Published in final edited form as:

Biotechnol Prog. 2009 ; 25(5): 1402–1410. doi:10.1002/btpr.217.

Purification of Hemoglobin by Tangential Flow Filtration with Diafiltration

Jacob Elmer, David R. Harris, Guoyong Sun, and Andre F. Palmer

Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH 43210

Abstract

A recent study by Palmer et al. (2009) demonstrated that tangential flow filtration (TFF) can be used to produce HPLC-grade bovine and human hemoglobin (Hb). In this current study, we assessed the quality of bovine Hb (bHb) purified by introducing a 10 L batch-mode diafiltration step to the previously mentioned TFF Hb purification process. bHb was purified from bovine red blood cells (RBCs) by filtering clarified RBC lysate through 50 nm (stage I) & 500 kDa (stage II) hollow fiber (HF) membranes. The filtrate was then passed through a 100 kDa (stage III) HF membrane with or without an additional 10 L diafiltration step to potentially remove additional small molecular weight impurities. Protein assays, SDS-PAGE, and LC-MS of the purified bHb (stage III retentate) reveal that addition of a diafiltration step has no effect on bHb purity or yield; however, it does increase the methemoglobin level and oxygen affinity of purified bHb. Therefore, we conclude that no additional benefit is gained from diafiltration at stage III and a three-stage TFF process is sufficient to produce HPLC-grade bHb.

Keywords

hemoglobin; purification; tangential flow filtration; oxygen carrier; blood substitute; diafiltration

Introduction

As the demand for blood increases more rapidly (6–8% per year) than the supply of donated blood (2–3% per year)¹, there is an increasing need for the development of a viable red blood cell (RBC) substitute that can be mass produced. RBC substitutes currently in development include liposome-encapsulated Hb^{2–5} and cross-linked/polymerized Hb.^{6–9} Several of these hemoglobin-based oxygen carriers (HBOCs), such as Hemopure (Biopure, Cambridge, MA)^{10,11} and PolyHeme (Northfield Labs, Evanston, IL)¹² have shown some promise in clinical applications. However, in order for these products to meet the growing demand for RBC substitutes, a plentiful source of pure hemoglobin (Hb) is needed for the large-scale production of HBOCs.

While the majority of RBC protein is Hb (32–36 g/dL), other major proteins (such as carbonic anhydrase, actin, and catalase) are also present in RBCs.¹³ Several methods exist in the literature to purify Hb from RBC lysate. These include aqueous-phase extraction¹⁴, heating in the presence of reducing agents (which causes impurities to precipitate)¹⁵, and various types of chromatography^{16–18}. As an alternative to these methods, our laboratory has developed a simple method to purify Hb using tangential flow filtration (TFF)¹⁹.

The TFF process involves three filtration steps. Clarified RBC lysate is first filtered through a 50 nm (stage I) hollow fiber (HF) membrane, followed by HF membranes with molecular weight cutoffs (MWCO) of 500 kDa (stage II) and 100 kDa (stage III). RBC debris and any viruses present are removed by the 50 nm and 500 kDa HF membranes²⁰, while Hb passes through into the filtrate. Hb is retained in the stage III retentate, while other small molecular weight impurities (SMWI), such as endotoxin and Hb dimers, are removed in the filtrate. Hb obtained by TFF has a purity and activity similar to HPLC-grade Hb, along with methemoglobin (metHb) levels < 1%.¹⁹

The purpose of this study is to determine any possible benefits that an additional diafiltration step might have on the TFF process. Diafiltration should increase transmission of impurities through ultrafiltration membranes by continuously or intermittently adding buffer to maintain the retentate volume, thereby lengthening the total filtration time. It has been previously used to remove impurities from blood as an alternative to dialysis²¹ and can be used in other industrial applications²². In this study, diafiltration was applied during stage III to enhance transmission of any residual SMWIs through the 100 kDa HF membrane and further increase the purity of the bHb product.

