DESIGN AND IMPLEMENTATION OF A METHODOLOGY FOR THE INTRAMOLECULAR AND INTERMOLECULAR CROSSLINKING OF STROMA FREE HEMOGLOBIN AS AN INTRAVENOUS OXYGEN CARRIER

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Proyecto de Grado presentado como requisito parcial para optar al Titulo de Ingeniero Mecánico

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INTRODUCTION

Hemoglobin is the major protein in blood responsible for the transport of oxygen. However, there are two major reasons why hemoglobin molecules extracted from red blood cells can not be used as a intravenous oxygen carrier, the first is that hemoglobin breaks down into dimers after infusion, and therefore is toxic for the body; and the second reason is that hemoglobin outside the red blood cell has no accessibility to the cofactor 2,3 DPG, which facilitates oxygen bonding in the lungs and oxygen release in the tissues. Different biotechnological solutions to this problem is proposed in this work, the first is the reaction with pyridoxal 5' phosphate which is analogous to the cofactor 2,3 DPG and eases the bonding and release of oxygen. The second solution is the polymerization of hemoglobin with glutaraldehyde to form soluble polyhemoglobin which does not break down into dimers, and because it increases the size of the diluted particle, it decreases the oncotic pressure exerted by hemoglobin.

Another great characteristic of this intravenous oxygen carrier is the possibility to sterilize it, which removes the microorganisms responsible for AIDS, Hepatitis etc. And also since there are no group antigens, there is no need for blood

crossmatching and typing. This saves time and facilitates on spot transfusions like the infusion of salt solutions. Furthermore, this intravenous oxygen carrier can be lyophilized and stored for long periods of time as a stable dried powder that can be reconstituted with a salt solution just before use.

1. OBJECTIVES

This project was developed having as reference the following objectives:

1.1. Global objective

To design and implement a methodology for the intermolecular and intramolecular crosslinking of stroma free hemoglobin, so it can be used as a intravenous oxygen carrier.

1.2. Specific objectives

- To design and implement a methodology for the intermolecular crosslinking of stroma free hemoglobin.

- To design and implement of a methodology for the intramolecular crosslinking of stroma free hemoglobin.

- To design and implement physicochemical tests of the crosslinked stroma free hemoglobin.

- To characterize the crosslinked stroma free hemoglobin.

- To Appropriate foreign technology to the technology conditions present in Colombia.

2. THEORETICAL BACKGROUND

2.1. Blood substitutes

The advantages of a blood substitute that can be easily stored and infused safely at any time, in any place regardless of the quantity and the blood type are evident for any person. Because of the different and complex functions of blood, to think in the development of a total blood substitute is long way from today, but specific characteristics can be supplied by different substitutes, which combined can make a general blood substitute. The function of each blood component and its correspondent substitute of each function are listed in table1[1].

Table 1. Roles of blood components and their substitutes

Blood components	Roles	Substitutes
Plasma proteins		
Albumin	Maintenance of blood volume	Plasma expander
Fibrinogen	Coagulation factor	(dextran, hydroxyethyl starch)
Globulin	Immune antibody	Antibiotics

[1] Reference 1 pg 117-118

Plasma Electrolytes (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^-, HPO_4^{2-})			
55%		Osmoregulator, Carbon dioxide carrier	Electrolyte replenisher
	Lipids (fatty acids, cholesterol, neutral fat, lecithin)		
		Nutrition	Lipid microsphere
	Carbohydrate	Nutrition	Glucose injection
	Red blood cell		
	Hemoglobin	Oxygen and carbon dioxide carrier	**Artificial red cell**
Cells	Carbonic Anhydrase	Enhancement of carbon dioxide elimination	
45%			
	White blood cell	Phagocytosis, antibody production	Antibiotics, chemotherapeutic agents
	Platelet	Coagulation (hemostasis)	Blood coagulants

Therefore if a red blood cell or an intravenous oxygen carrier is developed, the demands for blood substitutes would be completely established.

2.1.1. Potential areas of application[2]

Because the available intravenous oxygen carriers just persist for 30 hours[3] in circulation, their present potential clinical uses can be narrowed to five different areas which are summarized in the outline below.

[2] Reference 2 pg 3-5

2.1.1.1. Surgery

This is specially important in cardiopulmonary bypass surgery, trauma surgery, cancer surgery, orthopedic surgery and other elective surgery. For example in trauma surgery the blood replacement in some cases may be up to 100 units, where a intravenous oxygen carrier can be used and at the end of the surgery, autologous blood can be infused.

2.1.1.2. Emergency resuscitation of traumatic blood loss

Examples include car and other accidents; disasters like earthquakes, plane accidents and others; civilian and noncivilian causalities in major or minor conflicts. In these cases, intravenous oxygen carriers can be used to replace blood loss in hemorrhagic shock. This is important in situations when there are no time or facilities for cross-matching or lack of sufficient supply of donor blood for immediate use.

2.1.1.3. Short term red blood cell support for other conditions

One example is severe hemolytic anemia. Another example is temporary support until bone narrow transplantation or recombinant human erythropoietin starts to

[3] Reference 2 pg 3

take over and it also stimulates erythropoiesis in the bone narrow. Another example is temporary support when very rare blood groups are not immediately available.

2.1.1.4. Medical application where intravenous oxygen carriers may be more effective than red blood cells

Intravenous oxygen carriers in solution would be superior to red blood cells in perfusion through obstructed vessels. Examples include myocardial infarction or stroke. The potential uses of intravenous oxygen carriers in organ preservation and in those situations where hypothermia is used are other examples, this is because red blood cells do not release oxygen properly at low temperatures, compared with intravenous oxygen carriers that can be prepared with acceptable P_{50} at low temperatures.

2.1.2. Decreasing the risks of infection

At present, the substitute of hemoglobin is donated blood, blood transfusion from donated blood can cause different problems like hemolysis by mismatching, coagulation and infection, even though this is been well treated today, but at a high

cost. First of all, blood can not be sterilized at present, transmission of infection is still possible at the following rates[4]:

Table 2. Rates of transmission of different infections in donated blood units[4]

Infection	Probability of transmission
HIV	1/225,000 units
Hepatitis B	1/200,000 units
Hepatitis (others)	1/3,300 units
HTLV I/II	1/50,000 units

Today a blood unit, after all the screening test and regulatory requirements are done, has a cost of about U$250 and not including the lawsuits by those who received tainted blood.[5]

2.1.3. Decreasing the load on the blood system

The minimal level of red blood cells in patients before giving transfusion in increasingly low, because the probability and risks of becoming infected. This can cause impair tissue oxygenation in patients with cardiovascular or pulmonary

[4] Reference 2 pg 6

[5] PICARD, ANDRE "*The Red Blood Cell Man*" De: McGill News Vol 76 – Number 4 – Winter 1996 – pp22-25

impairments, so intravenous oxygen carriers may provide a wider margin of safety for these and all patients[6].

To infuse autologous blood after surgery, requires to collect 4 to 6 units of the patient several weeks before the surgery, but when major surgery like trauma, cardiovascular surgery, orthopedic surgery and others occur, these do not allow the time interval to collect the blood from the patient[6].

Now days, the increasing needs from an increasingly aging population that needs more surgical and medical treatments including chemotherapy, transplantation and HIV therapy, has caused the margin between blood supply and demand to become very narrow, not including unexpected demands on the blood system caused by car and airline accidents, major disasters like earthquakes and conflicts and wars[6], which our country Colombia suffers at present. The possibility of plant scaling the production of intravenous oxygen carriers, is the solution, it will not only diminish the margin between blood supply and demand but it will also lower the costs of blood units, not only because of the type of production but also because the elimination of the screening tests done for virus detection. The only missing piece is where to obtain the hemoglobin needed for the production, at first hand it will be from donated blood, but other groups are working with bovine hemoglobin, recombinant human hemoglobin and transgenic human hemoglobin.

[6] Reference 2 pg 7

2.2. Oxygen carriers

Different investigations in possible oxygen carriers done during the last decades, has narrowed the research in two red blood cell substitutes which are being used and some of them are in clinical trials and ready to become accessible to people: modified hemoglobin and perfluorocarbons, unlike natural red blood cells, this can be pasteurized, filtered and chemically cleaned to make them sterile. In addition, because the substitutes do not have blood group antigens, crossmatching and typing are not required. This saves time an money and allows in spot transfusions in emergencies reducing the time of response in an emergency, which can be the key to save a life of an injured person. Furthermore, this can be stored for more than one year, compared with about one month for donor blood using standard methods[7], that can be improved by very expensive and laborious storage methods in which blood is frozen for long term storage[8], this procedure is done for rare blood types like are B- and AB.

2.3. Hemoglobin

"The equilibrium equation for the oxygen binding to a material (M) with oxygen affinity can be simply represented by:

[7] CHANG T.M., "Artificially Boosting the Blood Supply" Chemistry and Industry, 17 april 2000, p 281-285.
[8] Reference 2 pg 8

$$aM + bO_2 \underset{K}{\longleftrightarrow} (M)_a(O_2)_b$$

$$K_{(constant)} = \frac{[(M)_a(O_2)_b]}{[M]^a (P_{O_2})^b}$$

Because K is an oxygen binding constant, oxygen binds to M in a high pO_2 and dissociates from M at low pO_2. The oxygen partial pressure P_{50} at which half of the M molecules bind oxygen is often used to express the oxygen affinity for this material M. In the case of a red blood cell as a natural oxygen carrier, the material with a high oxygen affinity is hemoglobin".[9]

The different investigations done in the search of the best oxygen carrier has showed that none of the compounds known by humanity right now transports oxygen as efficiently as hemoglobin, which can be readily extracted from red blood cells. Perutz[10] has carried out extensive studies on the structure function of hemoglobin. In a short description, hemoglobin is encapsulated in a red blood cell in order to work during circulation without any problems, and it is surrounded by proteins, lipids and cholesterol, the sum of this three group of components is called stroma[11]. Hemoglobin is a tetramer of four subunits: two α units and two β units that are joined together by noncovalent bonds resulting from hydrogen bonding

[9] Reference 1pg 9
[10] PERUTZ M.F., Proc. R. Soc. Land. B, 1980, 208, 135
[11] Reference 1

and Van der Waals forces[12]. A schematic representation of the hemoglobin molecule is shown in figure 1.

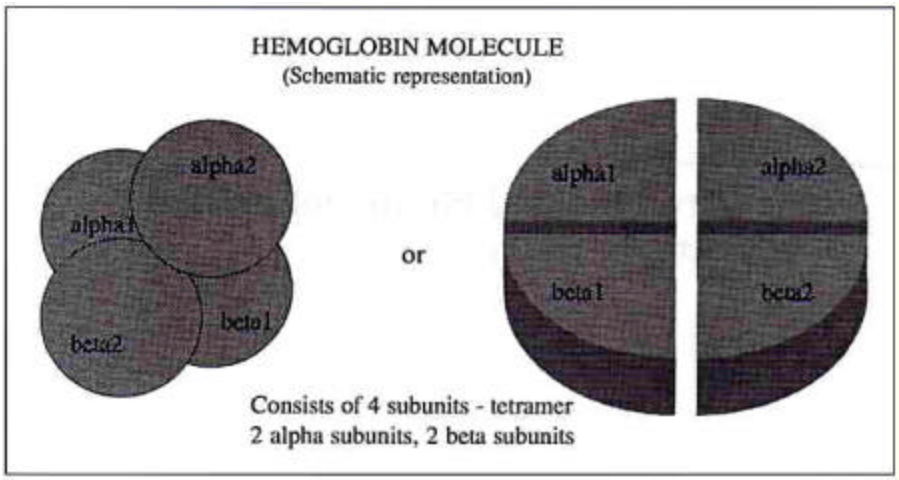

Figure 1. Schematic representation of the hemoglobin molecule[13]

Each subunit contains an heme group (iron-protoporphyrin IX), which is the binding site of oxygen, it has a the central iron with a low oxidation state (bivalent) that enables reversible oxygen binding. Because the hydrophobic atmosphere in the hemoglobin pocket, the iron does not oxidize to a trivalent state and then, it does not loose the ability of reversibly oxygen binding, when this happens, it binds oxygen so tightly so to preclude oxygen transfer, this hemoglobin in its trivalent state is called methahemoglobin[14].

[12] Reference 3col 3, 20
[13] Reference 2 pg 10
[14] Reference 1pg 6-8

"Hemoglobin constitutes about 90% of the total protein in a red blood cell"[15]. "The cell can carry 23 ml of oxygen per 100 ml of blood, this is about 80 times higher than the physical solubility of oxygen in plasma"[16]. Hemoglobin is in the "oxy", relaxed, or "R-state" when it carries oxygen (oxyhemoglobin). To release oxygen, the oxygen undergoes conformational change with a 15° rotation, the molecule is then in the "deoxy", tense, or "T-state" (deoxyhemoglobin).[17]

The cofactor 2,3-diphosphoglycerate (2,3-DPG) causes this conformational change because of its allosteric capabilities. Thus, in the presence of 2,3-DPG hemoglobin can release oxygen more readily at higher tissue oxygen tension[17].

2.3.1. Hemoglobin inside the red blood cell

Inside the red blood cell, hemoglobin is a tetramer, an the membrane retains 2,3 DPG in the cell to bind to hemoglobin. The hemoglobin concentration inside the red blood cell is 35 g/L (14 g/L in whole blood) which is possible because hemoglobin inside the cell does not exert osmotic pressure in the plasma outside, only the complete red blood cell exert osmotic pressure. Red blood cells have a lifetime in circulation of about 100 days[18].

[15] Reference 3 col. 3 : 33-34
[16] Reference 1pg 8
[17] Reference 2 pg 10

"The diameter of a red blood cell is 8.5μm, an the shape of the cell is a centersunken, disk-like vesicle, the thickness of which is a minimum of 1μm and a maximum of 2.4 μm"[19] as shown in figure 3.

