywords: Dendrimer, streptokinase. Conjugation, potency, characteristic, Protein corona

INTRODUCTION

Streptokinase is a group of extracellular proteins (lunar) with a molecular mass of 47 kDa and 415 amino acids [1] were used for the treatment of circulatory disorders. It is produced and extracted by some streptococci beta-hemolytic strains [2,3]. It as a fibrinolytic agent can interact with plasminogen and converts it to plasmin (the active protease which degrades fibrin) [4]. Streptokinase acts as a therapeutic protein with strong thrombolytic activity. However, using it as therapy is limited because of short biological half-life [5]. This results in short biological half-life and limited therapeutic potential of this protein. Due to rapid elimination from the circulatory system, i.v. infusion of the drug for 12–72 hours is generally required. Studies reported that recombinant streptokinase (rSK) has the same benefits as natural SK [6]. Several approaches were taken into consideration for improving the effectiveness and duration of SK action and reduce its side effects [7]. PEGylation causes a change in protein, peptide or non-peptide molecule through linking one or more polyethylene glycol (PEG) chains. The benefit of this modification increased half-life of streptokinase, reduced toxicity and immunogenicity, enhanced stability and solubility of the drug and decreased degradation by enzymes. Moreover, PEGylation can increase the potentials of peptides and protein as therapeutic agents. PEG molecule can form a complex bond with free molecule including amines, thiols, or carboxylic acids existed on the protein surface [5]. Recently, dendrimers are considered as potential drug delivery vehicles. They are branched polymers with highly reactive used for covalent conjugation of drugs, ligands, and/or antibodies. Moreover, anionic dendrimers in comparison to cationic dendrimers have lower toxicity with a longer clearance time. Dendrimers have mainly been conjugated to small molecules

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than macromolecules. Unlike PEG polymer, dendrimers with their compact structure may not wrap around proteins to mask the enzyme's active sites but might still increase the protein stability by occupying the antigen recognition sites. Therefore, conjugating of SK with dendrimer retains drug enzymatic activity and improves its stability by increasing its circulation time [8]. Therefore, according to the above reasons, the production of dendrimer-recombinant streptokinase protein conjugates and the evaluation of its biological activity was the aim of the study.

Materials and Methods

Materials:

Purified recombinant streptokinase (without human albumin) was a gift from the Pasteur Institute of Iran (Tehran, Iran). The plasmin-specific substrate (S-2251), used to estimate the protein activity, was obtained from Chromogenix (Germany), BSA was acquired from Sigma (St. Louis, MO) and all other chemicals were obtained from Merck (Darmstadt, Germany).

Methods:

- #### Synthesis of G2 dendrimer and protein Loading

Dendrimer-G_2 was prepared as described previously [9]. Briefly, 2 ml or 3.7 mmol of polyethylene glycol in the DMSO solvent was used. Then, 3.7 mmol DCC was added as an activator and placed for 15 minutes on a magnetic stirrer. Afterward, 3.7 mmol acid citric was added and placed on a magnetic stirrer for 1 hour. Then, 3.7 mmol DCC was added again to the reaction. After 15 minutes of mixing, 6ml of citric acid and 10 ml of anhydrous DMSO solvent were added to the reaction. After mixing for an hour, the reaction was completed by adding 30 ml of distilled water to the reaction. Purification of synthesized G2 dendrimer was performed using a Sephadex G-75 column (Merck, Germany). 0.6 gr Sephadex powder was dissolved in 20 ml of distilled water, after 24 hours, it was transferred to a column and washed with distilled water. Impurities of the G2 dendrimer solution were removed with filter paper and transferred to the column. The purified solution was exited from the end of the column and collected. This step was repeated until all impurities were deleted. The pure G2 dendrimer was lyophilized.

- #### Recombinant streptokinase - G2 dendrimer polyethylene glycol-citrate conjugates

In the first, 200 mg G2 dendrimer was dissolved in 15 ml DMSO. Then 373 mg DCC and 200µl acid sulphuric were added to it and mix. Afterward, 15 ml recombinant streptokinase was added to the reaction and the reaction lasted for 168 hours. The reaction was terminated by adding 5 ml of distilled water. The solution was passed through a filter paper to remove impurities. Then, purification was done using a Sephadex G-75 column (Merck Germany). The samples were divided into 0.5 ml and then lyophilized.

