DEVELOPMENT OF DELIVERY SYSTEM FOR TRANSFERRIN USING CHEMICAL MODIFICATION APPROACH

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ABSTRACT

Proteins are very important molecules as therapeutics. They have several advantages over small molecule drugs but their use is limited by their circulation short half life, immunogenicity and physicochemical instability. Several approaches have been developed to improve the pharmacokinetics of proteins. One that is clinically proven is the covalent conjugation of poly ethylene glycol (PEG) to the protein. This is known as PEGylation. Problems associated with PEGylation include product heterogenicity and low conjugation efficiency. A new approach is applied in this study which is site-specific PEGylation and it is called disulfide bridging PEGylation. The main principle is to reduce a native disulfide bond in the protein molecule followed by PEGylation using a bis-alkylation PEG reagent that can insert a three carbon bridge that connects to the two sulphur that had formed the disulfide. In this study controlled reduction and disulfide bridging PEGylation of transferrin (which has 19 disulfides some that are close to the surface and are accessible) was conducted. For transferrin (Tf) partial reduction was achieved using (0.5 mM) DTT which is considered to be mild reducing conditions. No denaturant was required for the partial reduction of transferrin. Mono-PEGylation was achieved with a small amount of di-PEGylation being observed. Holo-Tf (which is Tf bound to iron) and apo-Tf (which is Tf iron free) were compared to see if bounded iron had any effect on protein conformation that could influence disulfide reduction or PEGylation. Both forms of Tf give the same results by Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS PAGE) indicating that iron had no effect. Using higher equivalents of the PEG reagent was unnecessary; we obtained good mono-PEGylation

by adding 2 equivalents of PEG to the protein. The results approved by Size exclusion chromatography –High performance liquid chromatography (SEC-HPLC) analysis. These results demonstrate the possibility of controlled reduction of multiple disulfide proteins under mild conditions which may improve the stability, prolong the half life and improve pharmacokinetics of Tf to present it as medicine.

INTRODUCTION

Proteins are very complex molecules. They have many key roles in regulating the biological functions inside the human body [1]. Therapeutic proteins are used for replacement therapies when specific endogenous protein is deficient [2]. There are some obstacles in the development of proteins as medicines, such as short half life, route of administration, high molecular weight, low solubility, low stability, hydrophilicity, purification and immunogenicity and this leads to poor pharmacokinetic properties [3-5]. To overcome these difficulties in the development of proteins as medicines (mainly their short half life) and to optimise their pharmacokinetic properties, several approaches have been developed such as, PEGylation, FC fusion, slow release formulations, protease-resistant variants, protein fusion and protein cross linking [6-12].

PEGylation is the covalent attachment of polyethylene glycol (PEG) to the protein molecule. PEGylation is introduced to increase the half life of proteins. PEG is a neutral, hydrophilic, polymeric molecule that is considered non immunogenic and non toxic [9]. PEGylation is conducted by nucleophilic addition of electrophilic PEG reagents. PEG is unreactive by itself so must be modified so that it can be conjugated to a protein [13-16]. These active groups on PEG can react with amino groups (alpha or epsilon) of lysine of the protein (lysine accounts for 10% of amino acids in a typical protein) [4,9]. Due to the presence of different lysines in the protein molecule, multiple PEG molecules may attach to a single protein causing heterogeneity and positional isomerism. Each positional isomer has different activity and may lead to batch to batch variability. Site specific PEGylation has been introduced to overcome these problems. One of the methods utilized for site specific PEGylation is Disulfide Bridging PEGylation. This technique involves 2 steps, first; reduction of disulfide bond to release its two thiols, and second; using bis-alkylating PEG reagent to rebridge the released free thiols [17,18]. This technique requires the reduction of native disulfides which required (in proteins other than transferrin) unfolding for

proteins with multiple disulfides which leads to loos their activities. Therefore controlled reduction of proteins is required to avoid unfolding and to avoid losing their activities[19-21].

Transferrin (Tf) is a glycoprotein that is widely distributed in the blood. Tf has 19 disulfide bonds and some of them are close to the surface [15], which makes them ideal to investigate the possibility of control reduction of disulfide bonds. Investigating the possibility of reduction of Tf using mild conditions may help in developing the PEGylation of Tf. Our aim in this research is to investigate the possibility of controlled reduction of Tf using mild conditions and by controlling the number of disulfide bonds that are opened we can control the PEGylation process to achieve a higher amount of site-specific mono-PEGylation and minimise heterogeinicity and positional isomerism and this may contribute to the development of proteins with improved solubility and pharmacokinetic properties to be presented as medicin.