Materials and Methods

Lysis of RBCs

Each round of Hb purification started with 1 L of bovine RBCs (bRBCs) diluted 90:10 (bovine blood: 4 % citrate solution). RBCs were initially harvested by centrifugation (3716g, 30 min., 4°C). Plasma proteins and acellular Hb were washed away by resuspending the cells in a 0.9 % w/v saline solution, centrifuging (3716g, 30 min, 4°C) the mixture, and subsequently discarding the supernatant. This wash step was repeated twice. After the final wash step, bRBCs were resuspended in phosphate buffer (PB; 3.75 mM, pH 7.2) to a final total volume of 2 L and lysed on ice for one hour.

TFF of Hb

A 3-stage filtration process was used to purify bHb from bRBC lysate (Figure 1). The bRBC lysate was first passed through a glass wool column to remove large cellular debris. The solution was then sequentially passed through 50 nm, 500 kDa MWCO, and 100 kDa MWCO HF membranes. Table 1 shows the technical specifications of each HF membrane. In this study, 50 kDa HF cartridges were not used, since the majority of bHb is retained on the 100 kDa HF membrane. The retentate was continuously recycled during each stage, while the filtrate was collected and directly applied to the next stage. When diafiltration was applied at stage III, the recycled retentate was kept at a constant volume of approximately 1 L until a total of 10 L of PB had been added to the retentate bottle. 10 mL samples of filtrate and retentate were collected at each stage for further analysis. HF filtration cartridges were sterilized immediately after each use with 0.5 M NaOH for 1 hour and stored in a 0.01 % SDS/0.02 % sodium azide solution at 4°C. Before use, each HF cartridge was rinsed thoroughly with deionized H₂O and tested to ensure that the physical integrity of the HF cartridge was maintained. The entire filtration process was performed on ice to minimize methemoglobin (metHb) formation. Each purification process was repeated three times to acquire suitable statistics.

Hb, MetHb, and Total Protein Concentration

UV-visible spectroscopy was used to determine the Hb and metHb concentrations at each stage of the purification process using the cyanomethemoglobin method^{23–28}. The absorbance of the sample was first measured at 630 nm (L_1). The samples were diluted (by a factor of D_1) to yield absorbance readings of L_1 between 0.1–1.0. Three drops of 1:1 KCN

solution (10% KCN: 10 mM PB) were added to 3 mL samples and the absorbance was again measured at 630 nm (L_2). The metHb concentration was calculated using Eq. 1:

$$[\text{metHb}] = \frac{L_1 - L_2}{E_1} \lambda D_1 \quad (1)$$

Where $E_1 = 3.7 \text{ (cm mM)}^{-1}$ is the extinction coefficient of metHb at 630 nm²⁴ and λ is the path length, $\lambda = 1 \text{ cm}$. To measure Hb concentration, 3 drops of $\text{K}_3\text{Fe}(\text{CN})_6$ were added to diluted (D_2) 3 mL samples. After 2 minutes of incubation at room temperature, 3 drops of 10% KCN were added to the sample. The final absorbance at 540 nm was then measured to obtain the value of L_3 . The total Hb concentration was calculated using Eq. 2:

$$[\text{Hb}] = \frac{L_3}{E_2} \lambda D_2 \quad (2)$$

Where $E_2 = 11.0 \text{ (cm mM)}^{-1}$ is the extinction coefficient of Hb at 540 nm²⁴ and λ is the path length, $\lambda = 1 \text{ cm}$. Finally, the metHb level was calculated using Eq. 3:

$$\% \text{met} = \frac{[\text{metHb}]}{[\text{Hb}]} \times 100 \quad (3)$$

Total protein concentration was measured by the Bradford method using the Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL). The mass of total protein at each stage was then calculated by multiplying the protein concentration by the total volume collected.

Endotoxin Assay

The endotoxin level of purified bHb was measured with an endotoxin test kit (Pyrogen Plus, Lonza, Walkersville, MD).