The total volume of human blood is 5 liters and the amount of hemoglobin is 650 g, though there are some individual differences.[19]

"Furthermore, a red blood cell encapsulates various ions, regulator an enzymes, as well as hemoglobin. Apart from 2,3 DPG the red blood cell carries carbonic anhydrase as an enhancer of carbonic dioxide transportation, super oxide dismutase and catalase as quencher of active oxygens, and enzymatic systems to reduce the formation of methahemoglobin and glycolitic metabolism. These significant functions in a red blood cell help to maintain the oxygen and carbon dioxide carrying capacity, and regulate the acid-base equilibrium related to the functions of various ion channels."[19]

[18] Reference 2 pg 11
[19] Reference 1 pg 6

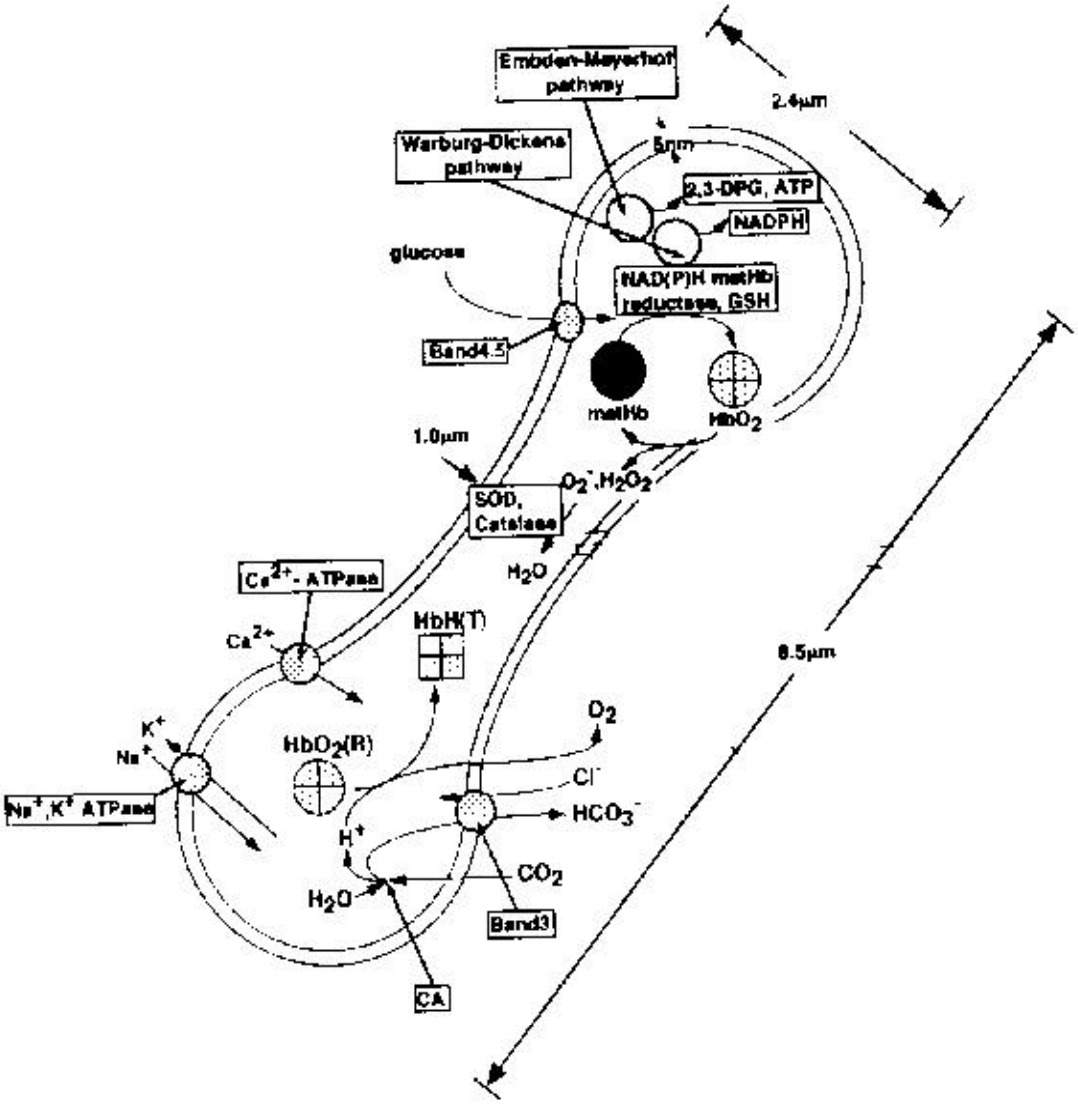

Figure 2. Schematic representation of the structure and function of a red blood cell.[20]

2.3.2. Hemoglobin as a red cell blood substitute

Obviously, the first reaction towards the development of a intravenous oxygen carrier is to infuse directly hemoglobin as an oxygen carrier. But hemoglobin

outside the red blood cell presents different problems which will be described as follows.

When erythrocyte hemolyzates have been administered by infusion, it has been found that stromal components are extremely toxic, resulting in coagulopathy and associated renal failure[23]. This was solved by Rabiner in 1967 when he prepared a stroma free-hemoglobin solution by ultrafiltration and centrifugation procedures[21,22]. Still other problems appear when stroma-free hemoglobine (SFHb) is used directly as an oxygen carrier. When taken out of the red blood cell microenvironment, hemoglobin binds oxygen too tightly, an therefore does not release the required oxygen to the tissues[23], this happens because of the absence of 2,3 DPG in the blood plasma, that regulates oxygen binding an release.

It also break down into two dimers, each one containing one alpha and one beta units, which are filtered quickly by the kidneys and removed from circulation. This dimers are toxic to the kidneys[24], cause renal failure and have a circulation time of only 2 hours.

[20] Reference 1 pg 8
[21] Reference 3 pg 4-5
[22] Reference 4
[23] Reference 3 col. 4 : 15-18
[24] Reference 2 pg 12

Nitric oxide plays an important role in controlling the vascular tone. Lowering nitric oxide results in vasoconstriction. Increasing nitric oxide results in vasodilatation. The intercellular junctions of the endothelial cell layer allow tetrameric hemoglobin to cross from the circulating blood, because of its size. Hemoglobin has also a high affinity for nitric oxide and acts as a sink in removing nitric oxide resulting in vasoconstriction[25].

The concentration of number of soluble particles decides the amount of osmotic pressure. Hemoglobin inside the red blood cell is separated from the plasma and not dissolved in plasma. They therefore do not exert an osmotic pressure. However free hemoglobin when infused as a solution will behave like protein dissolved in plasma and each free hemoglobin behaves like one solute particle increasing the osmotic pressure. To maintain the isoosomotic pressure with plasma only a solution of 70 g/L of free hemoglobin will have to be used[26], reducing the oxygen transport capacity since the normal concentration of hemoglobin is around 130 g/L.

As discussed earlier, hemoglobin inside the red blood cell has different interactions with super oxide dismutase and catalase and enzymatic systems to reduce the oxidation of the central iron, and therefore form methahemoglobin. When free

[25] CHANG T.M.S. "*Future Developments In Modified Hemoglobin As a Red Blood Substitute*" in www.medicine.mcgill.ca/artcell
[26] Reference 2 pg 35

hemoglobin is infused in blood plasma, it has no interaction with this compound and tends to form methahemoglobin.

2.4. What can be done to solve the problems of hemoglobin

2.4.1. Pyridoxilation of stroma free hemoglobin

"The affinity of hemoglobin is regulated by an organic phosphate such as 2,3 DPG. Benesch and Benesch showed in 1967, that in the presence of 2,3 DPG, hemoglobin has a higher P_{50}. This highly anionic organic phosphate is present in human red cells at the same molar concentration of hemoglobin. Hemoglobin in the red blood cell can therefore readily unload oxygen in capillaries supplying tissues."[27]

"The binding site for 2,3 DPG is important because it forms the basis of many modifications of hemoglobin to form modified hemoglobin intravenous oxygen carriers. The 2,3 DPG pocket is in the central cavity of deoxyhemoglobin, and consists of three positively charged residues in each β chain: the a-amino group, lysine EF6, and histidine H21. These positively charged groups in the central cavity of the hemoglobin molecule interact with the strongly negatively charged 2,3 DPG.

In this way 2,3 DPG stabilizes the deoxyhemoglobin quaternary structure by crosslinking the β chains. This shifts the equilibrium toward the T (tense) form."[28]

"The loss of 2,3 DPG and other organic phosphate ligands result in a hemoglobin solution with an oxygen affinity much higher than that of whole blood. The high affinity can be lowered by chemical modification of hemoglobin with pyridoxal 5' phosphate"[29]. "Benesch in 1975 made this important finding, and showed that pyridoxal 5' phosphate is an analog of the cofactor 2,3 DPG, and then improves the P_{50}."[30]

"Benesch discovered that a variety of pyridoxal derivates containing an anionic group in the 5' position react with the NH_2-terminal amino group of human hemoglobin. Their studies show that pyridoxal 5' phosphate reacts with the deoxy-hemoglobin tetramer to form a Schiff's base specifically with the NH_2-terminus of one β chain. This imine can be converted to the corresponding stable secondary amine by reduction with sodium borohydride to give the monosubstituted tetramer"[31].

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[27] Reference 2 pg 23
[28] Reference 2 pg 24
[29] Reference 5
[30] Reference 2 pg 21
[31] Reference 6

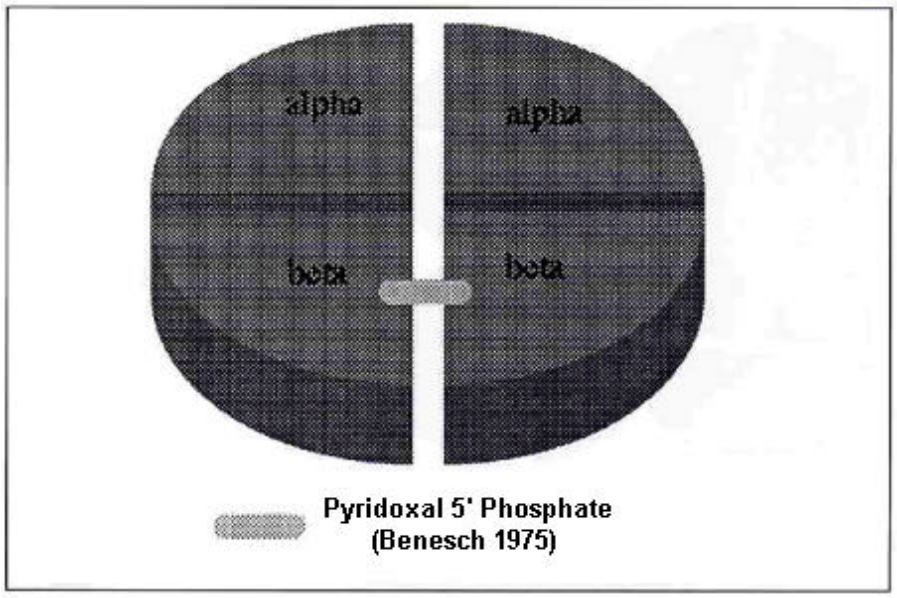


Figure 3. Representation of pyridoxilated hemoglobin.

2.4.2. polymerization of pyridoxilated stroma free hemoglobin

As explained earlier, the hemoglobin when infused directly breaks down into dimers which cause renal failure, the principal reason for crosslinking hemoglobin is to form a macromolecule, which will not break down into dimers, avoiding renal failure and increasing the time in circulation from 2 to 30 hours. It will also increase its molecular size, preventing the hemoglobin molecule to cross the in intercellular junctions of the endothelial cell layer and removing the nitric oxide, which is responsible of regulating the vasoconstriction.

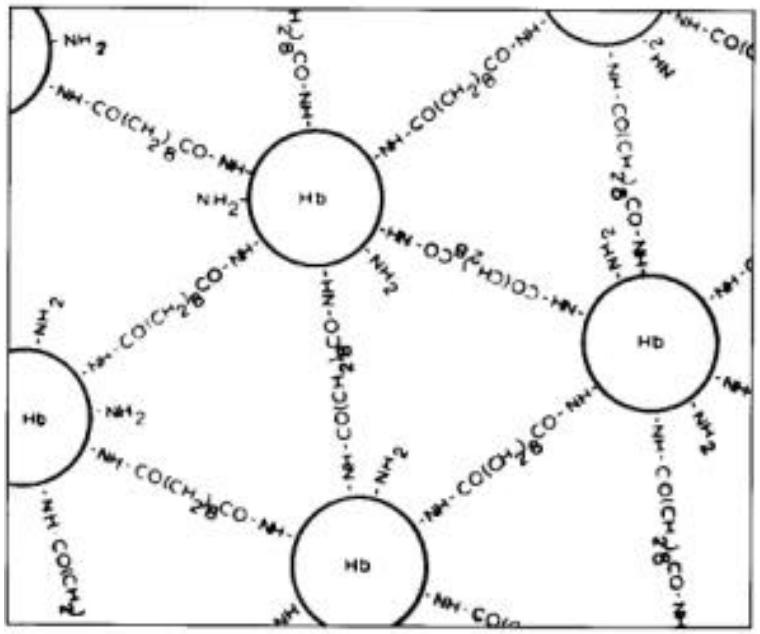


Figure 4. Crosslinked hemoglobin with a bifunctional agent (Sebacyl chloride).[32]

2.4.2.1. Crosslinking, regulating and quenching agents[33]

The bi- or polyfunctional agents suitable are preferably water soluble, and reactive with crosslinkable sites of hemoglobin, to yield a crosslinked water soluble product. The crosslinking agents used should not affect adversely the hemoglobin, its solubility or its function of reversibly binding oxygen for supplying it to tissues and organs. The bi or polyfunctional agents have al least two functional groups, and they can be the same or different. These groups are capable of reacting with and crosslinking amino groups and other crosslinkable sites on the hemoglobin

[32] Reeference 2 pg 16
[33] transcripted exactly from Reference 7

molecule. By amino groups is meant the N-terminal alpha amino group of the hemoglobin chains, and those of the basic amino acid residues such as lysine and arginine.

The functional groups of the cross-linking agent can be covalently bonded to each other or they can be separated by an aliphatic or by an aromatic ring. Exemplary aromatic stabilized fnctional groups are azo and halo activated with a nitro group. These include coumpounds having a heterocyclic ring with reactive groups bonded to the ring. For example, triazines of the formula:

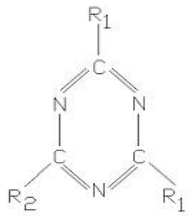


Figure 5. Triazine formula.[34]

wherein R_1 is halogen including fluoro, chloro and bromo, and R_2 is a nucleophilic substituent such as an aliphatic or aromatic group, a halogen, a lower alkyl of 1 to 8 carbons, and amino. Cross-linking agents embraced by this formula are 2-amino-4,6-dichloro-s-triazine and chloro-s-triazine. The cross-linking agents include aromatic stabilized agents prepared by the diazotation of an aromatic diamine, for example, benzidine and its derivatives with nitrous acid to yield bis-diazobenzidines of the formula:

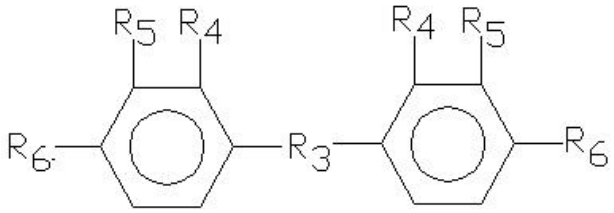


Figure 6. Bis-diazobenzidine formula.[31]

wherein R_3 is a member selected from the group consisting of a covalent bond, alkylene of 1 to 5 carbons, phenylene, ether, sulfone and secamine, R_4 is halogen or nitro, R_5 is hydrogen, nitro, lower alkyl of 1 to 8 carbons, sulfonate (SO_3H) and carboxylate, and R_6 is halogen, diazo (-N:N-), isocyanate (NCO), and isothiocyanate (NCS). Representative agents embraced by the formula include bis-diazobenzidine 2,2'-sulfonic acid, 4,4'-difluoro-3,3'-dinitrophenylsulfone and diphenyl-4,4'-diisothiocyanate.

Cross-linking agents suitable for the invention include compounds of the formula:

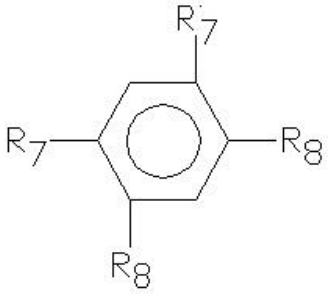


Figure 7. 1,5-difluoro-2,4-dinitrobenzene.[35]

[34] Reference 7
[35] Reference 7

wherein R_7 is halogen and R_8 is nitro, or hydrogen with at least one R_8 a nitro, as represented by the commercially available activated halogenated reagent 1,5-difluoro-2,4-dinitrobenzene.

