HAEMOGLOBIN (HB) LOADED WATER SOLUBLE HYPERBRANCHED POLYMER FOR THE DEVELOPMENT OF ARTIFICIAL OXYGEN CARRIER

BY

OOI HUI WEN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (TRANSFUSION SCIENCE)

ADVANCED MEDICAL AND DENTAL INSTITUTE
UNIVERSITI SAINS MALAYSIA

2019
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2019
DECLARATION

I hereby declare that this research has been sent to Universiti Sains Malaysia for the degree or Master of Science in Transfusion Science. It is not to be sent to any other universities.

With that, this research might be used for consultation and can be photocopied as reference.

Ooi Hui Wen

P-IPM 0048/18
ACKNOWLEDGEMENT

This project was never made possible without my supervisor, Dr Muhammad Azrul bin Zabidi, who has made this mission filled with information, knowledge and equipped me with experimental skills that could not be found anywhere otherwise and my co-supervisor, Dr. Ilie Fadzilah. Without their guidance, this project will never be completed. Not forgetting, the short grant (304/CIPPT/6313311), that funded the resources in this project.

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<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>λ</td>
<td>Lambda</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
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<td>%</td>
<td>Percentage</td>
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# LIST OF ABBREVIATIONS

<table>
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<tr>
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<tr>
<td>et al</td>
<td>and others</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g/dl</td>
<td>Gram per deciliter</td>
</tr>
<tr>
<td>hB</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HBOC</td>
<td>Haemoglobin Oxygen Carrier</td>
</tr>
<tr>
<td>HPG</td>
<td>Hyperbranched Polyglycidol</td>
</tr>
<tr>
<td>kDA</td>
<td>kilodalton</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mins</td>
<td>minutes</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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LIST OF EQUATIONS

Encapsulation efficiency % = \frac{\text{Post-encapsulation concentration}}{\text{Pre - encapsulation concentration}} \times 100

y = 2.2178x + 0.4608 (R^2 = 0.9427)

y = 289201x + 0.0452 (R^2 = 0.9987)
POLIMER LARUT AIR BERCABANG HIPER TERMUAT DENGAN HEMOGLOBIN UNTUK PEMBANGUNAN PEMBAWA OKSIGEN TIRUAN

ABSTRAK

Cabaran mengenai permintaan, bekalan dan penyimpanan darah yang dihadapi semasa proses pemindahan darah bagi tujuan rawatan telah mengalakkan usaha yang luas untuk menghasilkan darah tiruan, terutamanya sebagai pengganti sel darah merah. Aktiviti kajian lepas melibatkan gabungan pelbagai bidang termasuk bidang perniagaan, etika dan sains. Apabila sel darah merah semakin menghampiri tarikh luput, keutuhan membran sel semakin hilang. Walau bagaimanapun, kandungan molekul hemoglobin di dalam sel darah merah masih kekal. Hemoglobin bebas stroma adalah bertoksik dan boleh mengakibatkan kerosakan buah pinggan jika berada dalam sistem peredaran darah. Justeru, pengubahsuaian kimia diperlukan untuk penggunaan hemoglobin tersebut. Kajian ini melibatkan eksperimen yang menggunakan poliglisidol bercabang tinggi untuk mengurungkan hemoglobin bovin dan hemoglobin manusia yang diekstrak daripada darah penderma yang telah luput tarikh untuk menghasilkan mimik sel darah. Poliglisidol merupakan polimer bercabang tinggi dengan anggaran struktur dendrimerik yang sebanyak 50% ke 60%. Objektif utama adalah mengaji pergurungan hB dari darah pendermaan yang sudah lepas luput dan hB bovin untuk pengganti sel darah merah Ini dijalankan dengan mengasingkan hB dari darah diderma dari bank darah, pengurungan hB manusia and bovin dalam poliglisidol dan menilai kitaran kebolehulangan oksigen hB terperangkap dalam poliglisidol. Dapatan menunjukkan perbezaan penurunan kepekatan yang tidak signifikan (p > 0.05) antara hemoglobin bebas dan hemoglobin terperangkap untuk kedua-dua jenis hB (p = 0.019). Kecekapan enkapsulasi yang tinggi
dengan purata 88.1% dan 99.0% menunjukkan sekurang-kurangnya tiga molekul hB terperangkap dalam poliglisidol.

