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Purification of hemoglobin from red blood cells using tangential flow filtration and immobilized metal ion affinity chromatography

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ABSTRACT

Two methods for purifying hemoglobin (Hb) from red blood cells (RBCs) are compared. In the first method, red blood cell lysate is clarified with a 50 nm tangential flow filter and hemoglobin is purified using immobilized metal ion affinity chromatography (IMAC). In the second method, RBC lysate is processed with 50 nm, 500 kDa, and 50–100 kDa tangential flow filters, then hemoglobin is purified with IMAC. Our results show that the hemoglobins from both processes produce identical Hb products that are ultrapure and retain their biophysical properties (except for chicken hemoglobin, which shows erratic oxygen binding behavior after purification). Therefore, the most efficient method for Hb purification appears to be clarification with a 50 nm tangential flow filter, followed by purification with IMAC, and sample concentration/polishing on a 10–50 kDa tangential flow filter.

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1. Introduction

Approximately 1 in every 4 people will need a blood transfusion at some point during their lifetime. However, due to seasonal shortages and low supplies of rare blood types, donated blood is not always available. Blood shortages are even more severe in developing countries due to decreased donation, poor screening techniques, and a lack of proper blood storage facilities. Therefore, there is a significant need to develop an alternative to donated blood that is safe, stable, easy to store, and easy to manufacture.

Substantial progress has been made in developing hemoglobin-based oxygen carriers (HBOCs) that may be used as alternatives to donated blood. However, large scale production of these HBOCs requires a sufficient supply of highly pure hemoglobin (Hb). Hb may be easily obtained by lysing red blood cells (RBCs). However, RBCs also contain hundreds of soluble protein impurities [1,2] that must be removed from the stock Hb solution before HBOC synthesis. Several methods exist for purifying Hb from RBCs, including heat treatment [3], aqueous phase extraction [4], and anion exchange chromatography [5–7].

Tangential flow filtration (TFF) has also been used to effectively purify Hb from RBCs [8,9]. In the TFF process, Hb is filtered through 50 nm and 500 kDa TFF membranes (which remove viruses and cellular debris) and concentrated with a 50–100 kDa TFF membrane which also removes some small impurities. Despite the attractive features of TFF (low cost, simple and quick operation), recent results have shown that trace impurities (such as catalase) are present in the final TFF Hb product [10]. Therefore, we decided to add an affinity purification step to the TFF process to remove these unwanted impurities.

It has been previously shown that Hbs from many different species (human, bovine, chicken, etc.) have a very high affinity for zinc ($K_a = 1.3 \times 10^7\ M^{-1}$) [11,12]. The zinc ion tightly binds to β143His and β139Asp in the 2,3-diphosphoglycerate (2,3-DPG) binding pocket of human Hb (HbA), while potentially interacting with β93Cys as well [11,13]. There are also several histidines on the surface of HbA (6 on the α subunit and 7 on the β subunit) [14] which may bind zinc. At low concentrations, Hb binds zinc and remains soluble, but with a significant increase in oxygen affinity [11]. At high zinc concentrations (>10:1 molar ratio of Zn:Hb), Lehman et al. have shown that Hb may be selectively precipitated from RBC lysate and resuspended upon addition of EDTA, thereby partially purifying Hb [15–17].

Plomer et al. expanded on the zinc precipitation method by using immobilized metal ion (Zn^{2+}) affinity chromatography (IMAC) [18] to purify recombinant HbA (rHbA) from bacterial lysates [19]. In this technique, a chelating resin is used to sequester the Zn^{2+} ion which then binds Hb. The resin may then be washed to remove weakly bound impurities and purify the Hb. The final Hb product may be removed from the column with EDTA or imidazole, species which have a higher affinity for zinc ions. This technique is commonly used to purify rHbA from bacterial lysates [20–22], however, purification of Hbs from RBC lysate has not yet been fully examined.

Abbreviations: Hb, hemoglobin (any species); HbA, human adult hemoglobin; bHb, bovine hemoglobin; cHb, chicken hemoglobin; TFF, tangential flow filtration.

