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Short communication

Albumin removal from human fibrinogen preparations for manufacturing human fibrin-based biomaterials

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Abstract

Commercially available two component human fibrin sealants are commonly used to manufacture human fibrin-based biomaterials. However, this method is costly and allows little room for further tuning of the biomaterial. Human fibrinogen solutions offer a more cost-effective and versatile alternative to manufacture human fibrin-based biomaterials. Yet, human fibrinogen is highly unstable and contains certain impurities like human albumin. Within the context of biomaterials and tissue engineering we offer a simple yet novel solution based on classical biochemical techniques to significantly reduce albumin in human fibrinogen solutions. This method can be used for various tissue engineering and biomedical applications as an initial step in the manufacturing of human fibrin-based biomaterials to optimise their regenerative application.

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Keywords: Human fibrinogen; Human fibrin; Dialysis; Albumin; Biomaterials.

1. Introduction

Fibrin, an insoluble polymeric protein, is produced in response to bleeding during the last step of the blood coagulation cascade and is arranged in long fibrous chains [1]. The precursor of fibrin is fibrinogen, a soluble protein produced in the liver and found in blood plasma [2]. Tissue damage results in the cleavage of fibrinogen by thrombin and its assembly to form fibrin. Fibrin is known to play a pivotal role in wound healing, making it an ideal biomaterial choice for tissue engineering applications, aiming at restoring tissue structure and function [3,4].

Due to its poor mechanical properties and high biodegradability, fibrin, naturally a gel biomaterial, is often used in combination with other materials and/or chemically modified, i.e. cross-linking, to tune and control its physical properties. Thus, fibrin-based biomaterials are biocompatible, biodegradable, have high affinity towards biological surfaces, enhance cell attachment, tissue repair and support angiogenesis [1,4]. They also serve as suitable templates for controlled release of growth factors [5–7]. In addition, they have been used for stem cell delivery into the injury site, thus acting as cell carriers [8]. Due to its versatile nature, fibrin-based biomaterials have been tested as potential scaffolds to regenerate bone, cardiac tissue, cartilage, liver, nervous tissue, ocular tissue, skin, adipose tissue, tendons, and ligaments [9].

Fibrin requires the use of fibrinogen and thrombin as the starting materials for its preparation [10]. Commonly, commercially available two component human fibrin sealants are used to manufacture human fibrin-based biomaterials. However, this method is costly and allows little room for further modifications. Human fibrinogen solutions offer a more cost-effective and versatile alternative to manufacture human fibrin-based

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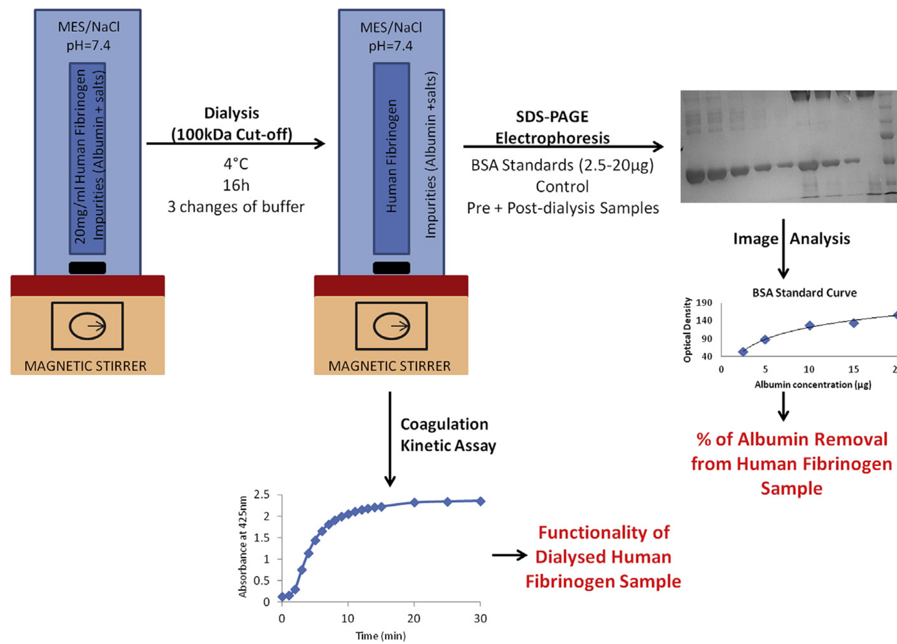


Fig. 1. Graphical summary of the method proposed in this paper for removal of albumin from human fibrinogen preparations for manufacturing human fibrin-based biomaterials.