SDS-PAGE

Undiluted samples from each stage of the purification process were mixed 1:1 with 5% β -mercaptoethanol Laemmli sample buffer and incubated at 95°C for 5 minutes. Each lane of the gel (12% acrylamide resolving, 4% stacking) was loaded with 30 μg of total protein. Gels were then run for 1 hour at 130 V, stained with Coomassie blue R250 (stain buffer, Bio-Rad) for one hour, and finally destained with destaining buffer (70% H_2O , 20% ethanol, 10% glacial acetic acid). All gels were scanned on a Gel Doc XR system (Bio-Rad) and analyzed with Quantity One 1-D software (Bio-Rad laboratories, Hercules, CA).

Hb-Oxygen Equilibria

A Hemox Analyzer (TCS Scientific Corporation, New Hope, PA) was used to measure the Hb- O_2 equilibrium curves of purified bHb and bRBCs. Samples were prepared by mixing 50–100 μL of purified bHb/bRBCs with 5 mL of Hemox Buffer (pH 7.4), 20 μL of Additive A, 10 μL of Additive B (not added to bRBC samples), and 10 μL of anti-foaming reagent (TCS Scientific Corporation). The samples were then allowed to equilibrate with air at a $p\text{O}_2$ of $145 \pm 2 \text{ mm Hg}$. After the sample was equilibrated, the gas stream was switched to pure nitrogen to deoxygenate bRBCs/bHb. The absorbance of oxy- and deoxy-Hb was then recorded as a function of $p\text{O}_2$. The resulting Hb- O_2 equilibrium curve was then fit to the Hill equation (Eq. 4) to regress A_o , A_∞ , P_{50} and n .

$$Y = \frac{Abs - A_o}{A_{\infty} - A_o} = \frac{pO_2^n}{pO_2^n + P_{50}^n} \quad (4)$$

Where Y is the fractional saturation of Hb, A_o is the absorbance at 0 mm Hg O_2 and A_{∞} is the absorbance at full saturation.

LC-MS

Mass spectroscopy was used to determine the molecular weight of the α and β globin chains of purified bHb. Purified bHb was sent to the Mass Spectrometry and Proteomics Facility of the Campus Chemical Instrument Center at The Ohio State University (Columbus, OH) for analysis. The chemical identity of purified bHb was assessed by measuring the molecular weight of the α and β globin chains using LC-MS. Separation of the α and β chains was achieved using a Dionex U300 HPLC system (Dionex Corporation, Sunnyvale, CA) connected in series to a Micromass A-TOF II Mass Spectrometry system (Waters Corporation, Milford, MA). The mobile phase was composed of 0.1 % trifluoroacetic acid (TFA) in deionized water as Buffer A and 0.1 % TFA in acetonitrile (ACN) as Buffer B. The flow rate was maintained at 25 μ L/min throughout all experiments. The separation was performed on a Discovery® BIO Wide Pore C18 HPLC column (1.0 mm $\times$ 10 mm, 3 μ m, Sigma Aldrich Inc., Atlanta, GA). The gradient was initiated with 30% Buffer B and was increased to 70% Buffer B over 60 min, then reduced to 30% Buffer B at 60.1 min and kept constant at 30% Buffer B until 90 min. Twenty μ L of sample ($\sim$ 10 μ g) was injected for each run.

Results and Discussion

Filter Performance

The permeate flow rate (PFR) and transmembrane pressure difference (TMP) at the beginning and end of each filtration stage are shown in Table 2. During each stage, the permeate flow rate decreased, but for different reasons. Large cellular debris are retained by the 50 nm HF membrane during stage I, which clogs the HF membrane's pores and simultaneously causes a large (6–8 psi) increase in TMP and increased resistance to permeate flow. During stage II (500 kDa HF membrane), the TMP actually decreases or stays roughly the same, since most of the large molecular weight components were retained in stage I. As the concentration of bHb in the retentate decreases in stage II, any resistance to flow caused by high concentrations of bHb also decreases. Another increase in TMP is observed during stage III, where bHb is retained by the 100 kDa HF membrane. Even though the tangential flow prevents most of the bHb from accumulating on the membrane surface and clogging the membrane pores, convective flow towards the membrane surface transports some of the retained bHb to the membrane surface where it forms a high-concentration protein “gel” layer. This phenomenon is known as concentration polarization, which leads to a higher osmotic pressure in the “gel” layer which increases resistance to permeate flow. Once the gel layer is established, the dominant driving force for transport becomes concentration gradients rather than TMP, and a decrease in PFR is observed despite an increased TMP. Nonetheless, no significant clogging was observed during any stage of the bHb purification process.