- #### Characterization, Shape and Surface morphology of nanoparticles

The size of G dendrimer and nanoconjugates were detected with DLS (Malvern zeta sizer Nano ZS, UK) instrument. The nanoparticles were dried in room temperature and sputter-coated with gold for SEM studies and suspended in acetic acid 0.3 %w/v with pH 3 with sonication for DLS measurements

- #### Evaluation of streptokinase loading capacity (LC) and encapsulation efficiency (EE)

To determine the LC and EE, the nanoparticles were centrifuged at 15000 rpm and 4 °C for 45 min. The amount of free Streptokinase in the supernatant was determined by the Lowry method (1951). In this study, a 0.7ml alkaline copper solution was added to a 0.5 ml sample. After mixing with vortex, it was incubated for 20 minutes in darkness. Then to each sample, 0.1 ml of the Folin solution was added. After mixing, it was again incubated for 30 minutes in darkness. Moreover, bovine serum albumin (BSA) was diluted ranging from 5 to 100µg/mL concentration. After reading the absorbance of the BSA series at 750 nm and drawing a standard curve, absorbance of samples was read at 750 nm and the concentration of samples was determined by placing values in the standard curve. LC and EE of the nanoparticles were calculated from equations (1) and (2) indicated below :

1) % EE=[(Total amount of streptokinase – the amount of free streptokinase)/Total amount of Streptokinase]*100
2) % LC=[(Total amount of streptokinase – the amount of free streptokinase)/weight of nanoparticles]*100

- #### Imaging with an Atomic Force Microscope (AFM)

G2 dendrimer and G2 dendrimer - recombinant streptokinase conjugates were prepared with a concentration of 100 micromolar in distilled twice water. One drop was put on lamella and placed in a desiccator for drying. Then two-dimensional and three-dimensional images were taken by the AFM microscope

- #### Fourier transform infrared spectroscopy (FT-IR)

This technique was utilized to study the secondary structure of polypeptide and proteins in aqueous solution. The samples of drug substances of recombinant streptokinase and nano-conjugated drug were mixed with potassium bromide in ratio 1 to 200. After, preparing the film, the absorption spectra of SK was measured in the region 400 - 4000 cm^{-1} (Jasco FTIR-410, Japan).

- #### Nuclear Magnetic Resonance (NMR) Spectroscopy (H-NMR)

In this method, 0.5 ml of DMSO solvent was added to the sample and vortexed for 2 minutes to mix. In this method, recombinant streptokinase with and without conjugation form

was analyzed and finally, both were compared (Bruker, Germany).

- ### Evaluation of the biological activity of recombinant streptokinase and G2 dendrimer-Streptokinase Recombinant:

In this technique, 50 µl of different concentrations of standard streptokinase and each sample were added to 50 µl of plasma in microplate wells and incubated at 37 ° C. Then, 50 µl of chromogenic substrate S2251 was added to wells. After incubation at 37° C, 25µl acetic acid is used and absorbance was read in wavelength 405 nm by ELIZA reader. Then, the results were obtained by standard curve and shown in terms of IU/ml.

- ### Protein corona formed in human Blood cells

The protein corona mass was determined through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Samples of Sodium dodecyl sulfate-polyacrylamide gel SDS-PAGE were prepared for analysis of SDS-PAGE. The SDS resolving and stacking gels were prepared by mixing components including water, degassed bisacrylamide, gel buffer, SDS, ammonium persulfate, and N, N, N0, N0-tetramethylethylenediamine. Then the gels were incubated for 15 minutes at 37 degrees centigrade. Moreover, the supernatant was diluted with a sample buffer (water, Tris–HCl [pH 6.8], glycerol, SDS, and bromophenol blue). Furthermore, B mercaptoethanol was added to sample buffer to cleavage disulfide bond and heated at 100°C for 2-3 min. Then, equal amounts of total proteins (14 mg/ml) were loaded on a 10% SDS-PAGE gel. Samples were loaded and stacked in the stacking gel, and electrophoresis was conducted at 18 mA per gel (90 V) until the bromophenol blue marker reached the bottom of the gel. Finally, the proteins were stained with Coomassie brilliant blue (0.025% Coomassie blue in 10% acetic acid) for 60 min on a rotary shaker and then destained for 30 min with fresh 10% acetic acid while being shaken. In the next step, separated proteins were transferred to a nitrocellulose membrane according to the procedure. Transfer of proteins to nitrocellulose was performed in transfer buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, 20 % methanol, pH 7.5) at 30 V, 40 mA overnight at 4 °C. Then, blocking of nitrocellulose was done with blocking buffer (1 % BSA in 50 mM Tris, 0.9 % NaCl, 0.05 % Tween 20, pH 7.5) overnight. Then, the nitrocellulose membrane was incubated for 2 hours in a buffer (Tris, NaCl, Tween 20, pH 7.5) plus the primary antibody (monoclonal anti streptokinase antibody). After incubation with secondary antibody (Complex-HRP conjugated), the bands of protein were observed in an HRP substrate di amino benzidine tetrahydrochloride (DAB). β-actin (dilution 1:10000) was considered as an internal loading control (dilution 1:10000) and the same membrane was probed with b actin.