MATERIALS AND METHODS

Materials

Human apo-transferrin, human holo-transferrin (MW 76-81 KDa) and DL-dithiothreitol (DTT) were purchased from sigma Aldrich. Novex Bis-tris 4-12% precast gels, SDS-PAGE kit, Ellman's reagent (5,5-dithiobis(2-nitrobenzoic acid)). Sodium dihydrogen orthophosphate dehydrates ($Na_2H_2PO_4.2H_2O$), ethylenediaminetetraacetic acid (EDTA), oxidised L-glutathione (GSSG), methanol, acetic acid, barium chloride, perchloric acid, iodine, sodium chloride, PD10 column, Eppendorf tubes. PEG bis-sulfone **2** (10 KDa and 30 KDa) synthesized in our laboratory. Double beam UV spectroscopy (U-2800 A) and SEC-HPLC system with UV detector.

Methods

Reduction of disulfide bonds using DTT(DL-Dithiothreitol)

Reduction of Tf was conducted with DTT at different concentrations. Tf was prepared as a solution of (1.0 mg/ml) in sodium phosphate buffer (0.1 M sodium phosphate, pH=7.0, 1.0 mM EDTA). Different molar concentrations of DTT used ranging from (1-100 mM). Typically the protein solution (1.0 ml) was added to the

required amount of DTT and incubated for a specific period of time (ranging from 30-120 minutes) at 20^0 C. The reduced protein solution (1.0 ml) was then added to pre-equilibrated PD10 column and allowed to elute completely onto the column (this is to remove DTT from the protein solution). The column was equilibrated with (25.0 ml) of the buffer used to prepare the protein solution. Fresh buffer (2.0 ml) was then added and discarded. Another aliquot of buffer (2.0 ml) was added and this fraction was collected. This collected volume (2.0 ml) contained the reduced protein without DTT. The molar concentration of the recovered protein in the sample was accomplished by measuring the absorbance by UV spectroscopy at 280 nm and the molar concentration of the protein in the sample was calculated by dividing the absorbance measured by the molar absorption coefficient of the protein (For Tf is 83451 M^{-1} cm^{-1}) [22].

Quantification of free thiols using Ellman's assay

To calculate the amount of free thiols /protein the Ellman's test was used. We prepared the Ellman's reaction buffer (0.1 M sodium phosphate buffer, 1.0 mM EDTA, pH=8). A solution of Ellman's reagent was prepared by dissolving (4.0 mg) of Ellman's reagent powder in (1.0 ml) of the reaction buffer. To a set of tubes that contain (2.5 ml) of the reaction buffer and (50.0 μl) of Ellman's reagent, (250 μl) of the protein sample was added (the number of tubes depend on the number of samples tested with an extra tube for the blank. The blank contains (250 μl) of reaction buffer. The solutions were allowed to incubate for 15 minutes at 20^0 C. Using plastic cuvettes, the absorbance was measured at 412 nm using a UV spectrometer. The molar concentration of thiols in the sample was then calculated by dividing the absorbance measured by the molar extinction coefficient of thiols which is 14150 $M^{-1}cm^{-1}$ [22]. The value obtained was divided by the molar concentration of the protein in the sample to obtain the number of thiols/protein.

PEGylation of protein using bis-sulfone PEG 2

The reduced protein solution was taken and the molar concentration of the protein in the sample was calculated. The amount of bis-sulfone PEG **2** that was needed was calculated by multiplying the molar concentration of the protein in the sample to be PEGylated by the number of equivalents of PEG required (to get 1:1, 1:2 protein: PEG) then multiplied by the molecular weight of PEG used (which is

either 10 KDa or 30 KDa). The stock solution available for 10 KDa PEG was (15.0 mg/ml) and for 30 KDa PEG was (10.0 mg/ml). The calculated mls needed for PEGylation was added to the protein solution and the mixture allowed to incubate overnight in the refrigerator at 4^0C. In order to recover the reduced non-PEGylated protein to avoid their interference reoxidation of reduced non PEGylated protein was made using a reoxidizing reagentwhich is (1.0 mM) oxidized glutathione (GSSG) which is added to the sample and incubated overnight in the refrigerator (4^0 C). Then the PEGylated protein tested on SDS-PAGE [18].