Walaupun kadar purata kitaran oksigen menunjukkan hanya lima kitaran oksigen untuk kedua-kedua sistem polyglisidol terperangkap dengan hB manusia (p = 0.109) dan hB bovin (p = 0.109), penggunaan poliglisidol bagi pengurungan hemoglobin menunjukkan kitaran kebolehulangan oksigen yang berpanjangan dan berterusan berbanding hemoglobin bebas yang hanya merekodkan satu kitaran sebelum penyahaktifan sepenuhnya. Perbandingan kecekapan pengurungan (p = 1.100) dan kitaran oxigen (p = 0.109) antara hemoglobin manusia dan bovin juga tidak menunjukkan perbezaan yang signifikan (p > 0.05). Dapatan menunjukkan bahawa hemoglobin bovin mampu berfungsi sebagai hemoglobin manusia. Perkara ini menujukkan hemoglobin manusia berkemungkinan boleh digantikan dengan hemoglobin bovin pada masa hadapan. Tambahan pula, darah bovin adalah mudah diperolehi.

Kajian sistem hemoglobin-poliglisidol ini menandakan satu langkah kejayaan menuju ke arah pembangunan untuk menghasilkan penggantian sel darah merah yang dapat berfungsi menghantar oksigen di dalam peredaran darah manusia.
HAEMOGLOBLIN (HB) LOADED WATER SOLUBLE HYPREBRANCHED POLYMER FOR THE DEVELOPMENT OF ARTIFICIAL OXYGEN CARRIER

ABSTRACT

The difficulties regarding demand, supply and storing of blood for therapeutic use transfusion have initiated extensive efforts to develop artificial blood especially red cell substitutes. The history of these hard work includes a complicated mixture business, ethics and scientific field. As time passes towards expiry, RBC membrane loses its structure integrity but the hB molecule remains intact. Free stroma hB is toxic as it can cause kidney toxicity therefore chemical modification is needed for the continuity of hB use. This study involves experimentation using hyperbranched polyglycidol to encapsulate bovine hB and human hB extracted from expired blood donation to produce red cell mimic. Polyglycidol is a hyperbranched polymer possessing around 50% to 60% of dendrimeric structure. The main objective of this study is to study nano-encapsulation of hB from expired blood human blood and bovine for development of red cell substitute by isolation of human hB from expired blood from volunteer donors, encapsulation of human and bovine hB in hyperbranched polymer called glycidol, and to evaluate reversible oxygen binding of encapsulated human and bovine hB. Findings showed no significant decrease of concentration (p > 0.05) between free-hB and encapsulated complex for both hBs (p = 0.109). These demonstrates high encapsulation efficiency with 88.1% and 99.0% indicating least three hB molecules were entrapped within interior chamber of the polyglycidol. For reversible oxygen cycles although average of five reversible cycles was not significant (p > 0.05) for both encapsulated human hB (p = 0.109) and bovine hB (p = 0.109), addition of polyglycidol for encapsulation of the hBs
prolonged the persistence of reversible oxygen cycle as compared to only one cycle recorded by the free-hB prior to its complete deactivation. The comparison of encapsulation efficiency (p = 0.100) and average of reversible oxygen cycles (p = 0.109) between encapsulated human hB and encapsulated bovine hB were not significant (p > 0.05) demonstrating that the performance of bovine hB was similar behaviour to human hB encapsulating and encapsulated in polyglycidol. These suggest that human hB could also be substituted by bovine hB as the latter is easier to obtain. The utilisation of hB-polyglycidol system signifies a noteworthy footstep in the progress of red cell substitute production with extended oxygen delivery in the circulation.
CHAPTER 1
INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Blood is a highly specialised body fluid that flows throughout the body by veins, arteries and capillaries. Blood has four main components which are red blood cells (RBC), white blood cells (WBC), plasma and platelets. RBCs constitute about 45% while the remaining of 55% consist of plasma, WBCs and platelets. Functions of the four components including regulation of immune response for fighting infections, to prevent bleeding and blood loss, carrying oxygen and nutrients to cells and bringing waste products to the kidney.

RBCs that are released into the circulation by the bone marrow is biconcave in shape, has the diameter of six to eight nm which is small enough to squeeze into smallest blood vessels and circulates in the body for only 120 days. A mature RBC in the blood lacks nucleus to enable storage of an important oxygen-binding protein called haemoglobin (hB). Haemoglobin is the protein that carries oxygen from the lungs to the cells and tissues around the body.