The method of detecting protein and nanoconjugate based on MASS (protein corona)

For analysis of control samples, laser mass spectrometer and MALDI matrix ionization (Application Applied Biosystems 4800 MALDI TOF/TOF) were used. Before the analysis of MALDI TOF, sample drug and nano-conjugate drug bypassing the chromatography pump based on pressure phase and manufacturer's instructions were desalinated by the C18 compressed column (Millipore, Bedford, USA).

- ### In vivo evaluation of the Nano cytotoxicity:

In this study, 5 rats were chosen. Nano-conjugate drug was injected to rat. Mice were killed 24 hours after the injection of a Nano-conjugate drug (1.4 mg/ml). Then the heart, brain, liver, and kidney were taken. Tissues of the brain, heart, kidney, and life were taken fresh from rats (n=5). The specimens were fixed in 10% neutral buffer formalin, then they are put in different concentrations of alcohol 70%, 80%, 90%, and 100%, then, they were immersed in xylene and afterward placed in paraffin in automatic tissue processor. Following fixation, the specimens were embedded on wax paraffin and sliced to 5 µm in thickness. The hematoxylin and eosin (H & E) were used to stain and analyze tissue sections as a histological method to identify various tissue types and the morphologic changes.

RESULTS:

Characterization of nanoparticles:

Figure 1 (A and B) shows AFM microscope related to dendrimer and dendrimer-SK. As shown in Figure 1, the particles were dispersed separately and uniformly over the lamellas. The size of the dendrimer and dendrimer-SK conjugates is shown in Figures 2 and 3, respectively. A comparison of figures showed that the size of dendrimer-SK conjugates was greater than the dendrimer size. For the characterization of dendrimer-streptokinase conjugates, the following techniques were used. The loading capacity is 90%, it means that 90% of the nanoparticles G2 weight is composed of the recombinant streptokinase. Each 1 mg nanoparticles contains 0.9 mg drug. % EE is 20%, it means that 20% of recombinant streptokinase is entrapped into the nanoparticles.

Fourier-transform infrared radiation (FT-IR) spectra analysis

To confirm conjugation, FT-IR was performed (Fig. 4). As depicted in that figure, the FT-IR spectra were due to PEG-streptokinase.

The presence of peaks in dendrimer-streptokinase conjugates at 3416cm^{-1}2000 cm^{-1}1355 cm^{-1} and 691cm^{-1} are relevant to the hydroxyl group (OH), carbonyl group (C=O), double bond group (C=C) and aromatic (C-N), respectively.

Nuclear magnetic resonance (NMR)

H-NMR spectra of SK and dendrimer-SK are shown in Figure 2. Results of HNMR spectroscopy showed the presence of multiple sharp peaks in the red spectrum compared to other spectra. It means that streptokinase was successfully

conjugated. HNMR data confirmed FTIR results which are indicated that streptokinase was conjugated. Purification of conjugates was achieved by gel filtration high-performance liquid chromatography (HPLC) HPLC analysis showed a sharp peak at 280 nm related to dendrimer-SK conjugates and three to four peaks indicating some impurities. Figure 7 shows the result of the Protein Corona (SDS-PAGE analysis of PEG streptokinase after purification). As shown in figure 7, the dendrimer-SK conjugates were shown as a thick single band (47 kDa) indicating dendrimer-streptokinase conjugates. Figure 8 shows the western blot analysis of streptokinase and *dendrimer-streptokinase* conjugates. As shown in figure 8, there was no significant difference between streptokinase and *dendrimer-streptokinase* conjugates in terms of protein expression.