biomaterials. Yet, human fibrinogen is highly unstable and contains certain impurities like human albumin and salts (i.e. L-arginine hydrochloride, sodium chloride, sodium citrate) to prevent its degradation [11,12]. Albumin is the most abundant protein in plasma and is structurally very stable due to the presence of 17 disulphide bonds. This allows albumin to remain stable under different conditions. However, its hydrophobic domains increase the hydrophobicity of the fibrin biomaterial (cells find it difficult to attach to hydrophobic surfaces) thereby reducing the applications for the biomaterial [13,14]. Most importantly albumin is also known to have an anti-coagulation effect on fibrinogen [14,15]. Due to the above mentioned reasons, albumin can have an inhibitory effect on fibrin activity during tissue regeneration and therefore its levels should be reduced from human fibrinogen solutions. Dialysis is commonly used to remove the salts present in protein solutions but albumin usually remains a concern due to its larger molecular weight. Hence, there is a need for a cost-effective and efficient method for removal of albumin from human fibrinogen solutions.

The aim of this work was to remove or significantly reduce the concentration of albumin present in human fibrinogen solutions using simple, classical biochemical techniques as an initial step in the manufacturing of human fibrin-based biomaterials. For this purpose, human fibrinogen was dialysed in dialysis tubing with a large molecular weight cut-off (100 kDa), which allowed albumin and other salts to pass through, leaving the fibrinogen in the tube. Protein samples (pre and post dialysis) were analysed using SDS-PAGE electrophoresis by differentiating between the molecular weight of the two proteins: human albumin has a molecular weight of approximately 67 kDa while human fibrinogen has an approximate molecular weight of 340 kDa. Finally, a simple and rapid coagulation kinetic assay was performed on the dialysed human fibrinogen to assess its functionality. Fig. 1 summarises the different steps of the method described in this paper. The ultimate goal of this work is to establish a simple, cost-effective and robust method for removing albumin from human fibrinogen solutions that can be universally used as a previous step in the manufacturing of human fibrin-based biomaterials intended for tissue repair.

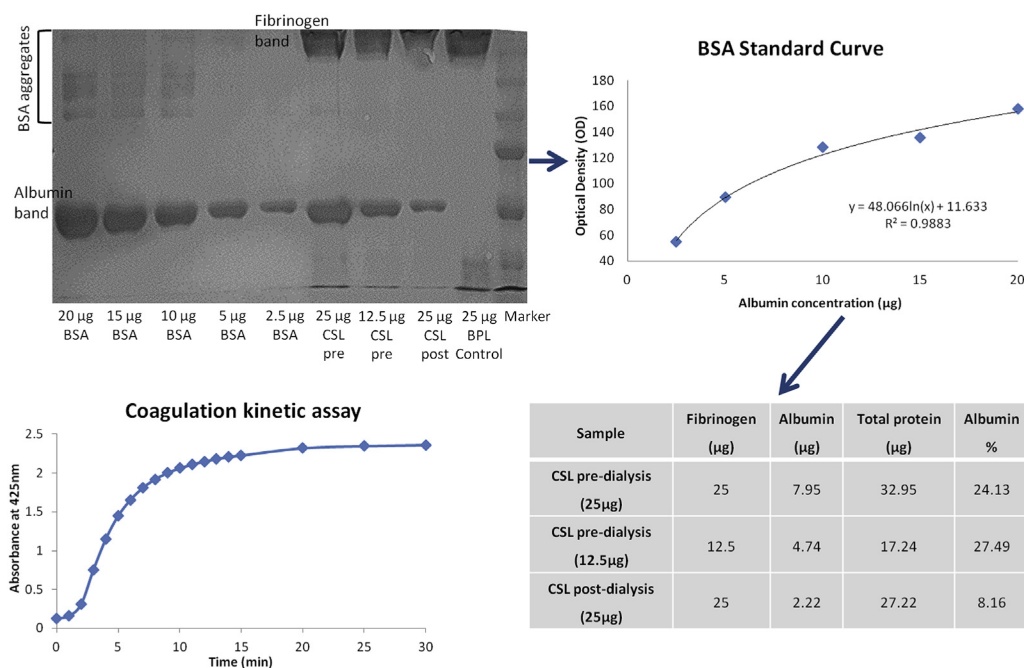
2. Materials and methods

2.1. Human fibrinogen solutions

Human fibrinogen from Bio Products Laboratory Ltd. (BPL, UK) was used as a control as it has a purity of approximately 100% and fibrinogen from CSL Behring Ltd. (RiaSTAP®, CSL Behring Ltd., UK), with an approximate purity of 65–75 %, was used as the test product. The powders (1 g of fibrinogen + X g of albumin + Y g of salts) were reconstituted as per manufacturers' instructions, in sterile deionised water (50 ml). Samples from the diluted CSL protein solution (20 mg/ml of fibrinogen + unknown concentration of albumin) were collected and used as pre-dialysis samples.