Total Protein and bHb Yield

The mass of total protein and bHb obtained after each stage of the filtration process is shown in Figure 2 and Figure 3, respectively. The overall yields of total protein and bHb with and without diafiltration are practically equivalent. Table 3 shows the percent recovery of bHb at

each stage of the filtration process. In both cases, roughly one third of the total bHb is lost during TFF. Most of the lost bHb is either retained during the first two stages (data not shown) or lost in the $\alpha\beta$ dimer form (which can pass through the 100 kDa membrane) during stage III. Significant losses during diafiltration in stage III (as high as 25%, data not shown) have been observed. This may be caused by the tendency of bHb to dissociate into $\alpha\beta$ dimers when diluted with the diafiltration buffer solution.

MetHb and Endotoxin Level

The extent of bHb oxidation or metHb level at each stage of the bHb purification process is shown in Figure 4. Overall, the metHb levels were below accepted standards of 1–2%, especially in the final product. There is an unusual spike in metHb levels in the stage I retentate, however, this may be a false reading, since the cellular debris in the stage I retentate scatter light and interfere with the absorbance measurements during metHb quantification. This phenomenon was also observed in our previous TFF study¹⁹. A slight increase in metHb level was also observed in the diafiltration product, due to the increased duration of the diafiltration step. In either case, however, the metHb level is low enough to make the purified bHb product suitable for HBOC synthesis.

The endotoxin levels of the bHb product (with and without diafiltration) are given in Table 4. Acceptable endotoxin levels are usually ≤ 0.5 EU/mL, however, it is important to note that the purified bHb is very concentrated ~ 300 – 400 mg/mL. Therefore, when it is diluted for HBOC synthesis, the endotoxin level will fall below acceptable levels. Instead of removing more endotoxin as expected, the additional diafiltration step produced samples having 2-fold more endotoxin relative to bHb samples that were not subjected to diafiltration. The additional endotoxin contamination may have come from the 10 L of PB that was used to perform the diafiltration. Therefore under these conditions, diafiltration increases the susceptibility of bHb to endotoxin contamination rather than allowing more endotoxin to be filtered out.

SDS-PAGE Analysis

Figure 5 and Figure 6 show the SDS-PAGE gels at each stage of the filtration process without and with 10 L of diafiltration, respectively. Heat exposure during SDS-PAGE sample preparation causes bHb to dissociate from its native tetrameric form into monomeric α and β subunits. The molecular weights of the α and β subunits of hemoglobin are close in molecular weight (~ 15 and 16 kDa, respectively), so they form a single band near the bottom of the gel. The band migrates further down the gel than anticipated due to overloading the gel with a large amount of protein. The highest amount of impurities appears in the stage I retentate. The same impurities are also present in the post glass wool samples as well; however, the stage I retentate samples are concentrated much more by TFF. Impurities also appear in the stage II retentate samples. The end product; however, from the stage III retentate appears to be pure with the exception of a single band around 30 kDa. The band around 30 kDa may correspond to bHb dimers (31 kDa) or other bRBC proteins such as flavin reductase (26.8 kDa) or carbonic anhydrase (~ 29 kDa)¹³. No appreciable difference is observed in the SDS-PAGE gels between non-diafiltration and diafiltration samples.

Oxygen Binding Equilibria

Overall, bHb activity is unaffected by the TFF process. The oxygen dissociation curves (Figure 7) of whole bRBCs and purified bHb samples are very similar. Purified bHb has a slight decrease in cooperativity (Figure 8) relative to bRBCs. This is to be expected as some allosteric effectors (such as Cl^-) in bRBCs are removed during the filtration process. The diafiltrated bHb also shows a decrease in P_{50} (Figure 8), which may be caused by the

slightly higher metHb levels relative to bHb that was not subjected to diafiltration. Overall, the oxygen binding affinity of purified bHb (with and without diafiltration) is maintained and is similar to bHb isolated from other purification methods^{19,28,29}.