MALDI-Mass

In the MALDI-mass technique, Mass=25874, and Score=65. It means that protein is 65% similar to bone marrow proteoglycan. The following spectra show LC-mass spectra. As shown by LC mass spectra, the peak observed at m/z 300 indicated conjugates.

Evaluation of Potency (Drug Activity)

The mean potency value of streptokinase and dendrimer-streptokinase conjugates is shown in Table 2. The result of Table 1 showed higher potency of dendrimer-streptokinase conjugates than streptokinase.

Cytotoxicity results

Histopathology of tissues including kidney, liver, myocardium, and lung after injection of PEG- streptokinase is shown in figure 10 (A, B, C, D). The result of pathology showed that injection of dendrimer-streptokinase conjugates did not change cell morphology. Moreover, the ratio of nuclear/cytoplasm was constant. Furthermore, the mitosis was normal.

Discussion

Streptokinase is an agent with a highly effective thrombolytic property. But its application in therapy is limited because of the short biological half-life in vivo. PEGylation of SK increases therapeutic attributes, but it is associated with some limitations [10]. Dendrimers are used as drug targeting vehicles, imaging, and many other biomedical applications.

In our study, the conjugation of SK was confirmed by FT-IR, NMR, and HPLC. Some studies reported that conjugation of SK can be characterized by SDS-PAG gel chromatography, isoelectric focusing and size exclusion chromatography. Nandini et al. evaluated various methods for detecting of SK conjugation and developed high-performance liquid chromatography based on size exclusion. It can determine the clinical properties of the protein, molecular size, polymer distribution as well as weight composition [11]. In our study, we used western blot as a valuable test for the analysis of proteins fractionated based on the molecular weight in SDS gel electrophoresis. The result of our study showed that no significant difference was seen between dendrimer-SK conjugates and streptokinase in terms of protein expression.

The SK-dendrimer may have a main effect on the activity of SK conjugates. Moreover, attachment of several numbers of dendrimer molecules to SK decreases the activity due to the masking of active sites of SK by dendrimer [8]. In our study, measurement of drug activity (potency) showed that the activity of dendrimer-SK conjugates is greater than SK. Wang et al. synthesized a series of streptokinase-poly (amidoamine) G3.5 conjugates with different amounts of dendrimer-to-protein. All of the SK conjugates were found improved stability in phosphate buffer solution compared to free SK. Mukhametova et al. reported that modification of SK including size, rigidity, and density of negative charge on the dendrimer surface affects the thrombolytic activity of dendrimer-SK conjugates. So that SK-G3.5 conjugates retain up to 85% of the thrombolytic activity compared with free SK [7].

Moreover, injection of dendrimer-streptokinase conjugates may be useful for the treatment of many previously difficult-to-treat conditions.

Conclusion

Dendrimers were useful for effective attachment to various proteins. It seems that high enzymatic activity of dendrimer-SK conjugates could be a favorable way for changing bioactive macromolecules with dendrimer. Furthermore, injection of dendrimer-streptokinase conjugates may be promising for the treatment of many conditions.

Disclosure statement

There is no conflict of interest. Moreover, the authors are responsible for the content and writing of this paper.

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Table 1: Gradient program table for high-pressure liquid chromatography apparatus

Time(min)	Mobile phase buffer A(V/V)	Mobile phase buffer B(V/V)
0-1	68	32
1-4	68→52	32→48
4-5	52	48
5-7	0	100
7-10	68	32

Table 2: The mean potency value of streptokinase and dendrimer- streptokinase conjugates