Detection of the PEGylated protein using SDS-PAGE(Sodium dodecyl sulphate-polyacrylamide gel)

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used for the analysis of the PEGylated samples using electric current applied to a gel matrix. Polyacrylamide provides a means of separation by size (obstruct large molecules while allowing small molecules to migrate). The samples are first prepared by adding (10.0 µl) of NuPAGE® LDS sample buffer to each micro-eppendorf tube followed by (30.0 µl) of the sample to be tested was added. Polyacryl amid gel (Novex® bis-tris 4-12% precast gel) was used. The gel washed with deionised water. Then (20.0 µl) of the prepared sample was added to each tube of the gel. Each of these precasts gels contains 10 tubes. The first sample was generally for See Blue® (prestained protein standards which contain several proteins with known mwt). The running buffer solution was prepared by mixing (100 ml) of running buffer (NUPAGE® MOPS SDS running buffer) with (900 ml) of deionized water. After charging the gel with the samples, the gel was placed in an electrophoresis tank and the tank filled to the level that cover the gel with the running buffer solution and electrical current was applied. Once the gel removed from the tank and the gel released and carefully placed in the fixing solution. Both fixing and staining solutions should be prepared 5 minutes before use. The fixing solution consists of water (40.0 ml), methanol (50.0 ml) and acetic acid (10.0 ml). After 10 minutes the gel was removed from the fixing solution and then placed in the staining solution. The staining solution consists of water (55.0 ml), methanol (20.0 ml) and stainer A (20.0 ml). After 5 minutes (2-3 drops) of stainer B was added and the gel left overnight. The gel was then removed and rinsed several times with water then scanned [18].

Barium iodide staining of gels

Barium iodide staining was used to detect PEG in the SDS-PAGE gel. In the case of a PEGylated protein the procedure was to first use cromassi blue to dye the protein as prescribed above followed by barium iodide staining to dye PEG. In the beginning the gel left in water overnight to remove excess cromassi blue stain then the gel was placed in (40.0 ml) of (0.1 M) perchloric acid for 10 minutes. Then (10.0 ml) of 5 % barium chloride solution was added to the gel and left for 10 minutes. After 10 minutes (2.0 ml) of (0.1 M) iodine was added to the gel and left for another 10 minutes. The gel was then removed and rinsed with water several times and left in water for 3-5 minutes to remove the excess iodine. The non-PEGylated protein remains blue while the PEGylated one becomes brown in color then scanned [18].

SEC-HPLC

Size exclusion chromatography-high performance liquid chromatography (SEC-HPLC) is a technique used for separation of the mixture components based on size. A HiLoad supredex 200 grade column was used for separation. The eluent buffer was sodium phosphate buffer (50 mM sodium phosphate, pH=7.0, 150 mM NaCl). The flow rate used was 1 ml/minute and the running time was 120 minutes. Samples were collected at the peaks and tested by SDS-PAGE. The column used in SEC-HPLC is packed with porous beads and the running buffer is forced through the column at a constant rate. The sample introduced into the running buffer stream going through the column. The porous beads packed in the column are with specific pore sizes; as the molecules flow through the column, they pass by these porous beads. If the molecules fit in the pore they are drown in the pore by diffusion, if not the molecules continue flowing with the buffer. A detector monitors the concentration of the samples exiting the end of the column; we get a relative distribution of molecular weight of the sample composition. Each peak in the SEC is an indication of the presence of certain molecular weight. Molecules with the higher molecular weight appear first on SEC followed by the smaller ones.

RESULTS AND DISCUSSION

Transferrin Tf is an extracellular protein with 19 disulfides some of them are accessible which makes it an ideal molecule to investigate the possibility of partial reduction and PEGylation. Tf binds iron in the blood and transports it throughout the

body. Tf receptors are widely distributed inside the human body and overexpressed in tumor cells which make Tf an ideal molecule to investigate targeting delivery system [23,24]. We hypothesized that Tf bound to iron might be more tightly folded and might display a different disulfide reduction product therefore both forms of Tf were examined which are apo-Tf (iron free Tf) and holo-Tf (iron loaded Tf), for the investigation of possible reduction of the lowest number of disulfide bonds followed by bis-alkylation PEGylation.

The reduction of holo-Tf was examined because it is the iron loaded form and it binds with the receptor in this form, using DTT as the reducing agent as explained in the experimental part. After reduction had been carried out the Ellman's test was conducted to quantify the number of thiols in the sample. The number of thiols/protein calculated was (12.93 thiols/protein) which suggest that 6 disulfide bonds had been reduced out of the 19 disulfide bonds in holo-Tf. To optimize the reaction conditions to achieve partial reduction we continued the reduction of holo-Tf with the same conditions using different molar concentrations of DTT (50, 30, 10, 1 mM). The result can be observed in (Table 1). We observed a reduction in the number of disulfide bonds reduced parallel to the reduction in concentration of DTT. As we increase the molar concentration of DTT, more DTT molecules will target the deeper bonds together with the surface bonds. This will reduce large number of disulfide bonds in Tf. We concluded from the results above that the best concentration of DTT solution to be used to achieve partial reduction is (1.0) mM.