Cross-linking agents suitable for the purpose of the invention also include compounds of the formula $(R_9)_2C=O$ wherein R_9 is hydrogen or halogen, and compounds of the formula $R_{10}–(CH_2)_n–R_{10}$ wherein R_{10} is the same or different and n is 1 to 8. The agents also include compounds having a functional group bound to an aromatic moiety either directly or through an alkylene bridge of the formula $R_{10}–(CH_2)_m–C_6H_4–(CH_2)_m–R_{10}$ wherein R_{10} is the same or different and m is 0 to 3. Cross-linking agents include the compounds having the functional groups bonded to a cycloalkyl as represented by the formula:

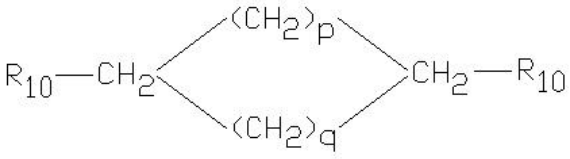


Figure 8. Cycloalkyl formula.[36]

wherein R_{10} is the same or different, p is 0 to 4, and q is 1 to 4. The cross-linking agents include compounds having functional groups bonded to an aliphatic chain interrupted with a nonfunctional group or having nonfunctional groups bonded to

the chain as represented by compounds of the formula $R_{10}–(CH_2)_x–R_{11}–(CH_2)_x–R_{10}$ wherein R_{10} is the same or different, R_{11} is selected from the group consisting of an ether bridge, a divalent amine and a sulfone, and x is an alkylene of 1 to 5 carbon atoms, with each x the same or different. Representative of the functional group embraced by R_{10} include isocyanate, vinyl, imine, isothiocyanate, isocyanide, aldehyde, epoxide, chloroformate, thiochloroformate, and imido lower alkyl ester, and thiolactones of the formula:

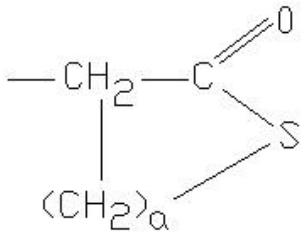


Figure 9. Thiolactone formula.[30]

wherein a is 1 to 3. Also, R_{10} can be an activated group formed by reacting the carboxylic acid with a thionyl halide or phosphorus halide, or an activated group formed by reacting an amide or an alkyl ester of the carboxylic acid with hydrazine and then with nitrous acid to yield the corresponding activated group COR_{12} wherein R_{12} is halogen or azide. The activated group can also be formed by reacting the carboxylic acid with N,N'-carbonyl diimidazole of the formula $R_{13}–N=C=N–R_{13}$ wherein R_{13} is the same or different and are a lower alkyl, a lower

[36] Reference 7

cycloalkyl, amino lower alkylene, and heterocyclic lower alkyl including morpholino ethyl. R_{12} can also be a

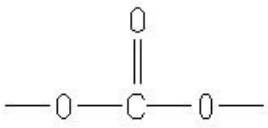


Figure 10. Lower alkyl formula.[37]

lower alkyl, and a

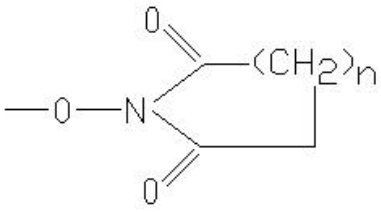


Figure 11. Another possibility for R_{12}.[34]

wherein n is 1 or 2.

Exemplary commercially available cross-linking reagents embraced by the above formula include divinyl sulfone, epichlorohydrin, butadiene diepoxide, ethylene glycol diglycidyl ether, glycerol diglycidyl ether, dimethyl suberimidate dihydrochloride, dimethyl malonimidate dihydrochloride, and dimethyl adipimidate dihydrochloride.

Representative of compounds bearing a functional isocyanate or isothiocyanate group are the compounds listed below. Additionally, the isocyanates or isothiocyanates can be synthesized by reacting an alkyl or aryl amine with phosgene or thiophosgene. The isocyanates used for cross-linking are diisocyanates and they react with the free amino groups of hemoglobin producing urea or thiourea cross-linked sites. Typical compounds include diphenyl-4,4'-diisothiocyanate-2,2'-disulfonic acid, toluene diisocyanate, toluene-2-isocyanate-4-isothiocyanate, 3-methoxydiphenylmethane-4,4'-diisocyanate, propylene diisocyanate, butylene diisocyanate, and hexamethylene diisocyanate.

Exemplary of cross-linking agents having an aldehyde or dialdehyde functionality include formaldehyde, paraformaldehyde, formaldehyde activated ureas such as 1,3-bis(hydroxymethyl)urea, N,N'-di(hydroxymethyl) imidazolidinone prepared from formaldehyde condensation with a urea according to the formula:

$$CH_2O+R_{16}HN–CO–NHR_{16} \rightarrow HOCH_2NR_{16}–CO–NR_{16}–CH_2OH$$

wherein R_{16} is hydrogen, alkyl, aryl or heterocyclic ring. Other dialdehyde cross-linking agents include dialdehydes of the formula OCH–R17–HCO wherein R_{17} is a member selected from the group consisting of a covalent bond and a straight or

[37] Reference 7

branched chain alkylene of 1 to 8 carbons. Dialdehydes embraced by the formula include gloxal, malonic dialdehyde, succinic dialdehyde, glutaraldehyde, adipaldehyde, 3–methyl glutaraldehyde, propyladipaldehyde, phthalic dialdehyde, terephthaldehyde and malonic dialdehyde.

$$RCO_2H \xrightarrow{activator} RCOX \xrightarrow{Hb-NH_2} RCON-H-Hb$$

Other cross-linking agents include derivatives of carboxylic acids and carboxylic acid residues of hemoglobin activated in situ to give a reactive derivative of hemoglobin that will cross-link with the amines of another hemoglobin. Typical carboxylic acids useful for this purpose have the formula $CO_2H(CH_2)_nCO_2H$, and $[(CH_2)_nCOOH]_3CH$ wherein n is 1 to 8. The carboxylic acids include citric, malonic, adipic and succinic. Carboxylic acid activators include thionyl chloride, carbodiimides, N-ethyl-5-phenyl-isoxazolium-3'-sulphonate (Woodward's reagent K), N,N'-carbonyldiimidazole, N-t-butyl-5-methylisoxazolium perchlorate (Woodward's reagent L), 1-ethyl-3-dimethyl aminopropylcarbodiimde, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate. The cross-linking reaction using a carboxylic acid can be represented by the equation:

Other cross-linking groups that can be used are prepared from esters and thioesters activated by strained thiolactones, hydroxysuccinimide esters, halogenated carboxylic acid esters and imidates.

The cross-linking reagent can be a dialdehyde precursor that readily forms a bifunctional dialdehyde in the reaction medium. Suitable dialdehyde precursors include acrolein dimer or 3,4-dihydro-1,2-pyran-2-carboxaldehyde which undergoes ring cleavage in an aqueous environment to give alpha-hydroxy-adipaldehyde. Other precursors, which on hydrolysis yield a cross-linking reagent, include 2-ethoxy-3,4-dihydro-1,2-pyran which gives glutaraldehyde, 2-ethoxy-4-methyl-3,4-dihydro-1,2-pyran which yields 3-methyl glutaraldehyde, 2,5-diethoxy tetrahydrofuran which yields succinic dialdehyde and 1,1,3,3-tetraethoxypropane which yields malonic dialdehyde and formaldehyde from trioxane. Similarly, the cross-linking reagents can be prepared by known synthetic procedures such as malonaldehyde from tetraethyl acetal, succinaldehyde from diethoxytetrahydrofuran, and adipaldehyde by the oxidation of cyclohexanediol.

In the above formula, the expression "an alkylene of 1 to 8 carbons" includes straight or branched chain alkylenes such as methylene, ethylene, propylene, isopropylene and hexylene. The expression "lower alkyl of 1 to 8 carbons" includes straight or branched chain alkyls such as methyl, ethyl, propyl, isopropyl, and hexyl.

The low molecular weight amine added to the polymerization vessel for regulating the cross-linking reaction, or for quenching it, is a mono-, di-, or multifunctional

agent, preferably a primary amine of the formula R-NH_2. The amine should be water soluble to assist in maintaining the solubility characteristics for the polymerized hemoglobin. Typical low molecular weight amines used to deactivate excess cross-linking agents are glycine, lysine, serine, threonine, alanine, ethanolamine, 2-aminoadipic acid and glutathione. Other compounds capable of deactivating the cross-linking agents are terminators such as bisulfites and diols capable of deactivating aldehydes, low molecular weight alcohols for deactivating activated carboxylic acids, activated halides and isocyanates, and sulfhydryls for deactivating epoxides and vinyls.

2.4.2.2. What crosslinking, regulating and quenching agent will be used?

The selection of the crosslinking, regulating and quenching agents, depended on the facilities for their acquisition, and the comparison that could be made with other groups that already polymerized stroma free hemoglobin, the groups of N.P. Kuznetsova[38], L.R. Sehgal[39], Alza Corporation[40] and T.M.S. Chang[41,42] use as crosslinking agent glutaraldehyde and as regulating and quenching agent lysine monohydrochloride, so this compound will be used in this work so that can a comparison be made afterwards with these other works. The final result is

[38] Reference 8
[39] Reference 9
[40] Reference 7
[41] Reference 2 pg 30-31
[42] Reference 10

polymerized pyridoxilated stroma free hemoglobin, a schematic diagram can be seen in figure 12.

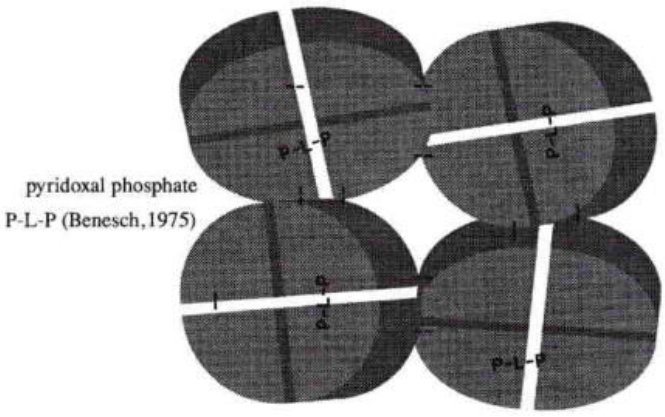


Figure 12. Final result, polymerized pyridoxilated stroma free hemoglobin.[43]

2.4.3. other processes used

During the production of polymerized pyridoxilated stroma free hemoglobin solution, it needs other processes different from the pyridoxilation and polymerization process, to prepare and adequate the hemoglobin solution for the next step.

2.4.3.1. Ultrafiltration

2.4.3.1.1. Definition[44]

[43] Reference 2 pg 21

Ultrafiltration is a low-pressure membrane process used to separate high molecular weight compounds from a feed stream. Ultrafiltration has larger pores than nanofiltration and reverse osmosis and is therefore the least costly of the three to operate. As a result ultrafiltration requires fewer membrane elements and lower pressures for operation. Ultrafiltration is useful for the separation of delicate materials, like proteins, since it is a non-denaturing method of separation. In general salts and low molecular weight species can pass through the membrane while suspended solids become concentrated on the other side of the membrane. Cross flow membrane filtration is commonly used for the concentration and purification of proteins and other biological particles from suspensions. Ultrafiltration is capable of concentrating bacteria, some proteins like hemoglobin, some dyes, and constituents that have a larger molecular weight of greater than 10,000 Daltons. Ultrafiltration is only somewhat dependent upon the charge of the particle and is much more concerned with the size of the particle.

2.4.3.1.2. Ultrafiltration process

The process is carried out in a ultrafiltration unit. It can be done in a dialysis unit that has to be bypassed and no dialysis fluid is entered in the filter. The pore diameter of the filter depends on the solution that is going to be ultrafiltered, and the size of the protein that is going to be concentrated.

[44] http://www.osmonics.com/products/page835.htm and http://www.rpi.edu/dept/chem-eng/Biotech-

2.4.3.2. Dialysis

2.4.3.2.1. Definition[45]

Dialysis is a procedure which separates electrolytes and colloids that are found in dissolutions, depending on their velocity of diffusion through a semipermeable membrane. The solution that is to be dialyzed is introduced into the semipermeable membrane and the electrolytes pass through the membrane that retains the colloids.

2.4.3.2.2. Dialysis process

The removal of excess reagents is very important. Dialysis against Ringer's lactate removes free glutaraldehyde and excess lysine and it also allows the electrolytes to be adjusted to physiological conditions[46]. The dialysis process is carried out

Environ/Projects00/memfilt/ultrafiltration.htm
[45] Reference 11 volume 4 pg 1207
[46] Reference 2 pg 31

using a dialysis membrane tube, like regenerated cellulose. is recommended to allow a good surface-volume relationship for an adequate dialysis process, for this, small volumes, for example 10 ml, are placed in the tubing and let in dialysis for at least 3 hours, within this time the dialysate equilibrate with the dialysis solution.

3. METHODOLOGY

3.1. Pyridoxilation of stroma free hemoglobin

The first modification done to stroma free hemoglobin is its pyridoxilation, this process, as explained earlier, will improve the P50 of the stroma free hemoglobin crosslinking intermolecularly the two β subunits forming a phosphate bridge between them.

3.1.1. Materials and equipment

3.1.1.1Reactives

- TRIS-HCl buffer (powder)
- NaOH (pellets)
- Pyridoxal 5’ phosphate (powder)
- Glucose (powder)
- Glutathione
- Ascorbic acid (powder)
- Gaseous nitrogen
- Sodium borohydride (powder)
- HCl (liquid)
- Distilled water

3.1.1.1.1. Equipment

- Balance weight
- pH-meter
- nitrogen fluxmeter connected to a bubble disperser by a hose.

- laboratory equipment like pipettes, glasses, erlenmeyers, volume flasks etc. Their capacities depend con the quantity of stroma free hemoglobin to be piridoxilated.
- Hemoglobin concentration meter.
- Magnetic agitator with its agitation bar.

3.1.2. Preparation of solution used

Besides the stroma free hemoglobin solution, a solution of NaOH 2 M is prepared, to adjust the pH of the hemoglobin solution to the desired point. During the whole process of piridoxilation and polymerization, is said that for each 500 ml of stroma free hemoglobin, no more than 30 ml would be used. This quantity should be prepared as follows:

Mesure 2,4 g of NaOH, add water until 30 ml are marked in the measuring flask.

3.1.3. Pyridoxilation process

All the process is carried out at 4°C, otherwise methahemoglobin formation is marked during the process. The stroma free hemoglobin solution is continuously agitated. Measure the concentration of hemoglobin in the hemoglobin meter. For each 100 ml of stroma free hemoglobin add 1.584 g of TRIS-HCl buffer (99.5%) to

obtain a concentration of 0,1 M. Adjust the pH of the solution to 7,4 with NaOH 2M or HCl. For each gram of hemoglobin present in the solution, add 16.79 mg of pyridoxal 5' phosphate so that the molar ratio is 4:1 to hemoglobin. Add glucose and glutathione to yield a concentration of 2 g/L, and ascorbic acid to yield a concentration of 1 g/L. These are added to minimize methahemoglobin formation. Check pH again and adjust to 7,4 with NaOH 2 M or HCl. Deoxygenate the stroma free hemoglobin solution with nitrogen for at least 2 hours, be careful because hemoglobin during nitrogen bubbling produces foam. Covalent linkage of pyridoxal 5' phosphate to hemoglobin is accomplished by adding 12.227 mg of $NaBH_4$ (96%) for each gram of hemoglobin present in the solution to yield a molar rate of $NaBH_4$ to hemoglobin of 20:1, diluted in a manageable but minimum volume of NaOH 0,001 M, which is prepared by diluting 2000 times a part of NaOH 2 M in distilled water.