2.2. Dialysis

The fibrinogen solutions were transferred into separate 100 kDa cut-off visking tubing (Spectra/Por® dialysis membrane, flat nominal width = 16 mm, diameter = 10 mm, Spectrum Laboratories Inc., US), sealed using dialysis clips and dialysed against 2-ethanesulfonic acid (MES)/NaCl buffer pH 7.4 (150 mM NaCl + 25 mM MES), with three changes over



Sample	Fibrinogen (µg)	Albumin (µg)	Total protein (µg)	Albumin %
CSL pre-dialysis (25µg)	25	7.95	32.95	24.13
CSL pre-dialysis (12.5µg)	12.5	4.74	17.24	27.49
CSL post-dialysis (25µg)	25	2.22	27.22	8.16

Fig. 2. Top left, SDS-PAGE gel. Top right, BSA standard curve obtained from the SDS-PAGE gel by image analysis. Bottom right, table showing amount (µg) and percentage of albumin in CSL product before and after the dialysis. Bottom left, coagulation kinetic assay showing functionality of dialysed human fibrinogen.

approximately 16 h, at 4 °C with gentle stirring in a 2 L cylinder. Post-dialysis solution was recovered using a pastette, the volume measured, and the solution diluted to give a theoretical 20 mg/ml fibrinogen concentration assuming that no fibrinogen was lost from the dialysis tube. This assumption was based on both the cut-off size of the tube (100 kDa) and the size of fibrinogen (340 kDa): human fibrinogen is 3.4 times larger than the pores of the dialysis membrane.

2.3. SDS-PAGE

To quantify the amount of albumin in CSL before and after dialysis, a non-reducing gel (8%) was run with a standard curve of bovine serum albumin (BSA) (Sigma, UK) (2.5–20 µg), 25 µg of fibrinogen pre-dialysis (CSL), 12.5 µg of fibrinogen pre-dialysis (CSL), 25 µg of fibrinogen post-dialysis (CSL) and 25 µg of control fibrinogen (BPL). 10 µl of each standard/sample was mixed with 3.3 µl of 3x non boil urea loading dye. The gel was run at 100 V until the molecular weight markers were separated and the bromophenol blue dye band reached the bottom of the gel. The gel was removed from the electrophoresis rig and transferred to Instant-blue stain (Novexin Ltd, UK). Bands were visualised after 15 min but left to increase in intensity for 1 h. The gel was photographed in an Epichemi™ II Darkroom (UVP Inc, US) and Optical density (OD) of bands was analysed using LabWorks™ image acquisition and analysis software (UVP Inc, US). Results were plotted into an OD versus BSA concentration graph to obtain a standard curve for BSA. The amount of albumin in CSL product before and after dialysis was calculated from the standard BSA curve.

2.4. Coagulation kinetic assay

In order to test the functionality of the dialysed fibrinogen a simple and rapid coagulation kinetic assay was carried out after the dialysis. Freshly prepared dialysed human fibrinogen solution was diluted to 2% and 500 µl added into a 1 ml cuvette (FB55147, Fisher Scientific, UK), with 450 µl of MES/NaCl pH 7.4 buffer and 2 µl of 1M $CaCl_2$. The coagulation assay was started immediately upon thrombin addition (human thrombin from TISSEEL®, Baxter International Inc., US, 10 I.U./ml in HEPES/NaCl pH 7.4), with the solution being thoroughly mixed by inversion using a fresh Parafilm M® cap before placing the cuvette in an M550 double beam UV/visible spectrophotometer (Camspec, UK). The Absorbance at 425 nm was recorded at 1 min intervals from time zero, until a plateau value was reached, which indicates complete coagulation.

3. Results

Fig. 2 shows results from the SDS-PAGE gel, run with pre and post-dialysis CSL samples along with BSA standards and BPL fibrinogen sample. The main band seen in the middle of the gel is albumin, which is approximately 67 kDa. On visually analysing the gel (Fig. 2), it was observed that the CSL product after dialysis had a reduced albumin band in comparison to the pre-dialysed albumin band. The amount of albumin before and after the dialysis step in CSL samples was calculated from the BSA standard curve.

Results showed that, when 25 µg of human fibrinogen pre-dialysis were loaded onto the SDS-PAGE gel, it was calculated that the CSL product contained 7.95 µg of albumin. So, the

total amount of protein pre-dialysis loaded onto the gel was $25 + 7.95 = 32.95$ μg. Therefore, in this case, the percentage of albumin pre-dialysis was $(7.95/32.95)*100 = 24.13\%$. When 12.5 μg of human fibrinogen pre-dialysis were loaded, it contained 4.74 μg of albumin, with a total amount of protein pre-dialysis loaded onto the gel of $12.5 + 4.74 = 17.24$ μg and a percentage of albumin pre-dialysis of $(4.74/17.24)*100 = 27.49\%$. Therefore, the average percentage of albumin in the CSL product pre-dialysis was $(24.13 + 27.49)/2 = 25.81\%$.