LC-MS Analysis

The RP-HPLC chromatograms of the bHb samples purified without and with diafiltration are shown in Figure 9A and Figure 10, respectively. The chromatograms are identical in each case, revealing only four major peaks. The electrospray ionization (ESI) mass spectra of each peak in the chromatogram (Figure 9B,C,E,G and Figure 10B,C,E,G) show good distributions of multiply charged ions. The ESI mass spectra of the first peak (Figure 9B & Figure 10B) reveal a base peak with a molecular weight of 616 g/mol, which corresponds to the heme group found in each of the bHb subunits. The deconvoluted ESI mass spectra of the second and third peaks (Figure 9D,F & Figure 10D,F) yield peaks with molecular masses of 15,053 g/mol and 15,954 g/mol, respectively. These values are equivalent to the theoretical molecular masses of the bHb subunits (α = 15,053 g/mol and β = 15,954 g/mol). Lastly, a base peak with a molecular mass of 31,906 g/mol is observed in the deconvoluted ESI mass spectra of the fourth peak (Figure 9H & Figure 10H). This mass is almost exactly twice that of the β monomer, suggesting that this peak represents a dimer of β subunits.

Overall, the LC-MS results show that the purified bHb product, with or without diafiltration, is essentially pure. Only four major species are observed, including the heme group, α and β subunits of bHb, and a β dimer. The chromatograms and ESI mass spectra from all samples are identical, showing no improvement in purity when diafiltration is used.

Conclusion

The results of this work reaffirm TFF as a suitable method for isolating HPLC-grade bHb from bRBC lysate for subsequent HBOC synthesis. However, the addition of a diafiltration step to the TFF process is both unnecessary and detrimental to the quality of the purified bHb product. Diafiltration lengthens the duration of the entire filtration process, causing an increase in bHb oxidation and a decrease in its P₅₀. The LC-MS results also show that no increase in purity is obtained when diafiltration is used. Therefore, diafiltration does not improve the overall quality of bHb purified with our three-stage TFF process and can be omitted in the future.

While diafiltration may be unnecessary in the purification of bHb, it is still useful in the purification of other proteins. It may also be more beneficial in the earlier stages of the bHb purification process. Diafiltration in these earlier stages may increase transmission of bHb through the 50 nm and 500 kDa membranes, thereby increasing the overall recovery of bHb.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health grants R01HL078840 and R01DK070862 to AFP.

References

1. O'Donnel E, Filipak S, Yanovsky A. The push for blood substitutes: is it all in vain? (Having my say). *Healthcare Purchasing News*. 2008; 32(1):56.
2. Li S, Nickels J, Palmer AF. Liposome-encapsulated actin-hemoglobin (LEAcHb) artificial blood substitutes. *Biomaterials*. 2005; 26(17):3759–3769. [PubMed: 15621266]