The nitrogen bubbling is done for at least another 12 hours, so the linkage between hemoglobin and pyridoxal 5' phosphate is accomplished. In Appendix I a flux diagram of the pyridoxilation process is shown.

3.2. Polymerization of pyridoxilated stroma free hemoglobin

The second modification done to the now pyridoxilated stroma free hemoglobin, is the intermolecular crosslinking of hemoglobin tetramers to form polymerized pyridoxilated stroma free hemoglobin. This process will prevent the dimerization of the hemoglobin molecule.

3.2.1. Materials and equipment

3.2.1.1. Reactive

- Glutathione
- L-lysine monohydrochloride
- Phosphate buffer (K_2HPO_4 and KH_2PO_4)
- Glutaraldehyde (liquid 25%)
- Distilled water

3.2.1.2. Equipment

- Balance weight
- pH-meter
- Magnetic agitator with its agitator bar.

- laboratory equipment like pipettes, glasses, erlenmeyers etc. Their capacities depend con the quantity of stroma free hemoglobin to be polymerized.
- Hemoglobin concentration meter.
- Ultrafiltration filter and dialysis equipment.
- Osmometer

3.2.2. Preparation of solutions used

The following solutions are prepared since they are used during the polymerization process.

3.2.2.1 Pyridoxilated stroma free hemoglobin solution

After the pyridoxilation process is carried out as described earlier, the hemoglobin concentration of the solution is under 16 g/dl which is the concentration that is used during the polymerization process. For this reason, a ultrafiltration process is carried out to increase the hemoglobin concentration to the working concentration of 16 g/dl.

3.2.2.1.1. Lysine solution

This solution is used as a regulating agent and quenching agent during the polymerization process. One milliliter of this solution is prepared as follows: add 239,84 mg of L-lysine monohydrochloride (99%) to yield a concentration of 1.3 M, add 87.09 mg of K_2HPO_4 and 68.04 mg of KH_2PO_4 to yield a buffer concentration of 0,5 M and add distilled water until the 1 ml mark is reached. Adjust the pH of the solution to 7,4 by adding either NaOH 2 M or HCl. For each 100 ml of pyridoxilated stroma free hemoglobin with an hemoglobin concentration of 16 g/dl, 19,85 ml of the lysine solution are needed.

3.2.2.1.2. Glutaraldehyde solution

This is the polymerization agent used. This solution is made by diluting a 25% glutaraldehyde stock solution in equal volumes with a 0,5 M phosphate buffer. One milliliter of the phosphate buffer is prepared as follows: add 87.09 mg of K_2HPO_4 and 68.04 mg of KH_2PO_4 to yield a buffer concentration of 0,5 M and add distilled water until the 1 ml mark is reached. After diluting the glutaraldehyde in the 0,5 M buffer, adjust the pH to 7,4 by either adding NaOH 2 M or HCl. For each 100 ml of pyridoxilated stroma free hemoglobin with and hemoglobin concentration of 16 g/dl, 3,3 ml of the glutaraldehyde working solution are needed.

3.2.3. Polymerization process

All the process is carried out at 4°C, otherwise methahemoglobin formation is marked during the process. The pyridoxilated stroma free hemoglobin solution is continuously agitated. All the quantities described below are based working with 100 ml of the pyridoxilated stroma free hemoglobin solution in a concentration of 16 g/dl. First adjust the pH of the pyridoxilated stroma free hemoglobin solution to 7,4 by adding NaOH 2 M or HCl and measure its osmolarity, then add 1,25 ml of the lysine working solution to achieve an hemoglobin lysine molar ratio of approximately 2:13. Add 0,15 g of glutathione to minimize the methahemoglobin formation to achieve a concentration of 1,5 g/l. Add 3,3 ml of the glutaraldehyde working solution to achieve an hemoglobin glutaraldehyde molar ratio of approximately 3:5. Measure the osmolarity of the solution periodically until it reaches the isoosmotic level of 300 mOsm/kg, usually a 12 to 18 hour period is needed. To quench the reaction add 18,6 ml of the lysine working solution, to achieve a molar relation of hemoglobin to lysine of 1:112 approximately, and continue stirring for an additional 12 hours. Centrifuge the polymerized pyridoxilated stroma free hemoglobin solution at 25,000 g for one hour. Other cleaning process may be also used, after adding the lysine quenching solution, ultrafilter the now polymerizated stroma free hemoglobin solution. Then reconstitute the supernatant or the ultrafiltered solution by dialysis against Ringer's lactate for 3 hours at 4°C. This will remove free glutaraldehyde and excess lysine and will also allow the electrolytes to be adjusted to physiological concentrations. In appendix II a flux diagram of the polymerization process is shown.

3.3. Evaluation of the physicochemical properties of modified hemoglobin

3.3.1. Hemoglobin concentration

3.3.1.1. Definition

As explained earlier, hemoglobin is the responsible for oxygen distribution in the tissues. Around 23 ml of oxygen per 100 ml of plasma is needed to achieve normal tissue oxygenation. Based on the oxygen carrying capacity of hemoglobin, its concentration in plasma has to be in the range of 13,5 to 17,5 g/dl for a male person and in the range of 12 to 16 for a female person[47]. The mean hemoglobin concentration in plasma is 14,7 g/dl. Its determination is obtained with the help of hemoglobin concentration meters which determine the hemoglobin concentration by spectophotometry.

3.3.2. Colloid osmotic pressure

[47] Reference 12 pg 48

3.3.2.1. Definition[48]

"Osmotic pressure across a membrane is exerted by an impermeable or unequilibrated solute. The necessary pressure to stop the osmotic movement of water to a solution, is known as osmotic pressure. It reflects the tendency of water to cross a membrane towards the solution"[49]. Under physiological conditions, plasma proteins are the only plasma solutes that can not move quickly across the capillary wall. Plasma proteins therefore exert a colloid osmotic pressure (oncotic pressure) across the capillary wall. Under physiological conditions, the net effect of oncotic pressure itself is the movement of fluid into the capillary.

The protein concentration of plasma is about 7 g/dl. Hemoglobin inside the red blood cell is separated from plasma and not dissolved in plasma. It therefore does not exert oncotic pressure.

The concentration of number of soluble particles decides the amount of oncotic pressure. Each free hemoglobin tetramer behaves like a particle. A molecule of polymerized pyridoxilated stroma free hemoglobin (Poly-PLP-SFHb) formed by particles ranging from 1 to four or more hemoglobins in each particle, will exert less oncotic pressure than free hemoglobin molecules. Generally speaking, 14 g/dl of Poly-PLP-SFHb solutions exert the same oncotic pressure as 7 g/dl of free

[48] Reference 2 pg 35

hemoglobin. They are therefore isooncotic with plasma. Modified hemoglobin solution are usually prepared to be isooncotic with plasma, as is the case of this work.

3.3.2.2. Determination

During the process of polymerization of pyridoxilated stroma free hemoglobin, the only solute present in the solution that exerts a significant colloid osmotic pressure is hemoglobin. The isoosmotic pressure of plasma is around 300 mOsm/kg, and blood varies between 275 and 295 mOsm/Kg. When the polymerization reaches this isoosmotic point, the process is stopped. This is measured with an osmometer.

3.3.3. Density

3.3.3.1. Definition

As it is well known, density is a relation between the mass and the volume of a body or a fluid, it represents the quantity of mass that is present by one volume unit. The density of human blood varies between 1,40 and 1,85 g/cm^3.[50]

3.3.3.2. Determination

[49] Reference 13 pg 160

The determination of the density of the polymerizated pyridoxliated stroma free hemoglobin solution or of any other fluid is done with the aid of a picnometer, these recipient, normally fabricated from glass, is gauged to a predetermined volume to which the picnometer is filled with the fluid. The picnometer is weighted before and after the fluid is filled in it, the difference of this two measurements is the weight of the fluid that occupies the volume of the picnometer. Therefore a the relationship between mass and volume determines the density of the fluid.

3.3.4. P_{50}

3.3.4.1. Definition

"Oxygen affinity with hemoglobin is characterized by P_{50}. P_{50} is the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen. Hemoglobin is saturated at 100% at a pO_2 around 108 mmHg[51]. P_{50} measures the ease of hemoglobin to in unloading oxygen as required in the capillaries supplying the tissues. The higher the P_{50} the more ease it has in unloading oxygen. Hemoglobin

[50] TELMISSANI O.A., KHALIL S., ROBERTS G.T. *"Mean Density of Hemoglobin per Liter of Blood: A New Hematologic Parameter With An Inherit Discriminant Function"*. Laboratory Hematology. 1999. 5:149-152.
[51] Reference 12

in red blood cells has a P_{50} of 26 mmHg" [52]. An oxygen dissociation curve is useful in measuring the many characteristics of hemoglobin (see figure 4).

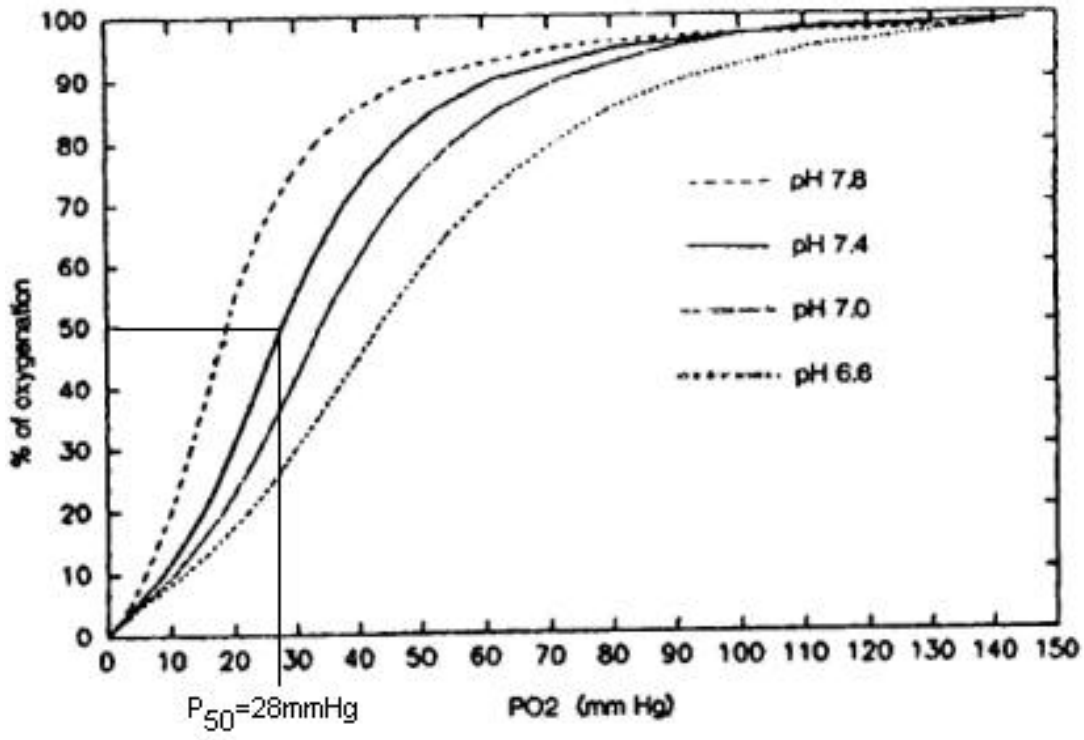


Figure13. Typical oxygen dissociation curve.[53]

3.3.4.2. Determination[54,55,56]

To obtain the P_{50} of any blood carrying solution, like the Poly-PLP-SFHb, oxygen dissociation curves Have to be obtained. The solution is tonometered with gas at

[52] Reference 2 pg 23
[53] Reference 2 pg 24
[54] Reference 9
[55] Reference 10

different oxygen concentrations and standard conditions of pCO_2 = 40 mmHg, T = 37°C and then the pH, pCO_2, pO_2 are measured in a blood gas analyzer, and the values corrected to standard conditions of pH and pCO_2 (pH = 7.4 and pCO_2 = 40 mmHg).

3.3.5. Hill coefficient

3.3.5.1. Definition

"Oxygen binds cooperatively to hemoglobin because the binding of oxygen to a hemoglobin molecule enhances the binding of further oxygen to the same molecule. The reason for this is that when oxygen binds to a subunit, there is a structural change within this subunit. Thus, there is communication between the heme groups of a hemoglobin molecule." [57]

"The quantitative measurement of cooperativity is the Hill coefficient n. The Hill coefficient increases with an increase in cooperativity. The maximum number of n is the number of binding sites for oxygen in a hemoglobin molecule, total of 4. A Hill coefficient of 1 means there is no cooperativity. For hemoglobin, the Hill coefficient is is normally 2.8."[57]

[56] Reference 5
[57] Reference 2 pg 25

3.3.5.2. Determination[58]

The Hill coefficient is determined from the dissociation curve at standard conditions of pH = 7,4 pCO_2 = 40 mmHg and T = 37°C, with the Hill equation:

$$\log\left(\frac{\%sat}{100-\%sat}\right) = n\log\ pO_2 - n\log\ P_{50}$$

Where %sat is the percentage of oxygen saturation at a fixed pO_2 pressure.

Various points from either side of the P_{50} point on the dissociation curve, are fitted to the Hill equation by linear regression. The slope of this line represents the Hill coefficient.

3.3.6. pH

3.3.6.1. Definition

The pH indicates the concentration of hydrogen ions in a dissolution, so it measures the acidity o the dissolution. It is defines as:

[58] Reference 9

$$pH = -\log\left[H^{+}\right]$$

Where [H+] is the concentration of hydrogen in moles/L[59]. The pH of hemoglobin lies between 7,35 and 7,45[60].

3.3.6.2. Determination

The determination is normally done with the help of a pH-meter which does the pH measurement based on the conductivity of the dissolution.

3.3.7. Bohr coefficient

3.3.7.1. Definition

“The affinity of hemoglobin for oxygen is modified by pH and carbon dioxide. Under physiological conditions, lowering the pH increases P_{50} and the oxygen dissociation curve is shifted to the right (see figure 4). Increasing carbon dioxide without changing pH, also increases P_{50} and shifts the oxygen dissociation curve to the right. This property of right shifting of hemoglobin due to lower pH or increasing

[59]"pH", *Enciclopedia Microsoft® Encarta® 98* © 1993-1997 Microsoft Corporation.

carbon dioxide is called the Bohr effect. This has important physiological significance. Active tissue such as contracting muscle, release more carbon dioxide and acid, so that the Bohr effect allows hemoglobin to unload more oxygen required by the active tissue." [61]

3.3.7.2. Determination[62]

The Bohr coefficient is determined by adjusting the pH of a tonometered sample (pCO_2 = 40 mmHg, T = 37°C) with an acid like lactic acid or chlorhydric acid, or a base such as sodium hydroxide, and then obtaining an oxygen dissociation curve as explained earlier at a range of pH from 6 to 8. A plot of log P_{50} vs. pH is made and the slope of the curve represents the Bohr coefficient.