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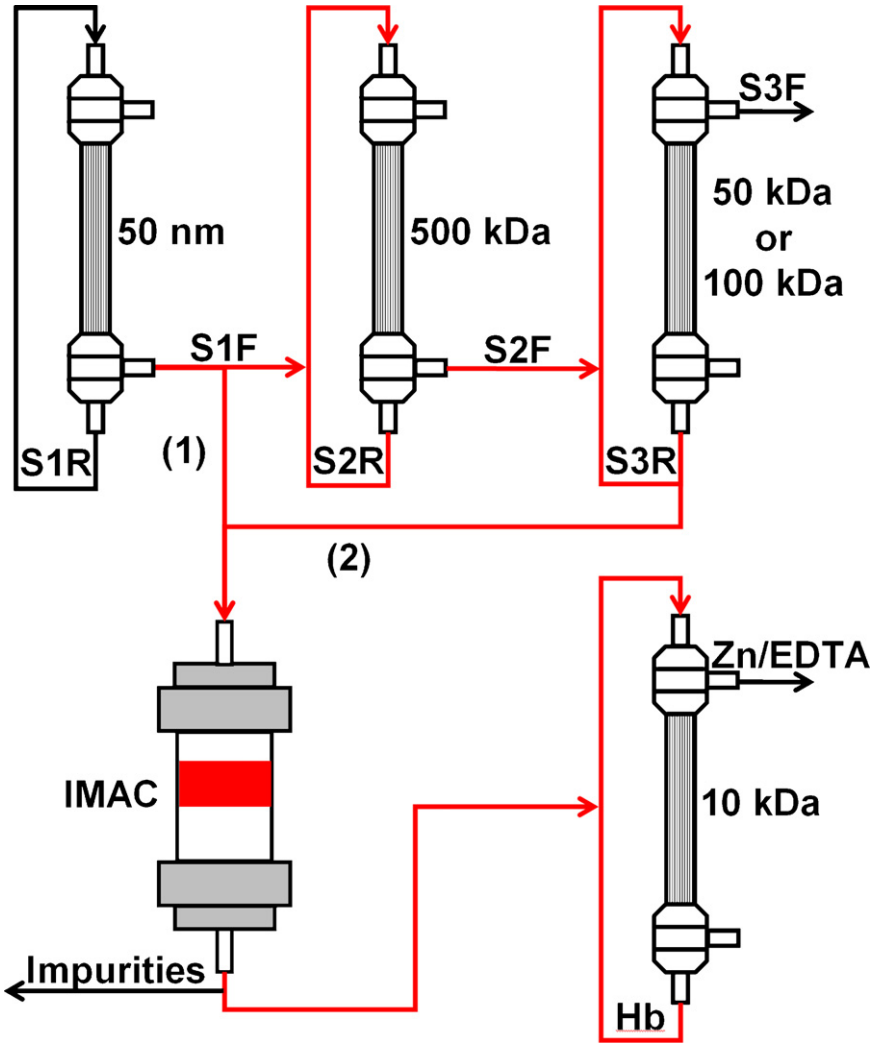

Scheme 1. Schematic of the purification processes. In (1), the RBC lysate is filtered through the 50 nm TFF filter and immediately loaded onto the IMAC column. In (2), the lysate is filtered through both 50 nm and 500 kDa filters, concentrated on a 50–100 kDa filter and then loaded onto the IMAC column. The purified Hb from both processes is finally diafiltered on a 10 kDa filter to remove excess zinc and EDTA.

In this study, we seek to determine the effectiveness of using IMAC in line with TFF to produce ultrapure human (HbA), bovine (bHb), and chicken Hb (cHb) from their respective RBC lysates. We have examined two types of processes (Scheme 1) in which (1) a single 50 nm TFF filter is used to prepare the lysate for IMAC and (2) a three-stage TFF process (filtration on 50 nm, 500 kDa, and 50/100 kDa) is used to filter and concentrate the Hb samples prior to IMAC. The relative purities and equilibrium oxygen binding characteristics of the Hb products obtained with these processes are also compared to determine the necessity of the 500 kDa and 50/100 kDa TFF stages.

2. Materials and methods

2.1. Red blood cell lysis

Blood from bovine (Quad 5, Ryegate, MT), human (American Red Cross, Columbus, OH), and chicken (King & Sons, Bradford, OH) sources were used as the starting material for this study. The blood was initially centrifuged at 3716 × *g* for 30 min (4 °C) to sediment the RBCs. The plasma supernatant was discarded and the RBC pellet was then washed three times by resuspending the RBCs in isotonic 0.9% saline solution and centrifuging at 3716 × *g* for 30 min at 4 °C. After the final saline wash and centrifugation step, the RBC pellet was resuspended in 3.75 mM phosphate buffer (PB, pH 7.2) and allowed to lyse at 4 °C overnight.

2.2. TFF of Hb

The RBC lysate was first clarified by passing it through a column packed with glass wool in order to remove large cellular debris. The clarified lysate was then passed through a three-stage TFF process (Spectrum Labs, Rancho Dominguez, CA) (Scheme 1). The first two TFF stages (50 nm and 500 kDa MWCO) removed viruses, large particles, and cell debris while Hb passed through in the filtrate. In the final TFF stage (25 kDa MWCO for cHb, 50 kDa MWCO for HbA and bHb), Hb was retained and concentrated, while impurities with smaller molecular weights (some proteins, 2,3-DPG, etc.) were removed in the filtrate. Samples for IMAC purification were taken from the filtrate at the end of Stage 1 (S1F) and from the retentate at the end of Stage 3 (S3R). Aliquots of each sample were then separated and frozen at −80 °C until ready for IMAC purification.

2.3. IMAC of Hb

A XK 50/30 column was packed with 200 mL of Chelating Sepharose Fast Flow resin (GE Healthcare, Piscataway, NJ). The resin was initially washed with ultrapure H_2O until the conduc-