Post-dialysis, 25 μg of human fibrinogen were loaded onto the SDS-PAGE gel (Fig. 2). It was calculated that it contained 2.22 μg of albumin, making a total amount of protein post-dialysis loaded onto the gel of $25 + 2.22 = 27.22$ μg, with a percentage of albumin post-dialysis in the CSL product of $(2.22/27.22)*100 = 8.16\%$.

The total reduction of albumin compared to 25 μg pre-dialysis was calculated as 7.95 μg - 2.22 μg = 5.73 μg. Therefore, the percentage of reduction of albumin was $(5.73/7.95)*100 = 72.08\%$. Compared to 12.5 μg pre-dialysis: $(4.74*2)$ μg − 2.22 μg = 7.26 μg. Thus, the percentage of reduction of albumin in this case was $7.26/(4.74*2)*100 = 76.58\%$. Therefore, the average percentage of reduction of albumin was $(72.08 + 76.58)/2 = 74.33\%$.

The coagulation kinetic assay of the dialysed protein assesses its functionality. As it can be seen from Fig. 2, the mix starts coagulating moments after addition of thrombin. The coagulation of the mix can be followed by measuring the Absorbance at 425 nm over time until after 20 min a plateau is reached indicating complete coagulation of the mix. This is a classical coagulation curve [16] showing that the human fibrinogen after our proposed dialysis method is functional.

4. Discussion

Fibrin, which is naturally a gel material, is an excellent biomaterial choice for tissue engineering as it is biocompatible, enhances cell attachment and migration, promotes angiogenesis and plays a pivotal role in important physiological processes such as wound healing [3,4,17]. However, depending on the specific application its mechanical and biodegradable properties may not be satisfactory and therefore it is often used in combination with other materials and/or chemically modified to enhance its physical properties [8,17–19]. The ideal fibrin biomaterial for tissue engineering applications would exhibit adequate mechanical support to resist in vivo forces [17,18]. The appropriate mechanical properties are specific to each tissue application. For example, an elastic modulus of 6.67 MPa has been reported for back skin [20] while an elastic modulus of 18.6 GPa was measured by micro-tensile testing for cortical bone [21]. Reported elastic modulus of fibrin gels are 0.93–6.49 kPa, depending on fibrin concentration [22].

Commercially available two component fibrin sealants are commonly used as the starting product to manufacture human fibrin-based biomaterials [1]. However, this option is expensive and allows little further modification of the biomaterial. Human fibrinogen solutions are a more cost-effective option as starting material as well as versatile in terms of further tuning of the biomaterial. Before they can be used, the impurities (salts and albumin) that accompany these products in order to stabilize fibrinogen [11,12] must be removed to optimise the tissue regeneration process. Salts are easily removed by dialysis but albumin, with its larger molecular weight, remains a concern. The aim of this work was to remove or significantly reduce the concentration of albumin present in human fibrinogen solutions using simple, classical biochemical techniques to establish a simple, cost-effective and robust method that can be universally used as a previous step in the manufacturing of human fibrin-based biomaterials intended for tissue repair.

Results showed that the introduction of a 100 kDa cut-off dialysis step considerably and effectively removed albumin from human fibrinogen solution with further room for optimisation depending on the manufacturing requirements. The advantages of the proposed method are: 1) dialysis, SDS-PAGE electrophoresis and UV/visible spectrophotometry are simple biochemical techniques that use common, easy-to-use equipment normally found in the laboratory; 2) efficacy and 3) cost-effectiveness. The present method is routinely used in our laboratory to separate albumin from human fibrinogen solutions prior to manufacturing fibrin-based biomaterials [23]. We consistently obtain a reduction of approximately 75% in the total albumin concentration of human fibrinogen preparations, thus showing its robustness. Further, optimising the 100 kDa cut-off dialysis step by either adding more changes or dialysing for a longer period of time could aid in depletion of higher percentage of albumin, depending on its need and application. We believe this method could be universally used as an initial step in manufacturing human fibrin-based biomaterials intended for tissue repair after acute trauma.

5. Conclusions

Within the context of biomaterials and tissue engineering we offer a simple yet novel solution based on classical biochemical techniques to significantly reduce albumin in human fibrinogen solutions. This method can be used for various tissue engineering and biomedical applications as an initial step in the manufacturing of human fibrin-based biomaterials to optimise their regenerative application.

Conflict of interest

There are no conflicts of interest to declare.

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