3.3.8. Viscosity

3.3.8.1. Definition[63]

[60] Reference 12 pg 76
[61] Reference 2 pg 25
[62] Reference 9

[63] "Viscosidad", *Enciclopedia Microsoft® Encarta® 98* © 1993-1997 Microsoft Corporation.

The viscosity is the property of a fluid to oppose to its flux when a force is applied to it. The force which with a layer of the fluid in movement drags with it adjacent layers determines the viscosity of the fluid.

Based on the molecular theory, when a fluid starts to move under the influence of gravity, the molecules of the stationary layers have to cross a frontier or limit to enter the flux region. Once this limit is crossed, this molecules receive energy from the molecules that are under movement and start to move. At the same time, the molecules that are in the layer that is moving transmit an impulse to the stationary molecules. The global result is that fluid reduces its velocity, and the stationary fluid starts to move, and so the layers in movement acquire a mean velocity.

For keeping a layer of fluid in movement at a greater speed than another layer, its necessary to apply a continuous force. The viscosity in poises is defined as the magnitude of the force necessary to maintain in equilibrium a velocity difference of 1 cm/s between layers separated by 1 cm. The viscosity is measure with the help of an equipment called viscometer.

4. RESULTS

4.1. Pyridoxilation of stroma free hemoglobin

The process was carried out at 4°C, with the help of a small refrigerator. The solutions used during the process were kept the most time possible in the

refrigerator, and magnetic agitator was placed inside the refrigerator for continuous stirring and the deoxigenation was possible with the use of a latex hose that took the nitrogen from the gas tank to inside the refrigerator. As shown in figure 14.

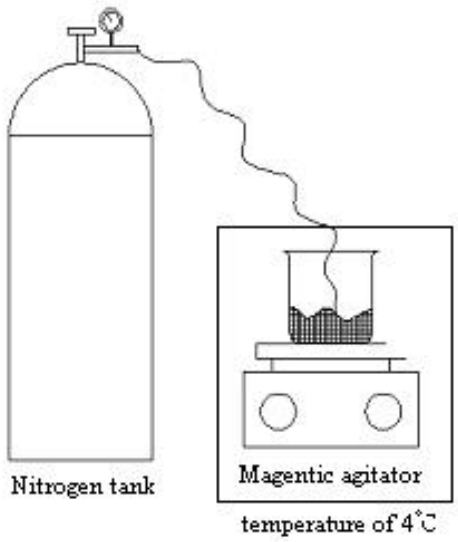


Figure 14. Schematic representation of the pyridoxilation process.

Following the method of preparation described above in the numeral 4.1, 485 ml of stroma free hemoglobin previously prepared as described in the reference 4 were used.

TRIS buffer (Applichem GmbH) was added as powder to obtain a concentration of 0,1 M. As is well known, molarity is defined as:

$$Molarity = \frac{moles}{liter}$$

So for the 485 ml of stroma free hemoglobin, 0,0485 moles of TRIS buffer are needed, the molecular weight of TRIS buffer is 157,6 g/mol with a purity of 99,5 %, so the quantity of TRIS buffer in grams needed is:

$$TRIS[g] = \left(157{,}6\frac{g}{mole}\right)(0{,}0485 moles)\left(\frac{100\%\, purity}{99{,}5\%\, purity}\right) = 7{,}682 g$$

In a balance weight 7,667 g of TRIS were measured, and this was added to the stroma free hemoglobin solution.

Then the pH was measured three times giving a pH with mean of 6,50 and standard deviation of $8{,}54 \times 10^{-2}$, which gives a 95% confidence interval of 6,50 $\pm$ 0,14 calculated with t-student statistical test with two degrees of freedom.

The pH of the solution had to be taken to a normal plasma pH of 7,4 by the addition of NaOH (Merck & Co. Inc.) 2M, 30 ml of this solution were prepared. To obtain a concentration of 2 M, 0,06 moles were needed:

$$moles = (molarity)(volume) = (2M)(0{,}03 liters) = 0{,}06 moles$$

The molecular weight of NaOH is 40 g/mol, so the quantity in grams of NaOH needed is:

$$NaOH[g] = \left(40\frac{g}{mole}\right)(0{,}06 moles) = 2{,}4 g$$

In a balance weight 2,391 g of NaOH were measured in a 100 ml volumetric flask, an taken to the 30 ml mark. This NaOH solution will be used throughout the pyridoxilation process, polymerization process and in obtaining the oxygen dissociation curves at different pH, that will be explained later.

With the help of a pipette, volumes of 0,5 ml were added until the pH of the stroma free hemoglobin solution was around 7,4. A total of 4,5 ml were used. The pH was measured three times giving a pH with mean of 7,42 and a standard deviation of $4{,}51 \times 10^{-2}$, which gives a 95% confidence interval of 7,42 $\pm$ 0,08 calculated with t-student, with two degrees of freedom.

The hemoglobin concentration was measured in a hemoglobin concentration meter (B-Hemoglobin Test System from Hemocue) and a concentration of 10,0 g/dl was the result. This means than in 485 ml of stroma free hemoglobin solution there are

$$Hb_{moles} = \frac{Hb_{grams}}{\left(M.W._{hemoglobin}\right)} = \frac{48{,}5g}{64{,}458g/mol} = 7{,}52x10^{-4} moles$$

48,5 g of hemoglobin, since the molecular weight of hemoglobin is 64,458 g/mol, the total moles of hemoglobin present ca be calculated as follows:

Pyridoxal 5' phosphate monohydrate (Applichem GmbH) has to be added to the stroma free hemoglobin solution in a molar rate of 4:1 to hemoglobin. So the moles

of pyridoxal 5' phosphate monohydrate needed are four times the moles of hemoglobin, this is $3x10^{-3}$ moles. The molecular weight of pyridoxal 5' phosphate is 265,16 g/mol with a purity of 98%, so the quantity of pyridoxal 5' phosphate monohydrate needed was:

$$pyridoxal[g] = \left(265{,}16\frac{g}{mole}\right)(0{,}003 moles)\left(\frac{100\%\, purity}{98\%\, purity}\right) = 0{,}814 g$$

In a balance weight, 0,816 g of pyridoxal 5' phosphate were measured and then added to the stroma free hemoglobin solution. The pH is again measured, with a result of a pH with mean of 6,97 with a standard deviation of $3{,}94x10^{-2}$ and a 95% confidence interval of 6,97 $\pm$ 0,07, so again we added NaOH to take the pH to normal conditions. 3 ml were needed to increase the pH from 6,97 to 7,4, giving a mean pH of 7,34 with a standard deviation of $4{,}02x10^{-2}$ and a 95% confidence interval of 7,34 $\pm$ 0,08 done with a t-student statistical test of two degrees of freedom.

The stroma free hemoglobin solution was deoxygenated for two hours with gaseous nitrogen (obtained from AGA FANO S.A.) as shown in figure 14. During the nitrogen bubbling, foam is produced so it has to be continuously shaken, so it would not overflow the recipient in which the stroma free hemoglobin solution is contained.

To accomplish the linkage of pyridoxal 5' phosphate to hemoglobin, $NaBH_4$ (Applichem GmbH) has to be added in a molar rate of 20:1 to hemoglobin, as calculated earlier, the stroma free hemoglobin solution has 7,52x10-4 moles of hemoglobin, so 1,50x10-2 moles of NaBH4 are needed. The molecular weight of NaBH4 is 37,83 g/mol with a purity of 96%. The quantity of NaBH4 needed in grams can be calculated:

$$NaBH_4[g] = \left(37{,}83\frac{g}{mole}\right)\left(1{,}5x10^{-2}\,moles\right)\left(\frac{100\%\,purity}{96\%\,purity}\right) = 0{,}591g$$

In a balance weight 0,602 g were measured and diluted in 3 ml of NaOH 0,001 M, which was prepared adding 1,5 μl of NaOH 2 M in 3 ml of distilled water. This was added in the stroma free hemoglobin solution.

The nitrogen bubbling was continued for 10 more minutes, afterwards, the latex hose was located such that the nitrogen entered in superficial contact with the solution, but did not touch the hemoglobin solution surface as shown in figure 15. This was done for 18 hours.

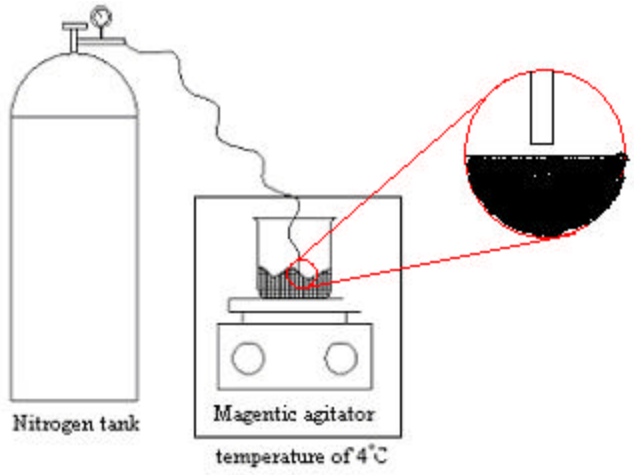


Figure 15. Deoxigenation process with nitrogen done to prevent foaming of the hemoglobin solution. The hose is placed just beneath the hemoglobin surface.

4.2. Ultrafiltration of the pyridoxilated stroma free hemoglobin solution.

After the pyridoxilation process, the hemoglobin concentration was measured three times again with an hemoglobin concentration meter (B-Hemoglobin Test System from Hemocue) giving a mean of 6,4 with a standard deviation of 5,8x10-2 and a 95%confidence interval of 6,4 ± 0,1 done with a t-student statistical test with two degrees of freedom. For the polymerization process, the concentration of the pyridoxilated stroma free hemoglobin solution has to be around 18 g/dl, so ultrafiltration is necessary. This was carried out in an hemodialysis machine Baxter 1550 with a filter cartridge C-110 of Baxter (this was facilitated by RTS Ltda in association with the Fundacion cardio infantil). The machine was bypassed, no dialysis solution was able to enter the process and the venous and arterial lines

were placed in a reservoir, so the pyridoxilated stroma free hemoglobin solution is in a closed cycle, as shown in figure 16.

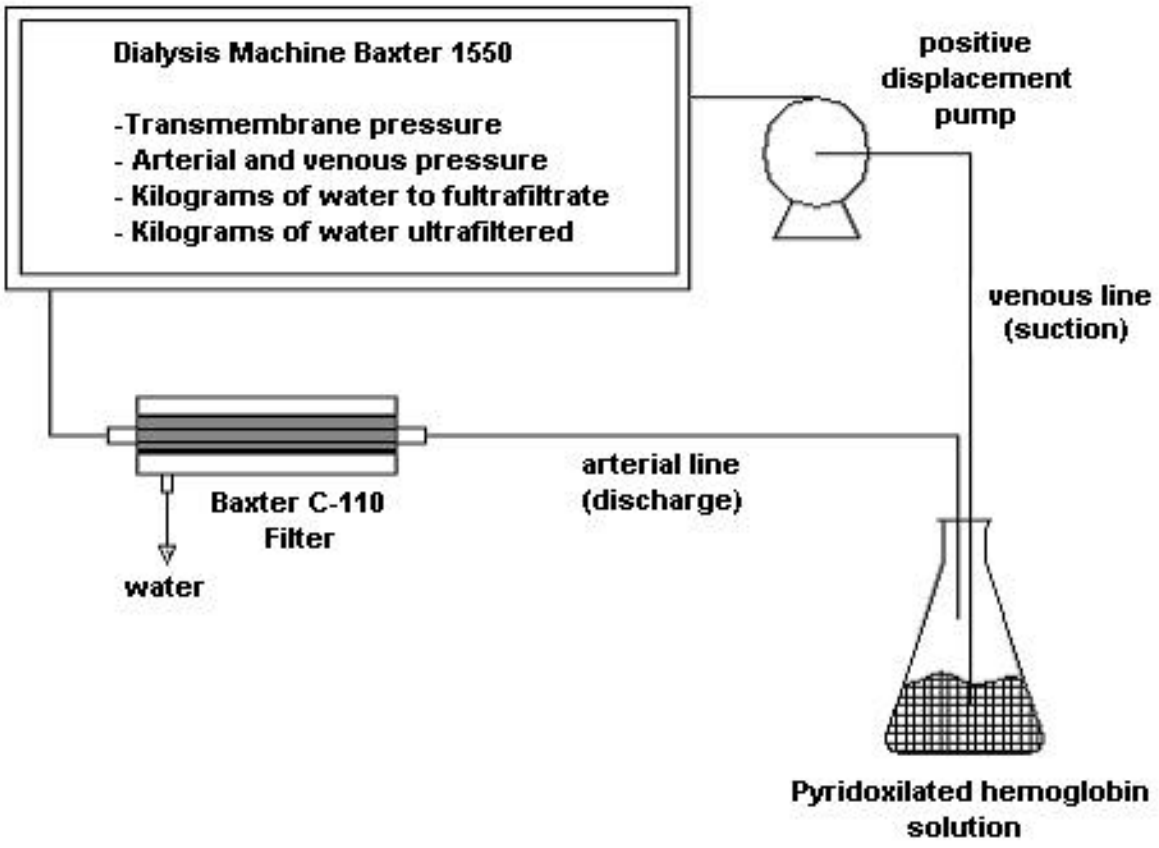


Figure 16. Ultrafiltration process done to the pyridoxilated hemoglobin.

The dialysis machine is programmed to remove the necessary kilograms of water. During this specific ultrafiltration process, the dialysis machine could not control correctly the quantity of water removed, because the little volume that was ultrafiltered, and air bubbles formed in the line. Even though the water to be removed was calculated to take the pyridoxilated stroma free hemoglobin solution

to an hemoglobin concentration of 18 g/dl, the final concentration of the pyridoxilated stroma free hemoglobin solution had a mean of 24,5 g/dl with a standard deviation of 2,08x10-1 and a 95% confidence interval of 24,5 ± 0,35 g/dl done with a t-student statistical test with two degrees of freedom, and a final volume of 105 ml, and it was diluted to 160 ml with the addition of Ringer's lactate solution, and having a final concentration for the polymerization process of 16,0 ± 0,4 g/dl at a 95% confidence interval done with a t-student statistical test with two degrees of freedom.

4.3. Polymerization of pyridoxilated stroma free hemoglobin

The process was carried out at 4°C, with the help of a small refrigerator. The solutions used during the process were kept the maximal time possible in the refrigerator. And during the polymerization process the pyridoxilated stroma free hemoglobin solution was kept inside the refrigerator.

From the ultrafiltration process, the working volume 160 ml of pyridoxilated stroma free hemoglobin solution has a concentration of 16,0 ± 0,4 g/dl at a 95% confidence interval done with a t-student statistical test with two degrees of freedom.