To confirm the number of disulfide bonds reduced as calculated according to Ellman's, Tf was PEGylated with 10 KDa bis-sulfone PEG **2**. Both apo-Tf and holo-Tf reduced with (1.0) mM DTT and PEGylated. SDS-PAGE followed by cromassi blue and barium iodide dying was used to confirm PEGylation. We can see in Figure 1 A (lane 5 for apo-Tf and 6 for holo-Tf) three forms of PEGylated Tf (mono, di, and tri-PEGylated forms) which suggests the reduction of three disulfide bonds. Barium iodide staining (stains only PEG with brown color) confirms the PEGylation in Figure 1 B (lane 5 and 6). Although there is more than one disulfide bond reduced we can see predominant mono-PEGylation with very little amount of di-PEGylation and hardly notice the tri-PEGylation as shown in Figure 1B.

We notice a difference between the calculated number of thiols/protein according to Ellman's test (which suggested reduction of 6 disulfide bonds) and the PEGylation on SDS-PAGE (which suggested reduction of predominant 2 disulfide

bonds). This may be due to the nature of Ellman's test. Ellman's test quantifies the molar concentration of thiols in the sample and by dividing the molar concentration of thiols over the molar concentration of the protein we calculate the number of free thiols available for each protein molecule. These calculations based on the assumption that all the protein molecules are reduced. In fact some of the protein molecules remain unreduced. This may give non-precise estimation about the number of free thiols that actually present in each protein molecule. Also Ellman's reagent interacts with any DTT available in the sample (because DTT contain free thiols in its structure) while SDS-PAGE technique is more precise. The results demonstrate no difference between holo-Tf and apo-Tf in reaction as seen in Figure 1 A (lane 5 for apo-Tf and 6 for holo-Tf), which means iron has no effect or interaction with both reduction and PEGylation process. Our results shows that solvent accessable disulfide bonds(surface disulfide bond) of both forms of Tf can be reduced in mild conditions. Partial reduction acheived since 2 of the 19 disulfide bonds in Tf reduced with predomenant mono-PEGylation.

To investigate the effect of the number of equivalents of PEG used; apo Tf reduced with 0.5 mM DTT in the same conditions mentioned above and PEGylated with 2 and 4 equivalent of PEG then tested on SDS-PAGE to confirm PEGylation. We can see in Figure 2 A and B (lane1 for 2 equivalent PEG and lane 2 for 4 equivalent PEG) that there is no observed difference between the two. Therefore increasing the number of equivalents of PEG added is unnecessary to have predominant mono-PEGylation.

We reduced apo-Tf using the same conditions that were used for reduction of holo-Tf and PEGylated with 30 KDa and 10 KDa bis-sulfone PEG **2** to see if there is a difference between the two forms of Tf and the results observed in Figure 3 (C and D). There is no observed difference in the results between holo and apo-Tf which confirms that iron has no effect on reduction and PEGylation.

To eliminate the possibility of other reactions in the sample during PEGylation, a control reaction was conducted where bis-sulfone PEG KDa 30 was added to both types of Tf without reduction incubated overnight in the refrigerator (4^0 C). SDS-PAGE of the control reaction shows an unexpected band similar in position to mono-PEGylation. This suggests that an interaction between bis-sulfone PEG **2** and holo-Tf and apo-Tf takes place as shown in Figure 4.

All the above results indicate that it is possible to control the reduction of multiple disulfide bonds in transferrin. We were able to reduce only two disulfides (with predominant monoreduction) with mild conditions, which is 0.5 mM DTT without the need of adding a denaturant. Also we conclude that iron has no effect or interference with either reduction or PEGylation of Tf. Using higher equivalent of PEG for PEGylation is unnecessary, and using the equivalents of 1:2 (protein:PEG) gives satisfactory mono-PEGylation.

SEC-HPLC

To confirm our PEGylation SEC-HPLC is used for separation of the sample components according to size. Using SEC gives an idea about the different compositions of the sample. We chose 3 samples to run on SEC. For each sample tested on SEC, samples from the peaks were collected and tested on SDS-PAGE. The first sample run on SDS-PAGE was the native holo-Tf. We run the native protein to confirm the time at which the peak for Tf appears, and to see if there is any contamination or aggregation in the sample. Figure 5 shows the peak for apo-Tf after 80 minutes.

The second sample to run on SEC was the holo-Tf reduced with 0.5 mM DTT and PEGylated with 2 equivalent 30 KDa PEG as shown in Figure 6. There are peaks at 55, 60 and 80 minutes. From the previous experiment we know that the peak for native holo-Tf appears at 80 minutes, so we suggested that the peak at 55 minutes is for di-PEGylation and at 60 minutes mono-PEGylation because in SEC larger molecules appear first. Samples collected at the peaks tested on SDS-PAGE and the results confirm our suggestion.