Variable	Mean ± SD
PEG-streptokinase	0.00003
streptokinase	0.00002

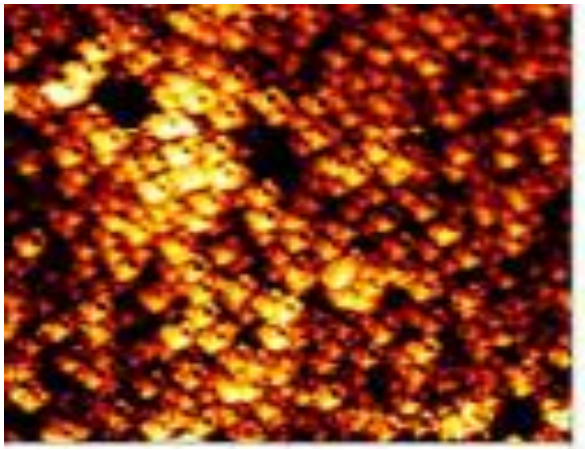

Figure 1(A): AFM picture related to the dendrimer

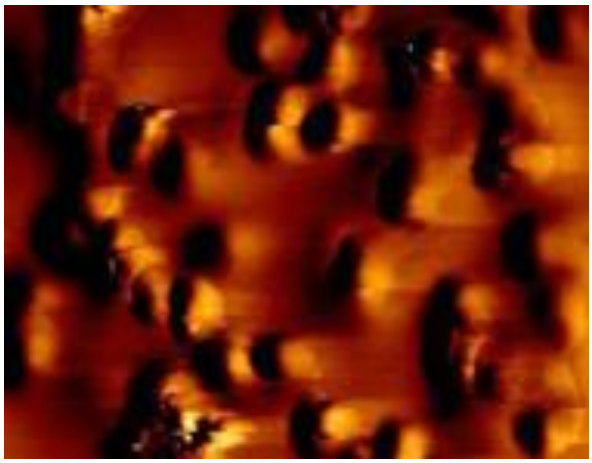

Figure 1(B): AFM picture related to dendrimer-SK

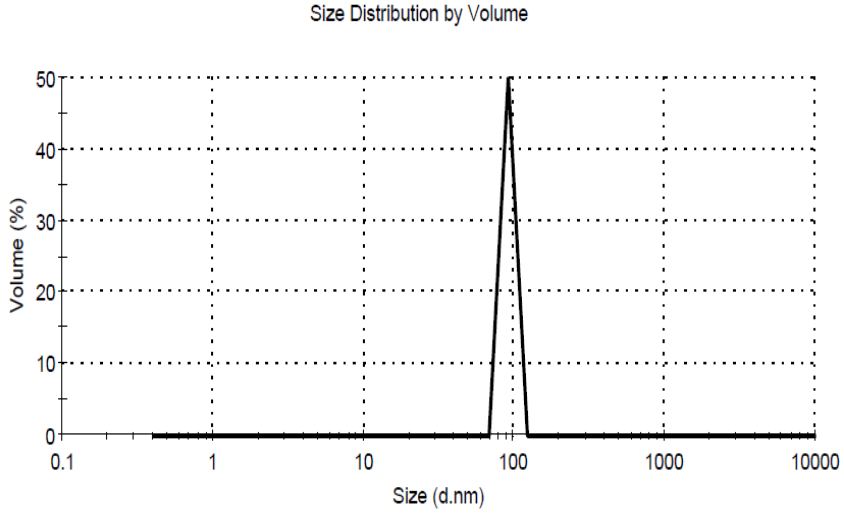

Figure 2: Size of dendrimer