Following the polymerization process described above in the numeral 4.2. First, the L-lysine monohydrochloride (Merck & Co. Inc.) was prepared. For the 160 ml pyridoxilated stroma free hemoglobin solution, a total volume of 32 ml are needed. For each milliliter of lysine solution, 239,84 mg of lysine are needed, to yield a concentration of 1,3 M, so for the 32 ml a total mass of 7,675 g are needed, in a weight balance 7,678 g are measured in a glass. Then the phosphate buffer has to be added, for each milliliter of lysine solution, 87,09 mg of K_2HPO_4 and 68.04 mg of KH_2PO_4 to yield a buffer concentration of 0,5 M are needed. In a weight balance 87,12 mg of K_2HPO_4 and 68,14 mg of KH_2PO_4 are measured in the same glass where the lysine is. Then this glass was taken to 32 ml mark with distilled water. The pH was measured giving a value of 6,37, so 5 ml of NaOH 2 M were added yielding a pH with a mean value of 7,35 with a standard deviation of 0,02 giving a 95% confidence interval of 7,35 $\pm$ 0,034 done with a t-student statistical test with two degrees of freedom.

Then the glutaraldehyde working solution has to be prepared. For the 160 ml of pyridoxilated stroma free hemoglobin solution, 5.3 ml are needed. The glutaraldehyde had to be diluted in phosphate buffer, 461.58 mg of K_2HPO_4 and 360.61 mg of KH_2PO_4 to yield a buffer concentration of 0,5 M, in a weight balance 0,463 g of K2HPO4 and 0,363 mg of KH2PO4 were measured and 3,8 ml of water measured in a pipette were added.1,3 ml of glutaraldehyde 50% were measured in a pipette and added to the buffer solution. The pH was measured giving a mean

value of 7,41 with a standard deviation of 0,02 giving a 95% confidence interval of 7,35 ± 0,034 done with a t-student statistical test with two degrees of freedom.

For the pyridoxilated stroma free hemoglobin solution the pH was measured giving a value of , the pH was adjusted by the addition of 2 ml of NaOH 2 M previously prepared.

The osmolarity of the pyridoxilated stroma free hemoglobin solution was measured three times giving a mean of 727 mOsm/kg in a osmometer (Fiske ONE-TEN Osmometer) with a standard deviation of 8 mOsm/kg, giving a 95% confidence interval of 727 ± 13 mOsm/kg.

The pyridoxilated stroma free hemoglobin solution was placed on the agitator at a speed of 200 rpm. Some of the lysine solution has to be added as a regulator, to achieve a hemoglobin lysine molar ratio of 2:13, 2 ml of the 32 ml lysine solution were supposed to be added. But because the solution was corrected with 5 ml of NaOH to give the appropriate concentration, then 2,3 ml were measured in a pipette and added to the pyridoxilated stroma free hemoglobin solution.

Then the glutaraldehyde working solution was added. 0,5 ml of this solution were added each hour, and a sample taken to measure osmolarity. The polymerization process was stopped after 14 hours when the osmolarity of the know polymerized

pyridoxilated stroma free hemoglobin solution was 312 mOsm/kg. To quench the reaction, all of the lysine solution was poured and the polymerized pyridoxilated stroma free hemoglobin solution was ultrafiltered the same way as described in the numeral 5.2 to eliminate excess reactants. In table 3, the decrease in osmolarity is shown and in figure 17 a graph of osmolarity vs. time is shown.

Table 3. Measure of osmolarity during the polymerization process

Time [h]	Osmolarity [mOsm/kg]
0	727
1	712
2	704
3	691
4	662
5	647
6	616
7	587
8	553
9	497
10	472
11	395
12	352
13	338
14	312

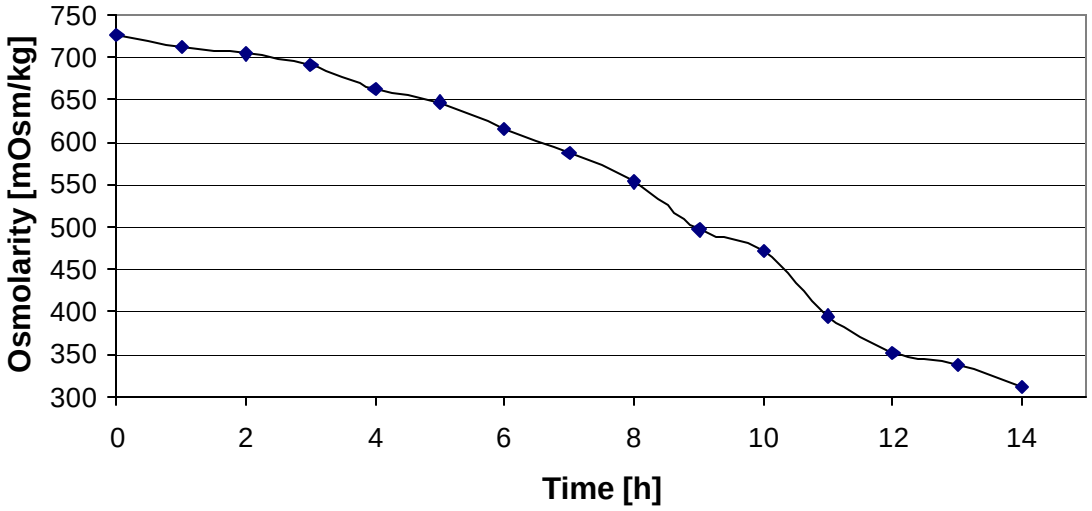


Figure 17. Osmolarity behavior during polymerization of pyridoxilated stroma free hemoglobin solution vs. time after addition.

After the ultrafiltration process was done, the hemoglobin concentration was measured giving a mean concentration of 22 g/dl with a standard deviation of 0,4 giving a 95% confidence interval of 22 ± 0,7 g/dl done with a t-student statistical test with two degrees of freedom, with a final volume of 99 ml. This was diluted to a concentration of 14,5 g/dl of hemoglobin adding 51 ml of Ringer's lactate solution.

4.4. Dyalisis of the polymerized pyridoxilated stroma free hemoglobin

The final process done to the polymerized pyridoxilated stroma free hemoglobin is dialysis against Ringer's lactate. This process removes free glutaraldehyde and lysine excess, that did not react during the polymerization process and its removal

is very important. This also allows the electrolytes to be adjusted to normal physiological concentrations.

The process is carried out at 4°C, and the dialysis solution is cold Ringer's lactate (Baxter Laboratories). The total volume of polymerized pyridoxilated stroma free hemoglobin is 150 ml so the dialysis process is divided into 8 partial dialysis of 20 ml each. The organic acetate cellulose membrane (Fisher Scientific) is prepared so all the traces of sulphur and heavy metals are removed, as described in the "Sensitive Assay Membrane Pre-treatment" found in the membrane's instruction bulletin. For each 20 ml of polymerized pyridoxilated stroma free hemoglobin solution, 16 cm of the membrane are used, a length that also permits the membrane to be clamped, and 2 liters of Ringer's lactate are used. The process is carried out for at least three hours, when equilibration occurs. At the end of the dialysis process, the polymerized stroma free hemoglobin solution had a concentration of 14,5 g/dl, and a pH with a mean of 7,166 and a standard deviation of 0,224, giving a confidence interval of 7,166 ± 0,184 and a final volume of 150 ml. The hemoglobin concentration and the ph were measured in 6 samples by the blood gas analyzer (Blood Gas Analyzer BMG 1312 Instrumentation Laboratory).

4.5. Evaluation of the physicochemical properties of modified hemoglobin

4.5.1. Oxygen dissociation curves

For the evaluation of the P_{50}, the Hill coefficient and the Bohr coefficient, oxygen dissociation curves have to be constructed. This curves were described earlier in numeral 4.3.4.1.

4.5.1.1. Equipment and reactive

- Magnetic agitator with is agitation bar and temperature controlled option.
- Gas blood analyzer.
- Syringes.
- Calibrated gas tanks with pCO_2 = 40 mmHg, and pO_2 ranging from 0 mmHg to 100 mmHg balanced in nitrogen.
- NaOH solution 2M.
- HCl 2 M.

4.5.1.2. Solutions used

The previously prepared solutions of NaOH 2 M and HCl 2 M will be used in this procedure to change the pH of the polymerized pyridoxilated stroma free hemoglobin solution to desired values, for the determination of the Bohr coefficient, the P_{50} and the Hill coefficient.

4.5.1.3. Calibrated gases

The calibrated gases that were used were supplied by Oxigenos de Colombia S.A. A total of six tanks of known oxygen composition and standard pCO_2 = 40 mmHg were acquired, with pO_2 ranging from 10 mmHg to 30 mmHg and balanced in nitrogen. Table 4 resumes the information of the composition requested and the composition delivered in 1 m^3 tanks.

Table 4. Calibration gases balanced in nitrogen

Tank	OXYGEN				CARBON DIOXIDE			
	Percentage		Pressure [mmHg]		Percentage		Pressure [mmHg]	
	Theoretic	Real	Theoretic	Real	Theoretic	Real	Theoretic	Real
1	1.32	1.35	10.03	10.26	5.26	5.30	40	40.28
2	1.77	1.85	13.45	14.06	5.26	5.18	40	39.37
3	2.24	2.25	17.02	17.10	5.26	5.34	40	40.58
4	2.70	2.77	20.52	21.05	5.26	5.30	40	40.28
5	3.16	3.21	24.02	24.40	5.26	5.27	40	40.05
6	3.68	3.68	27.97	27.97	5.26	5.25	40	39.90

4.5.1.4. Procedure for obtaining the oxygen dissociation curves.

Since for the determination of the Bohr coefficient, different pH's of the polymerized pyridoxilated stroma free hemoglobin solution have to be used, three different pH were prepared. The pH of the polymerized pyridoxilated stroma free hemoglobin solution after the dialysis process has a mean of 7,166 with a standard deviation of 0,2044, giving a 95% confidence interval of 7,166 $\pm$ 0,1502. Other two samples of

pH with mean of 6.961 standard deviation of 0,1016 giving a 95% confidence interval of 6,961 ± 0,0746 and mean of 6,824 standard deviation of 0,1378 giving a 95% confidence interval of 6,824 ± 0,1012 were prepared adding HCl 2 M.

Then, each of the three solutions were placed in the magnetic agitator. The temperature control of the agitator was placed in 50°C and the temperature checked constantly with a mercury thermometer to maintain a temperature near 37°C.

Each of the three polymerized pyridoxilated stroma free hemoglobin solutions was tonometered with the 6 different gas tanks for 10 minutes, starting with the tank which had less oxygen concentration and changing the tank until the last and highest oxygen concentration was bubbled. After the 10 minutes of bubbling passed, with a syringe, a bottom sample of each of the three polymerized pyridoxilated stroma free hemoglobin solutions was taken, and immediately a cap was placed on the mouth of the syringe to prevent direct contact with air, because the oxygen concentration of air is much higher than the oxygen concentration of any of the six tanks and oxygen diffusion in hemoglobin is very high, so it could gain oxygen from air modifying the known oxygen concentration and the results of oxygen saturation would be incorrect.

The pO_2, pCO_2, pH and Hemoglobin concentration of each sample was measured with the help of a blood gas analyzer (Blood Gas Analyzer BMG 1312 from Instrumentation Laboratory).

The pCO_2 measured was different from the standard condition of pCO_2 = 40 mmHg, so each sample had to be normalized. The normalization procedure was made as follows: if the blood gas analyzer pCO_2 result was of 32 mmHg and the pO_2 result was 45 mmHg, they were multiplied by a correction factor defined as:

$$C.F. = \frac{40 mmHg}{pCO_{2,measured}}$$

For this example a correction factor of 1,25 is used, so the normalized pCO_2, and pO_2 would be:

$$pCO_{2,normalized} = 40$$
$$pO_{2,normalized} = 56.25$$

To determine standard deviation of the population, for the first measured sample, three repetitions were made, giving a normalized standard deviation for the pO_2 of 1,42 this is shown in table 5.

Table 5[64]. Determination of the standard deviation (all pressures in mmHg**)**

[64] See Appendix III

Repetition	pCO_2	$pO_{2,hemoglobin}$	Normalization Factor	pCO_2	$pO_{2,hemoglobin}$
1	34.9	25	1.15	40	28.65
2	36.7	24	1.09	40	26.16
3	35	25	1.14	40	28.57
			Standard deviation		1.42

So for each measure, a 95% confidence interval in the pO_2 is ± 2,4

To determine the percentage of oxygen saturation in hemoglobin, it was assumed that at a pO_2 of 108 mmHg the saturation level of hemoglobin was 100%[65]. So to determine the percentage of saturation, the following equation was used:

$$\%Saturation_{Hemoglobin} = \frac{pO_{2,hemoglobin}}{108mmHg} x100$$

All the calculations previously describe are resumed in table 6, table 7 and table 8.

Table 6[66]. Hemoglobin solution with pH = 7.166 (all pressures are in mmHg)

					Normalization			
Sample	**$pO_{2,gas}$**	**pH**	**pCO_2**	**$pO_{2,hemoglobin}$**	**Normalization Factor**	**pCO_2**	**$pO_{2,hemoglobin}$**	**% sat O_2**
Tank 1	10.26	6.583	30.4	20 ± 2.4	1.32	40	26.32 ± 2.4	24.37 ± 2.2
Tank 2	14.06	6.805	60.2	59 ± 2.4	0.66	40	39.20 ± 2.4	36.30 ± 2.2
Tank 3	17.1	7.123	51.4	64 ± 2.4	0.78	40	49.81 ± 2.4	46.12 ± 2.2
Tank 4	21.052	7.684	54.1	79 ± 2.4	0.74	40	58.41 ± 2.4	54.08 ± 2.2
Tank 5	24.396	7.677	51.8	90 ± 2.4	0.77	40	69.50 ± 2.4	64.35 ± 2.2
Tank 6	27.968	7.125	42	83 ± 2.4	0.95	40	79.05 ± 2.4	73.19 ± 2.2

[65] Reference 12

Table 7[67]. Hemoglobin solution with pH = 6.961 (all pressures are in mmHg)

					Normalization			
Sample	**$pO_{2,gas}$**	**pH**	**pCO_2**	**$pO_{2,hemoglobin}$**	**Normalization Factor**	**pCO_2**	**$pO_{2,hemoglobin}$**	**% sat O_2**
Tank 1	10.26	6.886	35.53	22 ± 2.4	1.13	40	24.77 ± 2.4	22.93 ± 2.2
Tank 2	14.06	7.115	53	41 ± 2.4	0.75	40	30.94 ± 2.4	28.65 ± 2.2
Tank 3	17.1	6.816	54.7	52 ± 2.4	0.73	40	38.03 ± 2.4	35.21 ± 2.2
Tank 4	21.052	6.786	66.2	78 ± 2.4	0.60	40	47.13 ± 2.4	43.64 ± 2.2
Tank 5	24.396	7.344	67.2	98 ± 2.4	0.60	40	58.33 ± 2.4	54.01 ± 2.2
Tank 6	27.968	6.820	46.1	81 ± 2.4	0.87	40	70.28 ± 2.4	65.08 ± 2.2

Table 8[68]. Hemoglobin solution with pH = 6.824 (all pressures are in mmHg)

					Normalization			
Sample	**$pO_{2,gas}$**	**pH**	**pCO_2**	**$pO_{2,hemoglobin}$**	**Normalization Factor**	**pCO_2**	**$pO_{2,hemoglobin}$**	**% sat O_2**
Tank 1	10.26	7.114	50.6	28 ± 2.4	0.79	40	22.13 ± 2.4	20.49 ± 2.2
Tank 2	14.06	6.548	47.1	32 ± 2.4	0.85	40	27.18 ± 2.4	25.16 ± 2.2
Tank 3	17.1	6.548	47.9	37 ± 2.4	0.84	40	30.90 ± 2.4	28.61 ± 2.2
Tank 4	21.052	6.550	55.3	54 ± 2.4	0.72	40	39.06 ± 2.4	36.17 ± 2.2
Tank 5	24.396	7.093	58.3	72 ± 2.4	0.69	40	49.40 ± 2.4	45.74 ± 2.2
Tank 6	27.968	7.092	63.9	90 ± 2.4	0.63	40	56.34 ± 2.4	52.16 ± 2.2

From this tables, the oxygen dissociation curves for the three pH was plotted, see figure 18.