Running the control reaction (which consists of native holo-Tf and 30 KDa PEG) on SDS-PAGE shows unexpected band similar in position to mono-PEGylation as explained before. We decided to run the control reaction on SEC and observe the results. We can see in Figure 7 no signs of peaks at 55 and 60 minutes which suggest the absence of PEGylation. And the different small peaks at 62, 64 minutes appear. SDS-PAGE on the samples collected at the time intervals (62, 64, 80 minutes) shows only the native protein at 80 minutes, which eliminate the possibility of any PEGylation interaction between the native protein and the 30 KDa bis-sulfone PEG **2**.

The results obtained from SEC-HPLC are in consistent with our results for reduction and PEGylation of Tf obtained by SDS-PAGE and it is confirmed that predominant mono-PEGylation takes place and the conditions chosen for reduction and PEGylation of Tf using DDT are optimized.

Table 1. Number of thiols/protein as determined using Ellman's test for holo-Tf.

DTT mM	**Thiols/protein**
100	12.93
50	10.89
30	7.3
10	3.84
1	1.54

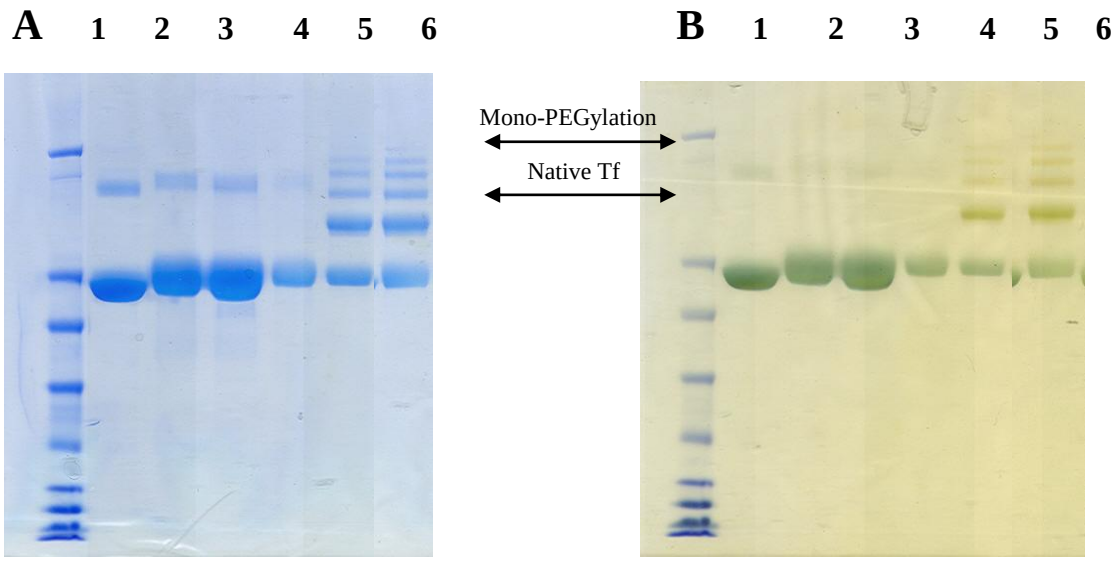

Figure 1. SDS-PAGE of holo-Tf and apo-Tf, **A** in cromassi blue and **B** in barium iodide stain. Lane **1:** native apo-Tf; lane **2:** apo-Tf reduced with 1 mM DTT; lane **3:** native holo-Tf; lane **4:** holo-Tf reduced with 1 mM DTT; lane **5:** apo-Tf reduced with 1 mM DTT+1eq PEG 10 KDa; lane **6:** holo-Tf reduced with 1 mM DTT+1eq PEG 10 KDa.

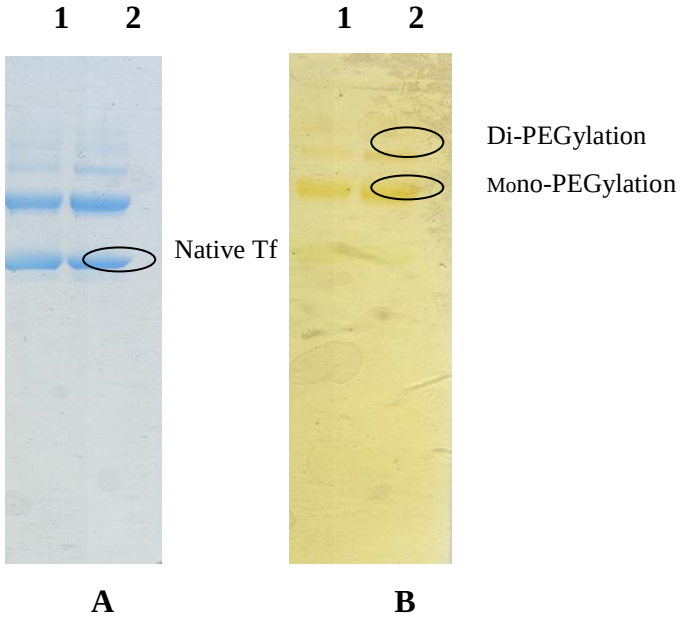

Figure 2. SDS-PAGE of apo-Tf, **A** in cromassi blue stain and **B** in barium iodide stain. lane **1:** apo-Tf reduced with 0.5 mM DTT+ 2eq. PEG 10 KDa; lane **2:** apo-Tf reduced with 0.5 mM DTT+ 4eq. PEG 10 KDa.