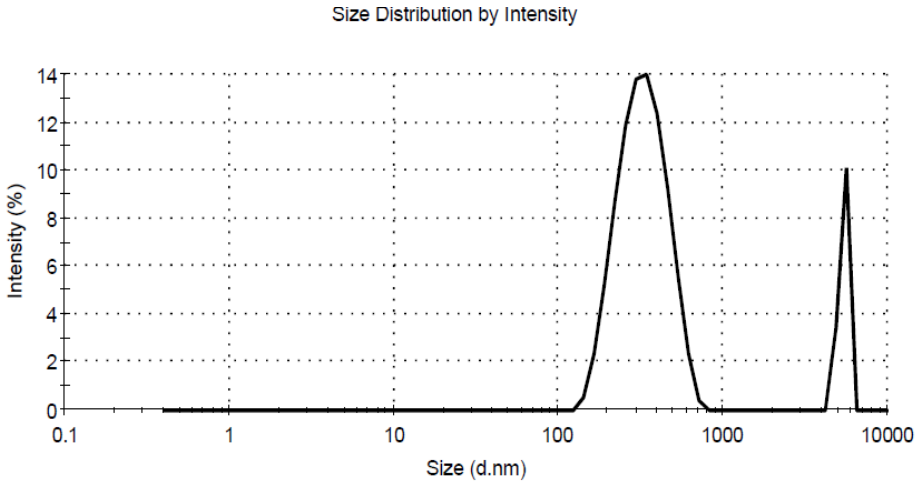

Figure 3: Size of dendrimer-SK conjugates

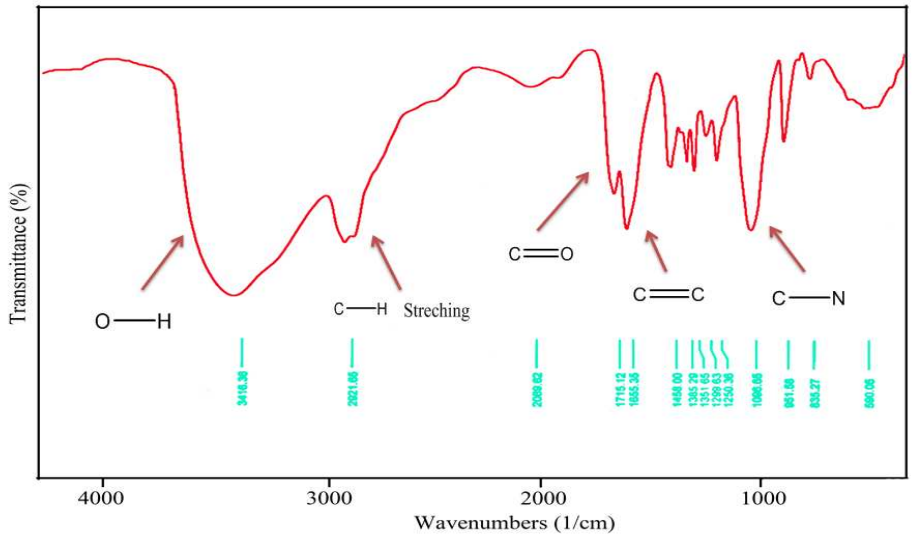

Figure 4: FT-IR spectra of PEG-streptokinase

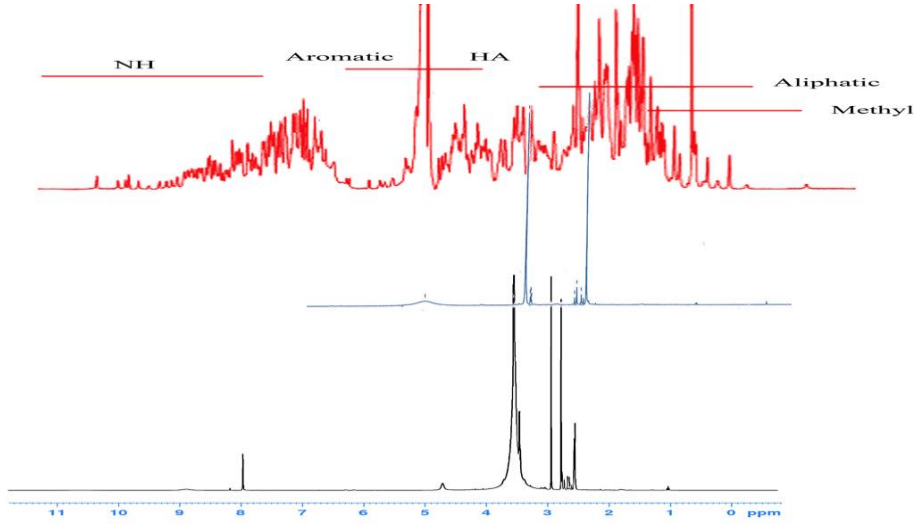

Figure 5: HNMR spectroscopy of dendrimer-SK and SK

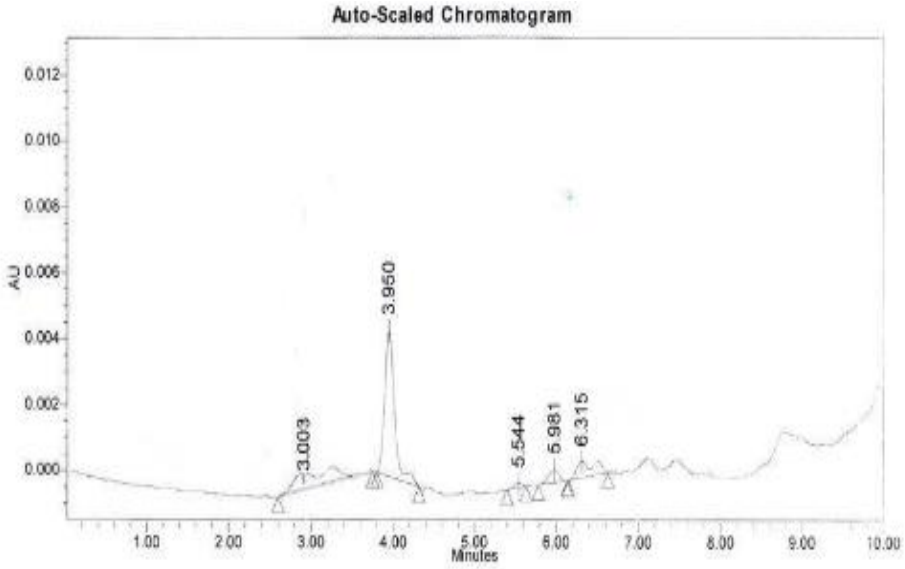

Figure 6: HPLC chromatograms of PEG-Stroptokines