[66] See Appendix IV
[67] See Appendix V
[68] See Appendix VI

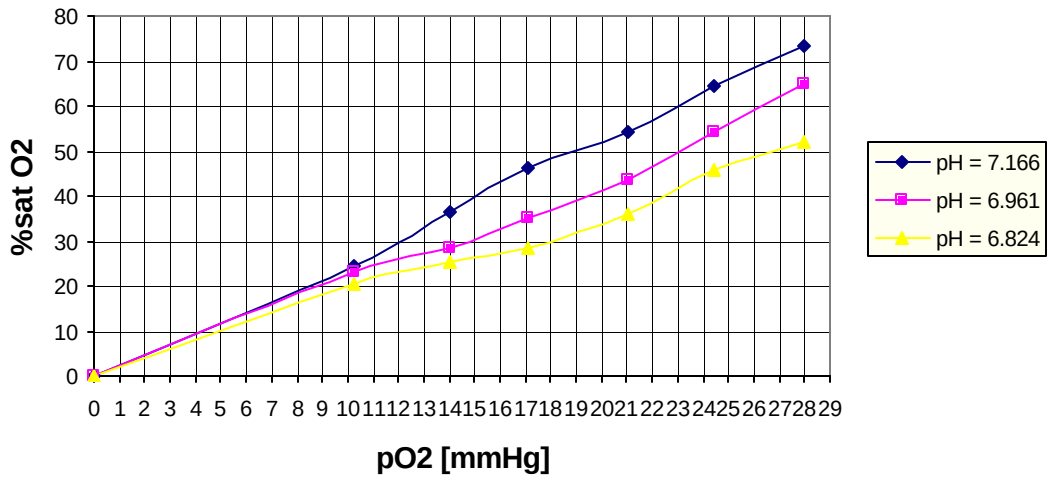


Figure 18. Oxygen dissociation curve of the polymerized pyridoxilated stroma free hemoglobin at different pH's.

4.5.2. Bohr Coefficient

The Bohr coefficient was determined by the adjustment in pH made to the three polymerized pyridoxilated hemoglobin solutions that were tonometered with the calibrated gas tanks. The P_{50} for each pH curve was determined from the graph of the oxygen dissociation curve (figure 18.) tracing a line at the 50% saturation point and finding the correspondent value of pO_2 when intersecting the pH graphs, this point represented the P_{50} for each pH curve. See table 9.

Table 9. P_{50} for the different pH oxygen dissociation curves for the determination of the Bohr coefficient.

pH	P_{50}	log P_{50}
7.166 ± 0.1502	19 ± 2.2	1.28

6.961 ± 0.0746	23.2 ± 2.2	1.37
6.824 ± 0.1012	26.7 ± 2.2	1.43

Then a plot of log P_{50} vs. pH was made and the slope of the curve ($\Delta \log P_{50}/\Delta pH$) represented the Bohr coefficient[69], as seen in figure 19.

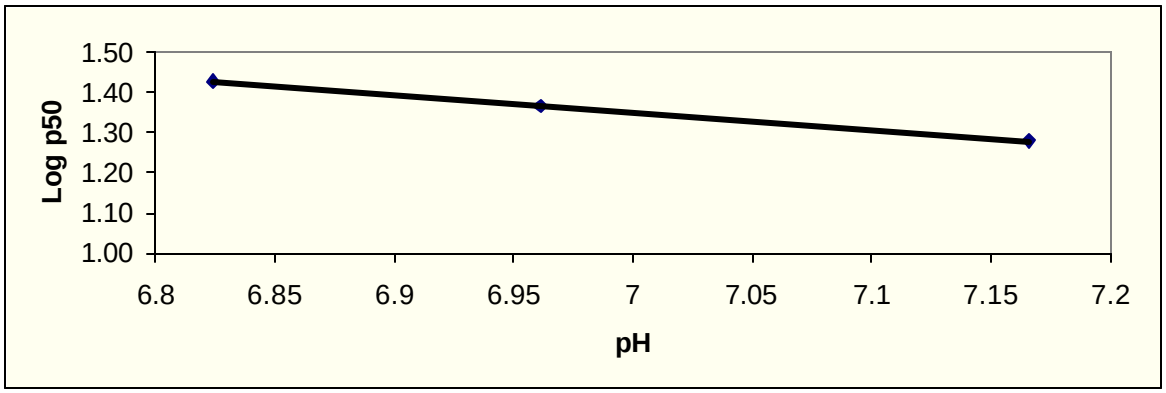


Figure 19. Determination of the Bohr effect in the polymerized pyridoxilated stroma free hemoglobin

A linear regression was made and the resulting equation was:

$$Log P_{50} = -0.4313\, pH + 4.3692$$
$$R^2 = 0.9998$$

To calculate the confidence interval of the Bohr coefficient, the definition of the slope in a linear regression has to be known:

[69] Reference 9

$$slope = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

$$y - \text{int} = \bar{Y} - b\,\bar{X}$$

Where for this case, x represents pH, y represents Log P_{50} and n represents the number of data fitted.

Remembering the propagation of error, when two different values are being multiplied or divided, the total error is defined as:

$$\%error_{total} = \sqrt[2]{\%error_{value1}^2 + \%error_{value2}^2}$$

And the % error is defined as:

$$\%error = \frac{(error)x100}{value}$$

And when the values are being summed or reduced, the total error is simply the square root of sum of the square of the errors.

In table 10 the error and percentage error for each value can be seen.

Table 10. Error and percentage error for different values for the

determination of the Bohr coefficient.

pH	error	%error	P_{50}	error	%error	log P_{50}	error	%error
7.166	0.1502	2.096	19	2.2	11.579	1.28	0.14806621	11.5789474
6.961	0.0746	1.0717	23.2	2.2	9.4828	1.37	0.12948593	9.48275862
6.824	0.1012	1.483	26.7	2.2	8.2397	1.43	0.11754025	8.23970037

Finally, the total error for the Bohr coefficient can be calculated and the 95% confidence interval for the Bohr coefficient is [-0.40,-0.47].

For the calculation of the y-intersect, Y = 1.36 and X = 6.984 so the interval in which the y intersect lies will be [4.1535, 4.6423]

4.5.3. Hill coefficient

The Hill coefficient is calculated from the dissociation curve of pH =7,166 that was the pH that resulted after the dialysis process of the polymerized pyridoxilated stroma free hemoglobin solution, with the help of the Hill equation:

$$\log\left(\frac{\%sat}{100-\%sat}\right) = n\log\ pO_2 - n\log\ P_{50}$$

First, the P_{50} was calculated as described in numeral 4.5.2, giving a value of 19 $\pm$ 2.2 mmHg. Then, one point in either side of the P_{50} on the dissociation curve were fitted by linear regression to the Hill equation, the slope of this line represents the Hill coefficient. See table 11.

Table 11. Determination of the Hill coefficient

pO_2	% sat	log (s/1-s)	log pO_2	log P_{50}	n
17.1	46.12	-0.07	1.23	1.28	1.48
21.052	54.08	0.07	1.32	1.28	1.60

The Hill coefficient has a mean value of 1,54 with a standard deviation of 0,0848, giving a 95% confidence interval 1,54 ± 0,379 done with the t-student statistical test with one degree of freedom.

4.5.4. pH

The pH was measured to six samples of the polymerized pyridoxilated stroma free hemoglobin, as mentioned earlier by the gas blood analyzer (Blood Gas Analyzer BMG 1312 Instrumentation Laboratory), giving a pH with mean of 7,166 and a standard deviation of 0,224, giving a confidence interval of 7,166 ± 0,184.

The pH of plasma lies in the 99% confidence interval of 7,40 ± 0,05[70]. Although the pH of the polymerized pyridoxilated stroma free hemoglobin solution is below, and out of this range, the pH for physiological usage is

[70] Reference 12 pg 76.

4.5.5. P_{50}

The P_{50} of the polymerized pyridoxilated stroma free hemoglobin solution was calculated extrapoling with aid of the Bohr coefficient to a physiological pH of 7,4. To obtain a maximum and a minimum P_{50}, the extrapolation was done with the maximum and minimum values of both the Bohr coefficient and the y-intersect of the linear regression equation, which with the Bohr coefficient was determined. For the maximum value of P_{50} the equation used is the maximal limit of the Bohr coefficient, the same is done with the minimal limit of the Bohr coefficient, this gives:

$$\log P_{50} = (Bohr)(pH) + y - \text{int}$$
$$P_{50} = 10^{(-0,4)(7,4)+4,1535} = 15,6 mmHg$$
$$P_{50} = 10^{(-0,47)(7,4)+4,6423} = 16,6 mmHg$$

4.5.6. Viscosity

The viscosity was measured in a viscometer (Brookfield Digital Viscometer model DV-II) with a number 6 disc and at a constant velocity of 100 rpm and at 25°. First, water was tested to calculate the correction factor to be applied. The water measurements gave a viscosity of 11,6 cp with a standard deviation of 0,2 and a 95% confidence interval of 11,6 ± 0,3 cp at 25°C. At this temperature the viscosity

of water is found to be 0,9 cp[71]. The correction factor for this viscosity measurement is 0,077 ± 0,002. The results of the four test that were made are found in table 12.

Table 12. Viscosity of Poly-PLP-SFHb @ 25°C

viscocity [cp]	normalized [cp]
70	5.43
60	4.66
60	4.66
70	5.43

This gives a viscosity with mean value of 5,05 and a standard deviation of 0,444, which gives a 95% confidence interval of 5,05 ± 0,523 cp done with a t-student statistical test of 3 degrees of freedom.

[71] www.pump.net

5. DISCUSSION

5.1. Evaluation of the physicochemical properties of modified hemoglobin

5.1.1. comparison with other authors

The comparison of the results was made with three different authors, Chang[72], Sehgal[73], and Alza Corporation[74]. The results of this three authors are shown in table 13.

Table 13. Result of the characteristics of Poly-PLP-SFHb of different authors

Parameter	author			Present work
	Chang	Sehgal	Alza Corporation	
Osmotic pressure [mOsm/kg]	312	280-310	300	357 ± 13
Hill coefficient	1.88	1.5-2		1.16-1.92
Bohr coefficient		-0.33 and -0.2		-0.40 and -0.47
P_{50} [mmHg]	15-18	14-16	22	15.6-16.6
pH	7.3-7.4	7.35-7.45	7.1	6.98-7.35
viscosity [cp]		4.5		4.53-5.57
density [gr/cm^3]				0.9-1.3

5.1.1.1. Osmotic Pressure

The final result of the osmotic pressure of the polymerized pyridoxilated stroma free hemoglobin solution after the dialysis process is 357 ± 13 mOms/kg. Compared with the other three authors, the final osmotic pressure is higher but not critical, concentrated blood units of red blood cells are around this value. It can be lowered if the polymerization reaction is allowed more time, although the degree of

[72] Reference 2
[73] Reference 9
[74] Reference 7

polymerization affects the P_{50}, the Hill coefficient and the Bohr effect[75], lowering the three parameters. It has to be decided what has less significance in physiological conditions, when this modified hemoglobin is infused, either to have a high osmotic effect or to level the osmotic pressure to the isoosmotic level and have a diminish in the levels of P_{50}, Bohr effect and Hill coefficient.

5.1.1.2. Hill coefficient

The final result of the measurement of hemoglobin cooperativity is the Hill coefficient. The result after polymerization of pyridoxilated stroma free hemoglobin of the Hill coefficient lies in the [1.16-1.92] interval. Following the method in reference 14, the ttest comparison of sample means, the comparison value is t and the degrees of freedom are:

[75] Reference 9

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_1^2}{n_1}}}$$

$$n = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_{12}^2}{n_2}\right)^2}{\frac{\left(\frac{s_1^2}{n_1}\right)^2}{n_1 - 1} + \frac{\left(\frac{s_2^2}{n_2}\right)^2}{n_1 - 1}}$$

Doing this calculations, and having means of 1,54 and 1,75, standard deviations of 0,0848 and 0,807 and having 3 datum and 30 datum respectively, the comparison value t = -1.2882 is obtained, with the degrees of freedom being $\nu = 1.369 \approx 2$, and the t value by tables of 4,303, so we conclude that there is not a significant difference between the two means at a 95% confidence interval. This result is comparable with both authors Chang and Sehgal. Compared with normal plasma, a big diminish in the Hill coefficient is shown when polymerization is done to pyridoxilated stroma free hemoglobin, since the value of the Hill coefficient of normal plasma is around 2,5[76], this is reflected in the loss of the sigmoidal shape in the oxygen dissociation curves and a diminish of slope increase near the P_{50}, if seen in figure 18. The oxygen dissociation curves in the range of 0 mmHg to 30 mmHg is almost linear not with a sigmoidal start, and there is no significant change

[76] Reference 2 pg 38.

in the slope near the P_{50}. This may be caused by the crosslinking effect, although is has been not well established[77].

5.1.1.3. Bohr effect

The final result of the Bohr effect after the polymerization of the pyridoxilated stroma free hemoglobin solution lies in the interval of [-0.40, -0.47]. Compared with the results of Sehgal, the Bohr coefficient is much higher. There are two possible explanations for this result: the first is that, as explained in numeral 5.1.1.1. The degree of polymerization changes the Bohr effect, because the terminal amino groups, histidine and cysteine, of hemoglobin are involved in controlling the Bohr effect, and they are also the ones that react with glutaraldehyde suffering modifications and therefore lowering the Bohr effect[78], since the degree of polymerization seems to be lower than the results of Sehgal, as shown by the higher osmotic pressure, this may show that less histidines and cysteines were affected by the polymerization process and therefore a higher Bohr effect is reflected in the final polymerized pyridoxilated stroma free hemoglobin solution. The second possible reason is that the three points of the three pH's tonometered are not significant, and more oxygen dissociation curves have to be constructed for more pH's varying from 6 to 8 and the range of pH's measured from 6.8 to 7.12 is not significant to reflect in the Bohr effect. Unfortunately not as much tests as the

[77] Reference 9

desired ones where carried out for different pH's and a wider range. For this reason more tests have to be carried out, and prove which of this two hypothesis is the right one.

5.1.1.4. P_{50}

The pyridoxilation process is responsible for elevating the P_{50} to better level much nearer the levels of normal plasma (28 mmHg). During the pyridoxilation process, no tests were carried out to monitor the reaction. Therefore, not until the end, when the oxygen dissociation curves were constructed, information about the pyridoxilation process was known. P_{50} is an indirect measurement of the pyridoxilation process, and tells if it was carried out. Other direct methods are known, as electrophoresis in acetate cellulose gels. Unfortunately no possibilities were present to carry out these tests. Although when compared with the three authors, the P50 is lies in the interval of 15-18 mmHg obtained by Chang. The result of the oxygen dissociation curves show a P50 in the 15.6-16.6 interval. This shows that the pyridoxilation process was carried out satisfactorily, with levels of pyridoxilation higher than those obtained by Sehgal.