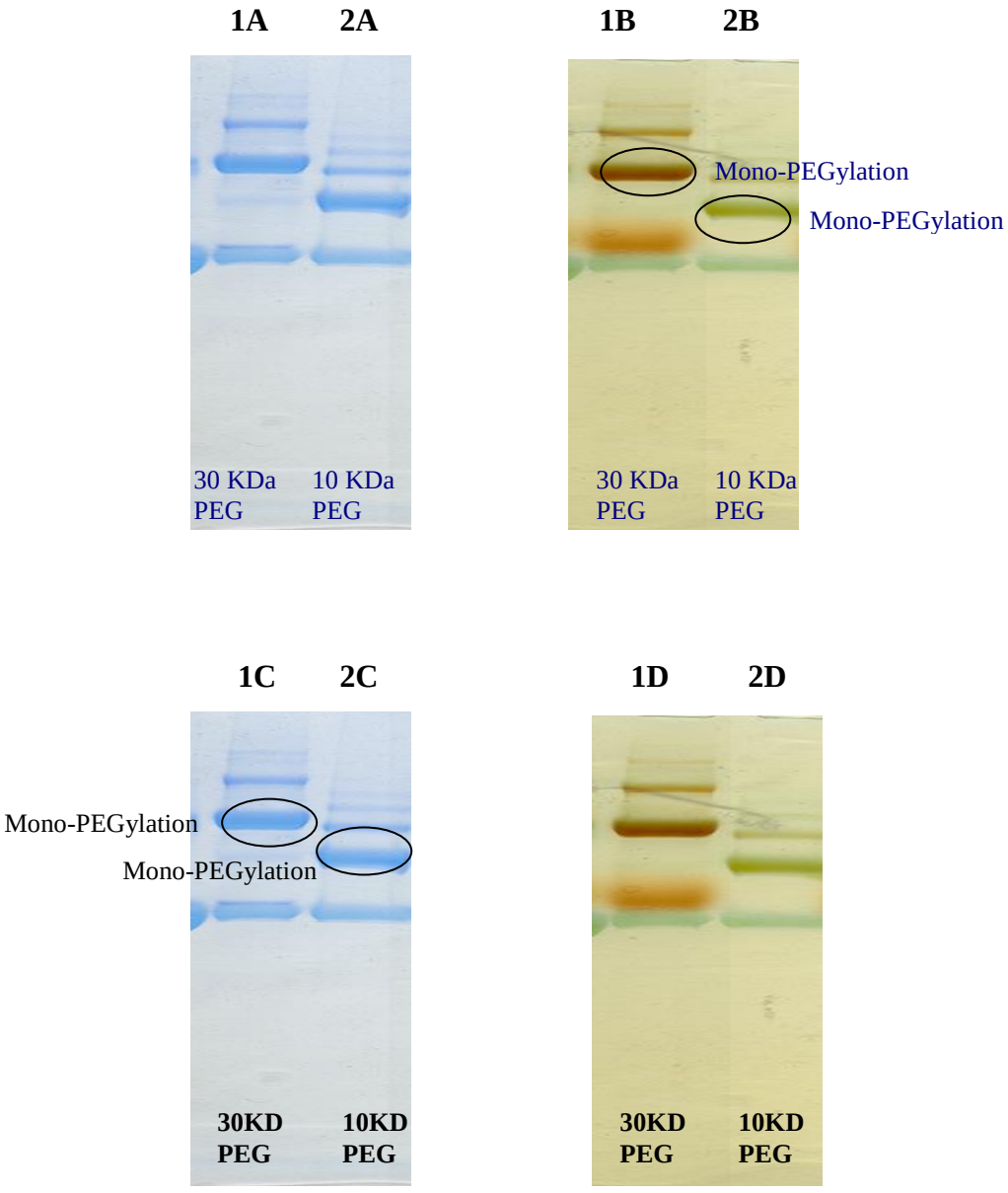

Figure 3. SDS-PAGE of holo-Tf, **A** in cromassi blue and **B** in barium iodide stain and apo-Tf **C** in cromassi blue and **D** in barium iodide stain. lane **1A:** holo -Tf reduced with 0.5 mM DTT+ 4eq. PEG 30 KDa; lane **2A:** holo-Tf reduced with 0.5 mM DTT + 4eq. PEG 10 KDa. Lane **1C:** apo-Tf reduced with 0.5 mM DTT+ 4eq. PEG 30 KDa; lane **2C:** apo-Tf reduced with 0.5 mM DTT + 4eq. PEG 10 KDa.