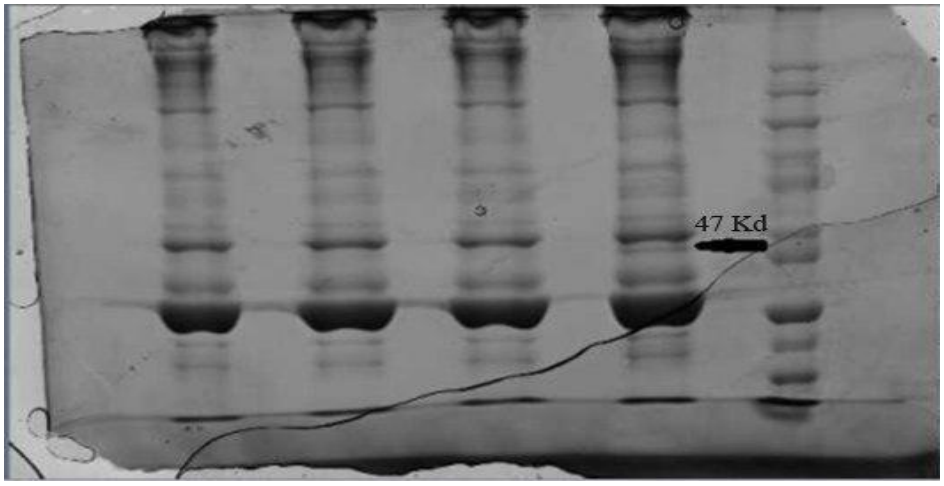

Figure 7: The result of corona protein (SDS-PAGE analysis of PEG streptokinase after purification)

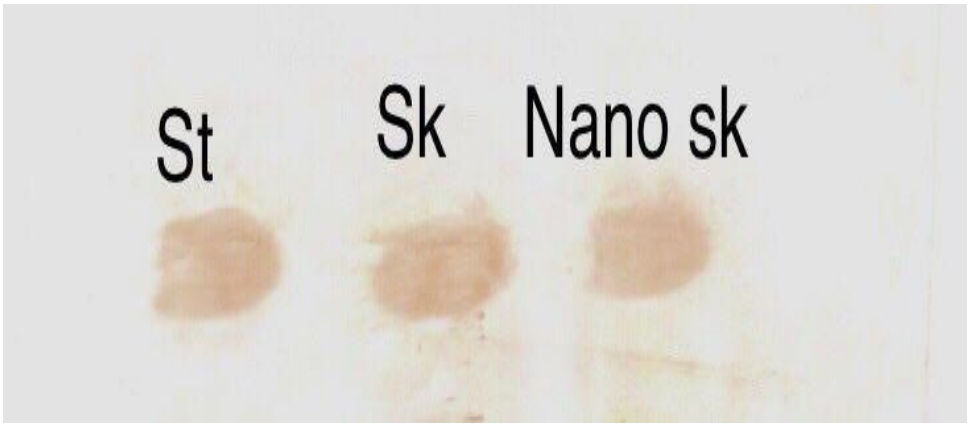

Figure 8: Western blot analysis of streptokinase and *dendrimer-streptokinase* conjugates (left: ST: standard, SK: streptokinase, Nano SK: dendrimer-streptokinase)