3. Arifin DR, Palmer AF. Determination of size distribution and encapsulation efficiency of liposome encapsulated hemoglobin blood substitutes using asymmetric flow field-flow fractionation coupled with multi-angle light scattering. *Biotechnol Prog.* 2003; 19:1798–1811. [PubMed: 14656159]
4. Arifin DR, Palmer AF. Stability of liposome encapsulated hemoglobin dispersions. *Artif Cells, Blood Substit, and Biotechnol.* 2005; 33:113–136.
5. Arifin DR, Palmer AF. Physical properties and stability mechanisms of poly(ethylene glycol) conjugated liposome encapsulated hemoglobin dispersions. *Artif Cells, Blood Substit, and Biotechnol.* 2005; 33:137–162.
6. Harris DR, Palmer AF. Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. *Biotechnol Prog.* 2008; 24:1215–1225. [PubMed: 19194934]
7. Eike JH, Palmer AF. Effect of glutaraldehyde concentration on the physical properties of polymerized hemoglobin-based oxygen carriers. *Biotechnol Prog.* 2004; 20:1225–1232. [PubMed: 15296452]
8. Arifin DR, Palmer AF. Polymersome encapsulated hemoglobin: a novel type of oxygen carrier. *Biomacromolecules.* 2005; 6:2172–2181. [PubMed: 16004460]
9. Dimino M, Palmer AF. High O₂ affinity hemoglobin-based oxygen carriers synthesized via polymerization of hemoglobin with ring-opened 2-chloroethyl- β -D-fructopyranoside and 1-o-octyl- β -D-glucopyranoside. *Biotechnol and Bioeng.* 2007; 97:462–472.
10. Sprung J, Kindscher JD, Wahr JA, Levy JH, Monk TG, Moritz MW, O'Hara PJ. The Use of Bovine Hemoglobin Glutamer-250 (Hemopure®) in Surgical Patients: Results of a Multicenter, Randomized, Single-Blinded Trial. *Anesth Analg.* 2002; 94:799–808. [PubMed: 11916776]
11. Levien LJ. South Africa: clinical experience with Hemopure. *ISBT Science Series.* 2006; 1(1): 167–173.
12. Gould SA, Moore EE, Hoyt DB, Ness PM, Norris EJ, Carson JL, Hides GA, Freeman IH, DeWoskin R, Moss GS. The life-sustaining capacity of Human polymerized hemoglobin when red cells might be unavailable. *J Am Coll Surg.* 2002; 195:445–455. [PubMed: 12375748]
13. Talamo F, D'Ambrosio C, Arena S, Del Vecchio P, Ledda L, Zehender G, Ferrara L, Scaloni A. Proteins from bovine tissues and biological fluids: Defining a reference electrophoresis map for liver, kidney, muscle, plasma, and red blood cells. *Proteomics.* 2003; 3:440–460. [PubMed: 12687612]
14. Lee CJ, Kan P. Hemoglobin purification. *US Pat.* 1993:5, 407, 579.
15. Li T, Yu R, Zhang HH, Wang H, Liang WG, Yang XM, Yang CM. A method for purification and viral inactivation of human placenta hemoglobin. *Artif Cells Blood Substit. Biotechnol.* 2006; 34:175–188.
16. Hsia CJC, Er SS, Hronowski LJ, Persaud K, Ansari MR. ATP-hemoglobin purification by ATP-agarose affinity chromatography. *J Chromatogr.* 1986; 381:153–157. [PubMed: 3771712]
17. Liao CY, Chang TM, Pan JP, Chen WL, Mao SJT. Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography. *J Chromatogr B: Analyt Technol Biomed Life Sci.* 2003; 790:209–216.
18. Wetz CE, Gawryl MS. Separation of hemoglobin from unpolymerized hemoglobin on hydroxyapatite using HPLC. *US Pat.* 1996:5, 691, 453.
19. Palmer AF, Sun G, Harris DR. Tangential Flow Filtration of Hemoglobin. *Biotechnol Prog.* 2008 accepted.
20. Azari M, Boosa JA, Burhop KE, Camacho T, Catarello J, Darling A, Ebeling AA, Estep TN, Pearson L, Guzder S, Herren J, Ogle K, Paine J, Rohn K, Sarajari R, Sun CS, Zhang L. Evaluation and Validation of virus Removal by Ultrafiltration During the Production of Diaspirin Crosslinked Haemoglobin (DCLHb). *Biologicals.* 2000; 28:81–94. [PubMed: 10885615]
21. Henderson LW, Besarab A, Michaels A, Bluemle LW Jr. Blood purification by ultrafiltration and fluid replacement (diafiltration). *Hemodialysis International.* 2004; 8:10–18. [PubMed: 19379396]
22. Lipnizki F, Boelsmand J, Madsen RF. Concepts of industrial-scale diafiltration systems. *Desalination.* 2002; 144:179–184.
23. Crosby WH, Munn JG, Furth ED. Cyanmethemoglobin method for estimation of hemoglobin. *US Armed Forces Med J.* 1964; 5:693–697.