[78] Reference 9

5.1.1.5. pH

The final pH result was of 7.166. Even though the final pH level is under normal plasma levels of 7.35-7.45, this represents no inconvenient because is can be changed by the addition of an acid or a base as explained earlier.

5.1.1.6. Viscosity

The final viscosity result had an interval of 4.53-5.57 cp, which lies in the interval obtained by Sehgal of 4.23-5.87 cp. Although the value obtained is comparable with that of whole blood[79], concerns about the effect on cardiac output have been raised. However, the viscosity is dependent on the size of the macromolecule obtained in the polymerization reaction, and future manipulations will be directed towards small polymer sizes and controllable viscosity.

5.1.1.7. Density

The density tests gave a result of 0.9-1.3 gr/cm3, the normal plasma density lies around 1.6 g/cm3, even though the final result is lower, the reason for this is that in the polymerized pyridoxilated stroma free hemoglobin solution, there is no presence of other proteins like albumin, which help to increase the total density,

this should not cause or affect any physiological conditions, as saline solution or Ringer's lactate do.

5.1.2. Cost comparison study

The following table describes all the reactives used and their costs, not including the costs of the different equipments used.

Table 14. Price of the reactives used in the production of one unit of Poly-PLP-SFHb

Reactive	Presentation	Price in dollars	Quantity used	price in dollars
NaOH	12 kg	$101.40	2.4 g	$0.02
TRIS HCl	5 kg	$418.95	43.72 g	$3.66
Pyridoxal 5' phosphate	25 g	$159.05	1.26 g	$8.01
HCl	18 L	$34.00	20 ml	$0.04
NaBH4	500 g	$98.45	0.917 g	$0.18
Nitrogen	6.5 m^3	$30.00	1.5 m^3	$6.92
Lysine monohydrochloride	5 Kg	$183.95	30.95 g	$1.14
glutaraldehyde	3L	$97.90	8 ml	$0.26
SFHb	1 unit	$19.00	1 unit	$19.00
Glutathione	25 g	$76.20	1.75 g	$5.33
Glucose	5 kg	$73.10	1 g	$0.01
Ascorbic Acid	2 kg	$110.20	0.25 g	$0.02

[79] Reference 9

Ringer's lactate	unit	$1.00	50 units	$50.00
			TOTAL	**$94.59**

As mentioned earlier, the final cost after all the screening test done to donated blood are made, the cost of a blood unit is around $250 dollars, compared with $95 dollars of polymerized pyridoxilated stroma free hemoglobin solutions. For a production plant of this type, around 50% of the production costs are the reactives used. This means that a donated blood unit costs more than $60 dollars over a unit of polymerized pyridoxilated stroma free hemoglobin solution, not saying that the reactives used are of biological grade and not A.C.S. grade, and that the quantities bought are not significant for a price reduction by the chemical fabricant.

6. CONCLUSIONS AND COMMENTS

An ideal intravenous oxygen carrier should have a normal oxygen carrying capacity. The pyridoxilation and polymerization processes carried out in this work normalizes the hemoglobin total concentration while maintaining oxygen capacity and osmolarity comparable to whole blood. Making this intravenous oxygen carrier a great substitute of donated blood.

Although the pyridoxilation reaction was carried out satisfactorily as seen by the P_{50} comparable with the results obtained by Sehgal and Chang , there was no direct method for controlling and determining the degree of pyridoxilation achieved after the 12 hours of deoxygenation with nitrogen. There is a direct method for determining the degree of pyridoxilation achieved by the process, the pyridoxilation reaction is determined by electrophoresis on cellulose acetate strips in EDTA-Tris-

borate buffer, at a pH = 8. The pyridoxilated fraction migrates at a faster rate[80]. It is recommended to acquire the technology to determine the pyridoxilation reaction kinetics, and have a better control over the pyridoxilation process, so optimization in costs and time process can be done. Normally the pyridoxilation rate is around 70%. The two fractions can be separated by batch processing in DEAE-Sephadex at different pHs, this could be a good approach in the aim of obtaining the maximal pyridoxilation rate[81], and therefore increasing the P_{50} of the polymerized pyridoxilated stroma free hemoglobin.

Although the P_{50} compared with blood is low, around 16 mmHg against 28 mmHg, it has been demonstrated that such a P_{50} permits a correct unloading of oxygen to the tissues in the absence of erythrocytes. Elevating the pyridoxilated fraction by batch purification may go against the Bohr effect as explained earlier, this demonstrates that the unloading of oxygen of this polymerized pyridoxilates stroma free hemoglobin is satisfactory and comparable to other authors.

Another inconvenient during the pyridoxilation process is the effect of foaming, it is recommended for next tests, that an antifoaming system is designed for the process, this requires a foam sensor, a positive displacement pump and an antifoam reservoir with its corresponding control lace. It is recommended that this system is designed.

[80] Reference 5

A explained earlier, the pyridoxilation reaction is more stable when done to deoxyhemoglobin, although 2 hours of nitrogen bubbling was done prior to the pyridoxilation reaction, there was no direct measure of the degree of deoxygenation, this could be done with a oxygen concentration meter, and the reaction should be started when a minimum of 5% oxygen is present in the hemoglobin solution.

The polymerization process, as seen by the diminish in the osmotic pressure, was carried out successfully, attaining a final hemoglobin concentration comparable to blood and isoosmotic with plasma. But the molecular weight distribution is not known, so the number of hemoglobin tetramers crosslinked forming a macro molecule is still a inconclusive. This requires also a technique to determine the molecular distribution of the polymerized pyridoxilated stroma free hemoglobin. Other authors use the technique of gel chromatography with Sephadex G-200. It is recommended to acquire this technology. A work in this area is being carried out by the author of this work.

Another excellent acquisition for the development of polymerized hemoglobins is an oncometer, this equipment determines the oncotic pressure exerted by hemoglobin as the only protein present in the polymerized pyridoxilated stroma

[81] Reference 5

free hemoglobin solution, osmotic pressure is also an indirect measurement of this parameter, on so inexact.

The pyridoxilation and polymerization processes have to be carried out at 4°C to control the methahemoglobin conditions. This was done with the help of a refrigerator with the set-point temperature at the desired temperature. A working laboratory at this temperature condition is recommended for further procedures.

No sterile conditions were used during the polymerization and pyridoxilation processes. In the future when infusing this modified hemoglobin solution in animals, a sterile condition during the whole process is strongly recommended, or at least, after infusing, filtering the hemoglobin solution through a 22 ?m sterile filter for subsequent animal test.

The process for the determination of the oxygen dissociation curves is to imprecise and time consuming. Because, when taking the sample into the syringe oxygen contact is present and therefore pO2 could be raised, and not known, and since the blood gas analyzer used does not determine the pO2, the saturation could not be determined correctly. What it was done was comparing the results with the result of Chang, and if the saturation level for the pO2 of the tank was similar, that test was used, otherwise it was discarded. Equipment for the determination of oxygen dissociation curves already exists (for example the Hem-O-scan), it is

recommended for further experimentation that this technology is acquired. So more exact and trustworthy curves are obtained.

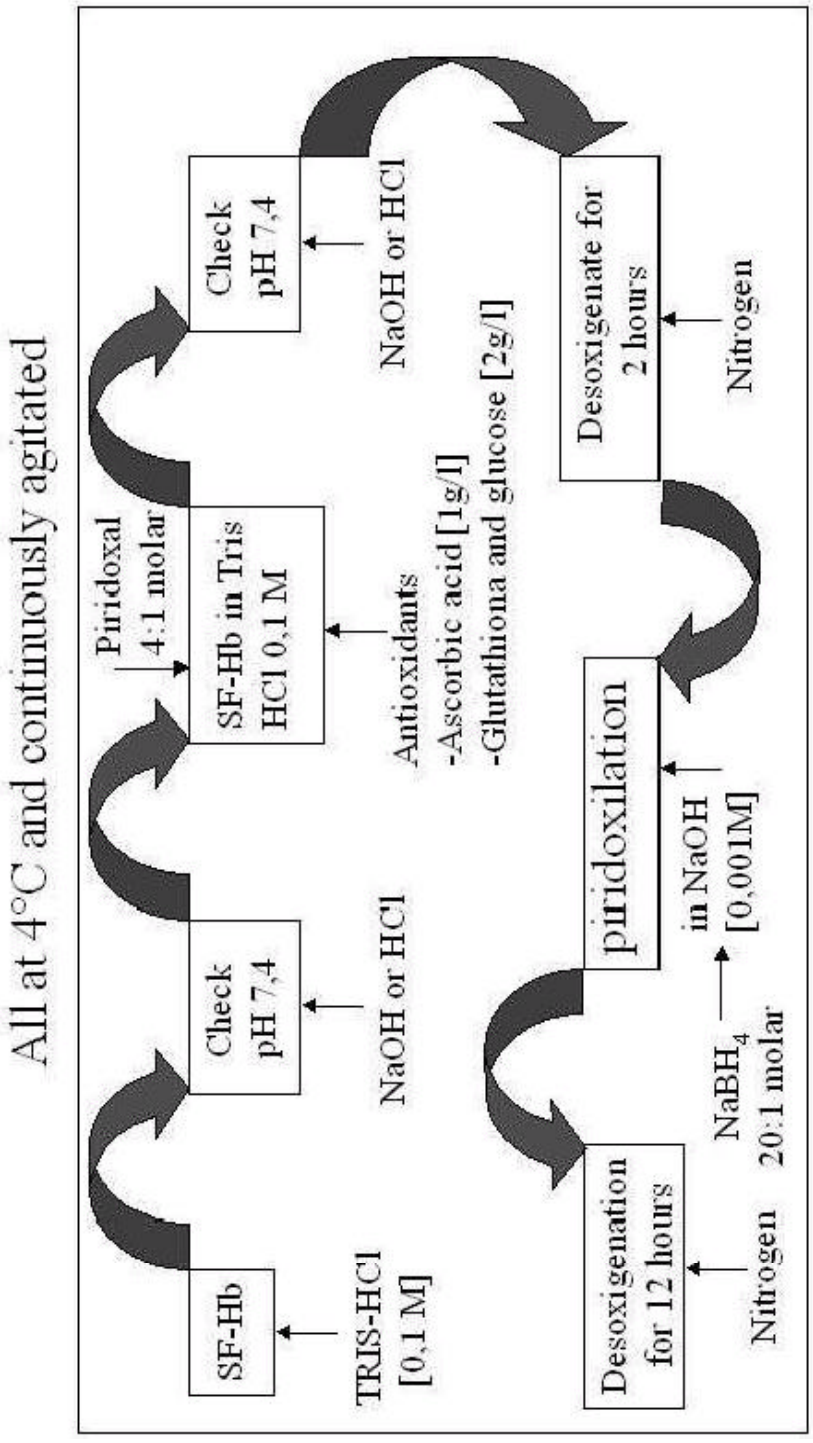
Although this is a starting point for the development of intravenous oxygen carriers based on modified hemoglobin in Colombia, the initial results are comparable with the parameters obtained by other authors, an therefore extremely satisfactory. There are some requirements previously mentioned, specially in the acquisition of technology for the determination of different parameters and the facilities to carry out such experiments and pilot plant tests .

A very important discussion point is the cost of production of such intravenous oxygen carriers, like the developed in this work, as it can be seen, the costs for the production are lower than actual donated blood units, and not saying they are much more secure.

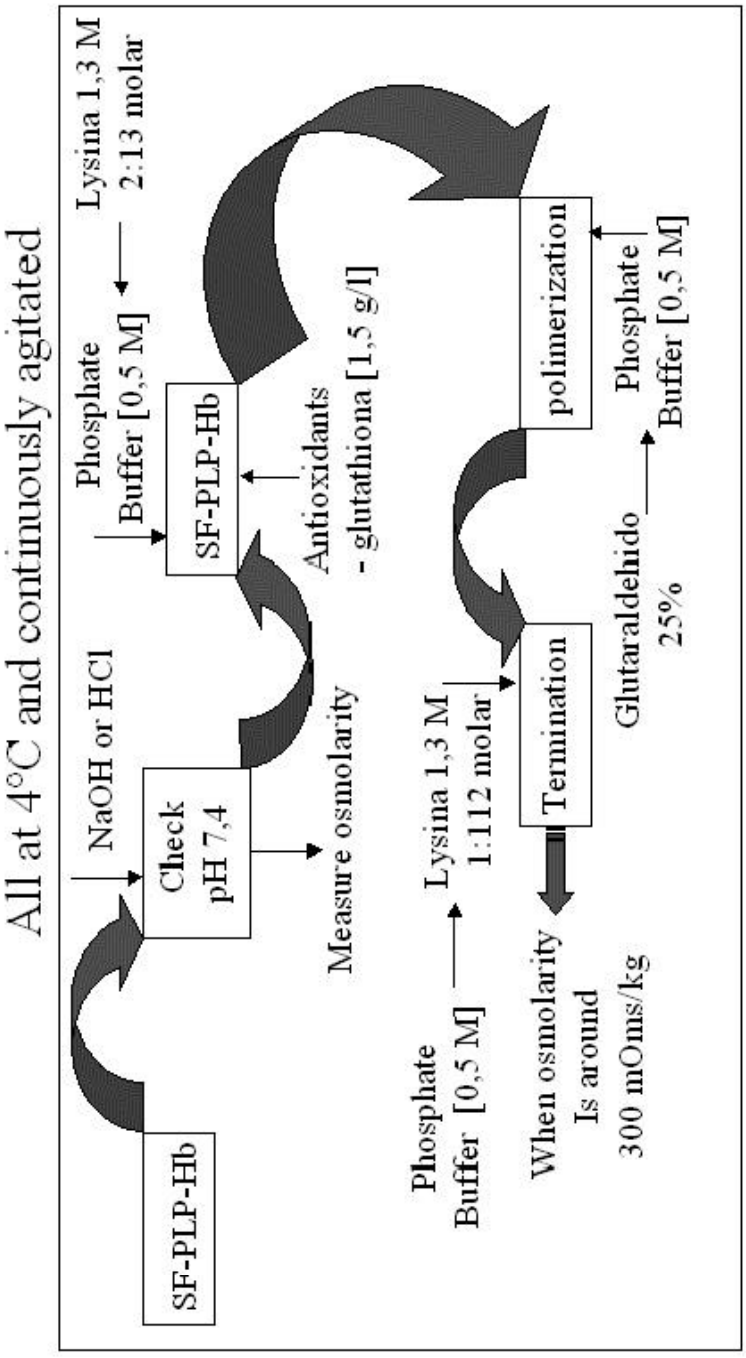
Further work in this area includes: production of conjugated hemoglobin, production of encapsulated hemoglobin, in vitro and animal test. It is the desire of this author and of the biomedical engineering group that his investigations is continued, for further scaling and plant production of intravenous oxygen carriers in Colombia. Future studies in these areas seem warranted because of the results obtained until now.

The perspectives in this area of study are very interesting, and following the optimization of the process described in this work, other modified hemoglobin solutions have to be tried out, like conjugated and encapsulated hemoglobin. And finally compare the result in production, cost and animal experimentation to decide with which of these oxygen carriers is suitable for the technologic and economic conditions present in Colombia.

Appendix I. Flux diagram of the pyridoxilation process.



Appendix II. Flux diagram of the polymerization process.



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