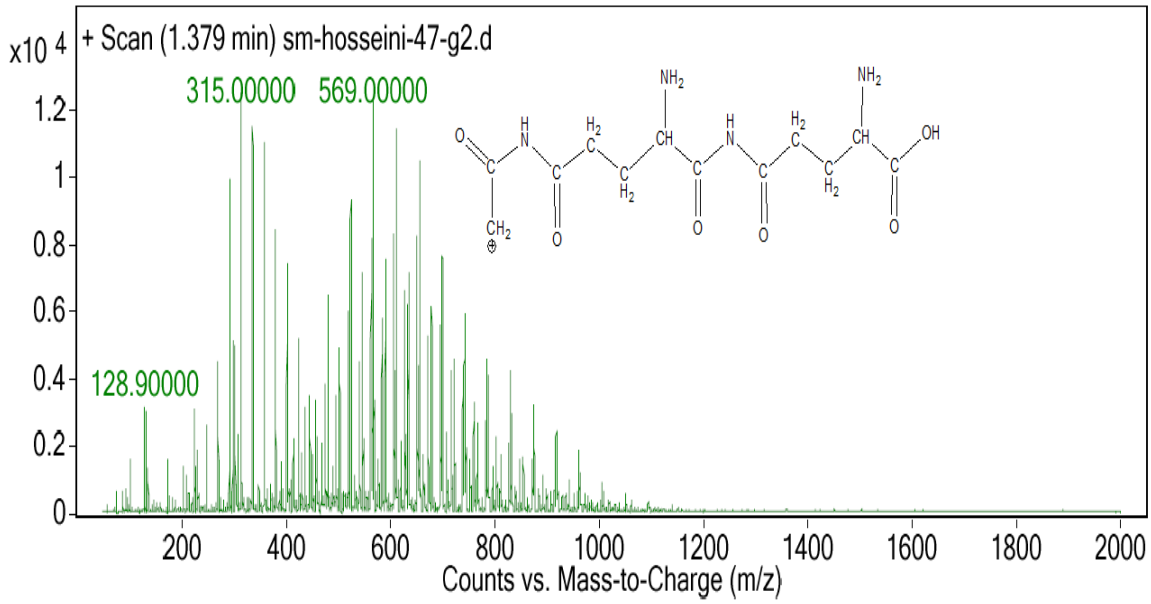

Figure 9: LC mass spectra of conjugated protein

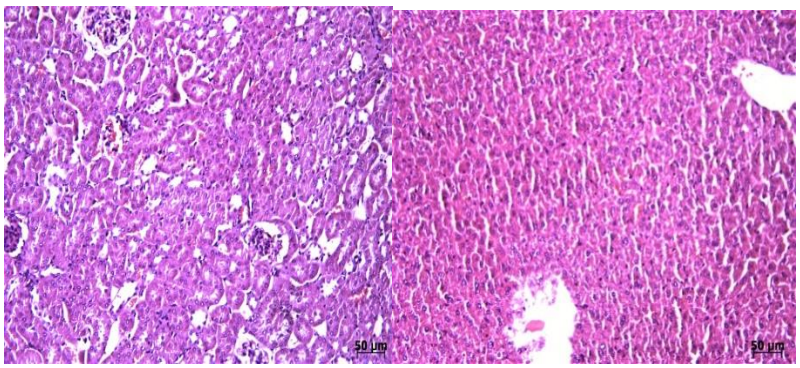

Figure 10A: Kidney conjugation **Figure 10 B:** Liver conjugation

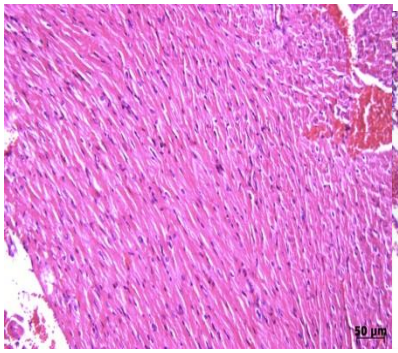

Figure 10C: Myocardium conjugation

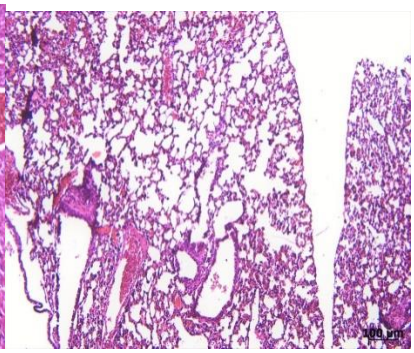

Figure 10 D: Lung conjugation