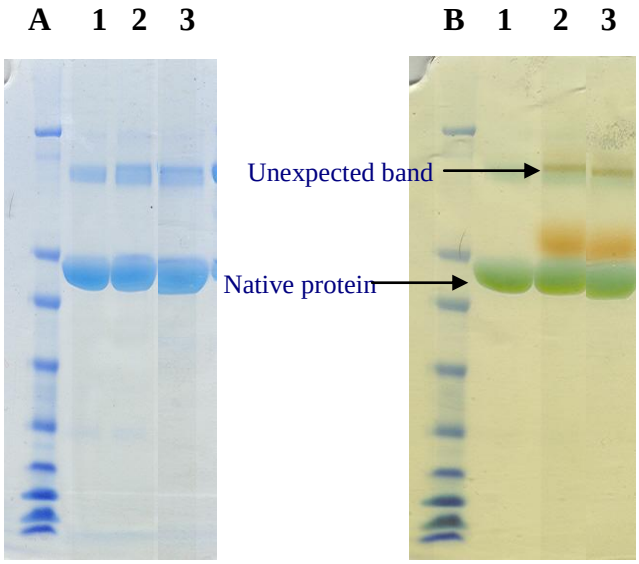

Figure 4. SDS-PAGE of holo and apo-Tf , **A** in cromassi blue and **B** in barium iodide stain. Lane **1:** native apo-Tf; lane **2:** native apo-Tf +30 KDa PEG; lane **3:** native holo-Tf+ 30 KDa PEG.

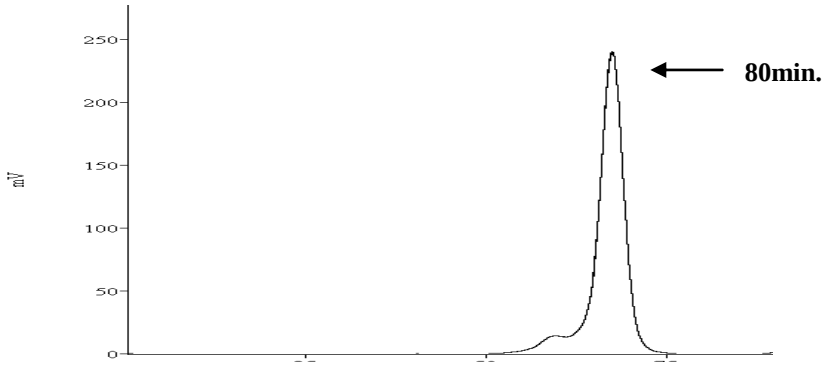

Figure 5. SEC-HPLC spectrum of native holo-Tf.

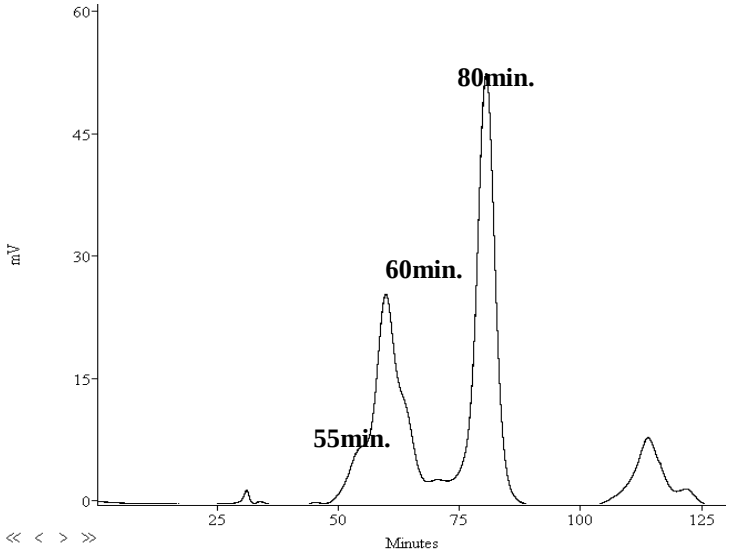

Figure 6. SEC-HPLC results of holo-Tf reduced with 0.5 mM DTT and PEGylated with 30 KDa PEG.