24. Zijlstra WG, van Kampen EJ. Standardization of hemoglobinometry. I. The extinction coefficient of hemiglobincyanide. *Clin Chim Acta*. 1960; 5:719–726. [PubMed: 13788465]
25. Eike JH, Palmer AF. Effect of Cl^- and H^+ on the oxygen binding properties of glutaraldehyde-polymerized bovine hemoglobin based blood substitutes. *Biotechnol Prog*. 2004; 20:1543–1549. [PubMed: 15458341]
26. Patton JN, Palmer AF. Physical properties of hemoglobin-poly(acrylamide) hydrogel-based oxygen carriers: Effect of reaction pH. *Langmuir*. 2006; 22:2212–2221. [PubMed: 16489809]
27. Arifin DR, Palmer AF. Physical properties and stability mechanisms of poly(ethylene glycol) conjugated liposome encapsulated hemoglobin dispersions. *Artif Cells Blood Substit Biotechnol Bioeng*. 2005; 33:137–162.
28. Dimino ML, Palmer AF. Purification of bovine hemoglobin via fast performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007; 856:353–357.
29. Sun G, Palmer AF. Preparation of ultrapure human and bovine hemoglobin via anion exchange chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2008; 867:1–7.

Figure 1.
Schematic of the multi-stage HF filtration system used in this study.

Figure 2.

Total protein recovered at each stage of the HF filtration process. Error bars represent the standard deviation from 3 separate rounds of Hb purification. Roman numerals correspond to the filtration stage, while (F) refers to the filtrate and (R) refers to the retentate of a particular stage. Diamond symbols represent situations where the entire retentate bottle was filled with foam with no liquid present, therefore it was impossible to measure the total amount of protein. Asterix symbols represent measurements of total protein that were outside the measurement range of the total protein assay.

Figure 3.

Total Hb recovered at each stage of the HF filtration process. Error bars represent the standard deviation from 3 separate rounds of Hb purification. Roman numerals correspond to the filtration stage, while (F) refers to the filtrate and (R) refers to the retentate of a particular purification stage. Diamond symbols represent situations where the entire retentate bottle was filled with foam with no liquid present, therefore it was impossible to measure the total amount of Hb. Asterix symbols represent measurements of total Hb that were outside the measurement range of the Hb assay.

Figure 4.

MethHb level obtained at each stage of the HF filtration process. Error bars represent the standard deviation from 3 separate rounds of Hb purification. Roman numerals correspond to the filtration stage, while (F) refers to the filtrate and (R) refers to the retentate of a particular purification stage. Asterix symbols represent measurements of methHb level that were outside the measurement range of the methHb assay.

Figure 5. SDS-PAGE results at each stage of the HF filtration process without diafiltration. Roman numerals correspond to the filtration stage, while (F) refers to the filtrate and (R) refers to the retentate of a particular purification stage.

Figure 6. SDS-PAGE results at each stage of the HF filtration process with 10 L of diafiltration at stage III. Roman numerals correspond to the filtration stage, while (F) refers to the filtrate and (R) refers to the retentate of a particular purification stage.

Figure 7.
Oxygen dissociation curves of bRBCs and purified bHb (stage III retentate).

Figure 8. Oxygen affinity (P_{50}) and cooperativity coefficient (n) of bRBCs and 100 kDa bHb retentate. Error bars represent the standard deviation from 3 separate rounds of Hb purification.

Figure 9.

(A) RP-HPLC separation of bovine α - and β -globin chains using a Discovery® BIO Wide Pore C18 HPLC Column (1.0 $\times$ 10 mm, 3 μ m, Sigma-Aldrich Inc., Atlanta, GA) with 0.1% TFA in deionized water (Buffer A) and 0.1% TFA in ACN (Buffer B) as mobile phases for bHb purification without diafiltration. The gradient was initiated with 30% B and increased to 70% B over 60 min, then reduced to 30% B at 60.1 min and kept constant at 30% B until 90 min. The flow rate was maintained throughout the experiment at 25 μ L/min. (B) ESI mass spectrum of the heme peak in the RP-HPLC chromatogram. (C) ESI mass spectrum of the α globin peak in the RP-HPLC chromatogram. (D) Deconvolution of the ESI mass spectrum of α globin to yield the molecular mass of the α globin chain. (E) ESI mass