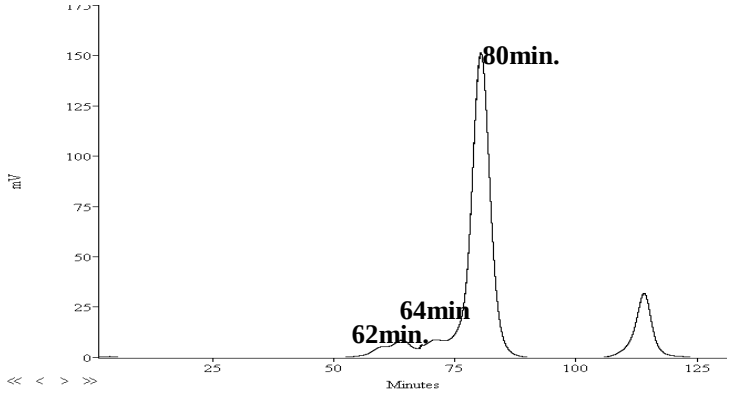

Figure 7 SEC-HPLC results of control reaction of holo-Tf with 30 KD PEG

CONCLUSION

The reduction of two disulfide bonds with predominant monoreduction out of the 19 disulfides of Tf with very mild conditions of the reaction without adding denaturants was observed. The two forms of Tf (holo-Tf which is iron loaded and apo-Tf which is iron free) were tested; the results demonstrate no difference in reduction or PEGylation of the two forms of Tf. These results suggest that iron has no

effect on reduction or PEGylation reaction of Tf. From the results observed in our experiments we concluded that it is possible to control the reduction conditions to achieve partial reduction in multiple disulfide proteins that have solvent accessible disulfide bonds and this give promising way to prepare reduced protein that may have better stability and solubility and improved pharmacokinetic properties to be used as medicines.

الخلاصة

البروتينات هي عبارة عن جزيئات مهمة جدا كعلاجات و لها العديد من المزايا ولكن يقل استخدامها بسبب قلة مدة حياتها في الدم ، والحياة الفيزيائية وعدم الاستقرار. وقد وضعت عدة طرق لتحسين الدوائية للبروتينات. واحد الطرق المثبتة سريريا هو الاقتران التساهمي مع بولي (جلايكول الإثيلين) (PEG) إلى البروتين.هذه العمليه المعروفه باسم بجيليشن PEGylation. المشاكل المرتبطة بهذه الطريقة هي تكون مركبات تغايرية وانخفاض كفاءة الارتباط . ويجري تطوير عدة استراتيجيات للتغلب على هذه المشاكل. واحد الطرق الجديده التي يجري وضعها هي انشاء جسر يربط جزيئتي السلفايد الموجوده بالبروتين مع PEG. في هذا النهج (للبجيليشن) يعتمد على وجود اواصر سلفا يمكن الوصول إليها . المبدأ الرئيسي هو اختزال اصرة ثاني كبريتيد السندات المحلية في جزيء البروتين تليها بجيليشن باستخدام كاشف PEG الذي يمكن أن يضيف جسر الكربون الثلاثي بين السلفر المتحرره من الاختزال ويعيد ربطها . في هذه الدراسة تم عمل اختزال مسيطر عليه مع بيجليشن للجسر الثنائي السلفايد للبروتين ترانسفيرين واللذي يحتوي على 19 ثنائي السلفايد قريبه من السطح باستخدام 0.5 ملم من دي دي تي واللذي يعتبر وسط مخفف للتفاعل ودون الحاجه الى استخدام عامل مساعد وقد تحقق الحصول على بروتين احادي البيجليشن مع كميه ضئيلة من ثنائي االحديد لبيجليشن. وتم اجراء التجربة على نوعين من الترانسفرين وهو الهولو ترانسفرين (واللذي فيه ارتباط مع الحديد) وابو ترانسفرين (الخالي من الحديد) لمعرفة ما اذا كان للحديد تاثير على عملية البيجليشن وقد تم استخدام تقنية الترحيل الكهربائي (SDS- PAGE) لتحليل النتائج والتي اشارت ان الحديد ليس له تاثير على سير التفاعل وانه لا داعي لاستخدام تراكيز عالية من مادة ال PEG لان حصلنا على بيجليشن ثنائي باستخدام كميتين مكافئتين من PEG فقط وتم اثبات صحة النتائج بواسطة استخدام تقنية طبقة الكروماتوغرافيه السائلة عالية الكفاءه من نوع (SEC- HPLC) واثبتت النتائج انه تم السيطرة على الاختزال الجزئي للسلفايد المتعددة الموجودة في البروتين ، الامر اللذي يؤدي الى تحضير بروتينات ذات اسقرارية عالية وذات مدة حرارية عالية وتحسن دوائية الترانسفرين ليتم استخدامه كعلاج دوائي.

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