spectrum of the β globin peak in the RP-HPLC chromatogram. (F) Deconvolution of the ESI mass spectra of β globin to yield the molecular mass of the β globin chain. (G) ESI mass spectrum of the β dimer peak in the RP-HPLC chromatogram. (H) Deconvolution of the ESI mass spectrum of β dimer to yield the molecular mass of the β dimer.

Figure 10.

(A) RP-HPLC separation of bovine α - and β -globin chains using a Discovery® BIO Wide Pore C18 HPLC Column (1.0 $\times$ 10 mm, 3 μ m, Sigma-Aldrich Inc., Atlanta, GA) with 0.1% TFA in deionized water (Buffer A) and 0.1% TFA in ACN (Buffer B) as mobile phases for bHb purification with 10 L of diafiltration at stage III. The gradient was initiated with 30% B and increased to 70% B over 60 min, then reduced to 30% B at 60.1 min and kept constant at 30% B until 90 min. The flow rate was maintained throughout the experiment at 25 μ L/min. (B) ESI mass spectrum of the heme peak in the RP-HPLC chromatogram. (C) ESI mass spectrum of the α globin peak in the RP-HPLC chromatogram. (D) Deconvolution of the ESI mass spectrum of α globin to yield the molecular mass of the α globin chain. (E) ESI

mass spectrum of the β globin peak in the RP-HPLC chromatogram. (F) Deconvolution of the ESI mass spectra of β globin to yield the molecular mass of the β globin chain. (G) ESI mass spectrum of the β dimer peak in the RP-HPLC chromatogram. (H) Deconvolution of the ESI mass spectrum of β dimer to yield the molecular mass of the β dimer.

Table 1

Technical specifications of the tangential-flow filtration cartridges used in this study.

Pore Size	Material	Surface Area (cm ²)	Modules	Part Number	Stage
50 nm	Polysulfone	1050	MiniKros® Sampler	M10S-360-01S	I
500 kDa	Polysulfone	1050	MiniKros® Sampler	M1-500S-260-01S	II
100 kDa	Polysulfone	1050	MiniKros® Sampler	M1AB-360-01S	III

Permeate flow rate and trans-membrane pressure difference at each stage of the filtration process without and with 10L diafiltration at stage III.

Table 2

Stage	Without Diafiltration				With 10L Diafiltration			
	Permeate flow rate (mL/min)		Trans-membrane pressure difference (psig)		Permeate flow rate (mL/min)		Trans-membrane pressure difference (psig)	
	Start	End	Start	End	Start	End	Start	End
I	45.60±2.20	21.30±4.25	2.92±0.40	10.67±2.02	38.00±5.29	17.53±1.29	3.81±0.96	9.87±1.21
II	29.40±3.84	21.40±4.23	2.10±0.34	2.78±0.49	57.00±4.58	29.40±2.40	3.56±0.27	1.23±0.19
III	13.50±1.34	4.30±0.54	8.34±1.30	10.74±2.34	11.50±3.50	2.67±0.44	6.55±2.80	9.97±2.78

Table 3

Hb recovery (%) at each stage of the filtration process.

Filtration Stage	Recovery (%)	
	No Diafiltration	10 L Diafiltration
Post wool	99.30±4.15	96.93±2.16
Stage I Filtrate	87.73±3.97	80.90±2.16
Stage I Retentate	n/a	6.90±2.82
Stage II Filtrate	81.07±3.84	78.07±2.78
Stage II Retentate	n/a	n/a
Stage III Filtrate	1.68±0.41	25.57±12.39
Stage III Retentate	66.38±3.82	63.20±11.75

Table 4

Endotoxin level of stage III retentate.

Stage III Retentate	Endotoxin Level (EU/mL)
No Diafiltration	1.0–2.0
10 L Diafiltration	3.0–4.0