Blood transfusion is a life-saving measure in medical procedure aiming to replace blood loss but also have inherent risk, danger and side effects. Transfusion transmitted infections of blood borne pathogens, such as viral hepatitis and HIV are some of the risks. Although fatal blood transfusion caused by incompatible cross-matching is uncommon, it remains the top reason of death from blood transfusion (Chen J. et al, 2009). The greatest risk of transfusion during trauma cases mostly happen when the immunological functions is upregulated. The transfusion alone can cause cytokine release thus initiating a systemic inflammatory response (Dunne et al., 2004).
Shortages of donated blood in blood banks are unfortunately common. The ability to meet the demand for blood is often limited. Blood shortage situation in Malaysia is manifested by the fact the only 2.2% of Malaysia population are donors whom mostly are repeated donors. (Lim, et al., 2018). Donated blood has a short shelf-life up to 42 days and must be kept in temperature between two to six degrees Celsius. As time progresses, stored blood loses 2,3-diphosphoglycerate (2,3-DPG) and increasing oxygen affinity causes the weakening of oxygen unloading in tissues. With the complications of side effect, shortages of donors and limitation of blood transfusion, it justifies the importance of developing blood substitutes.

A blood substitute in general is a substance that mimics blood cell particularly red cell to fulfil some functions of the RBC. The purpose of blood substitute is to provide an alternative treatment for blood transfusion from one person to another. The usage of blood substitute eliminates the risks of transfusion infections, fluid replacement in post-haemorrhagic therapy during trauma and most importantly a solution to the problem of blood shortage.

Research of blood substitute and engineered oxygen carriers have been continuously developing since the past years to find the best substitute for RBC in terms of optimum oxygen delivery, its biocompatibility, availability to deliver through small capillaries, easy storage and availability, can be mass produced and with lower production cost.

Blood substitutes are categorised into two categories. The haemoglobin-based oxygen carriers (HBOC) uses hB molecules which carry the oxygen loaded molecule in an engineered membrane to be transported to the cells. The perfluorocarbon-based oxygen carriers (PFCOH) uses perfluorocarbons, a chemical compound formulated into emulsion prior to transfusion.
This study aims to develop blood substitute of hB in an engineered membrane using hyperbranched polymer material known as polyglycidol, also known as polyglycerol. Global research on the usage of hyperbranched polyglycidol to be used as a nano-carrier have been developed for the past 20 years. The synthesis of copolymer of hyperbranched polyglycidol and other polymer such as poly(ε-caprolactone) has been successful for targeted drug delivery system (Li et al., 2012). The selected polymer system in this study will be loaded with hB molecules before efficiency and reversible oxygen binding profiling are furthered.

1.2 PROBLEM STATEMENT

Shortage of blood supply is always a problem in the medical world despite well-documented demand for blood transfusion. World Health Organisation (WHO) recorded estimation blood units of 112.5 million collected in 180 countries globally in 2016 with South-East Asia contributing only 16.7%. Yet, global ratio of donor to population is only at 2.5% whereas demand for blood is rising by 6-8% annually. This translates that only 7.9 units per 1000 people in South-East Asia which is lagging behind the minimum requirement set by WHO of 10 units per 1000 people (Mohamed et al., 2019).

This trend will continue further if additional restrictions are enforced particularly during disease outbreak given the fact that South-East Asia is located geographically prone to natural disaster and in tropical weather. High-cost lifestyle in this modern era leading to low birth rate together with increasing of ageing population are factors to the limitation of donor pool. Human blood transfusion faces several issues such as transmission of TTI, incompatibility due to antibody-antigen reactions, and availability in restricted or remote area. Therefore, artificial blood could possibly provide a solution to the problems mentioned earlier.
Most studies on blood substitutes have been focusing on few important characteristics of ideal red cell substitutes including prolonged half-life or stability to survive in the blood circulation before excreted, reversible oxygen binding capacity, biocompatibility with no compatibility testing and free of side-effects.

Blood substitute products such as hB based oxygen carriers (HBOC) and perfluorocarbon (PFC) during clinical trials ongoing in several phases were withdrawn from a few countries due to fatal side effects, and their application are limited to only certain medical procedures (Alayash, 2014). Free-hB from lysed RBC may cause extreme oxygen affinity, renal toxicity, renal failure, limited half-life and their structural integrity has been compromised (Macedo et al., 2018). Due to these reasons, naked hB molecule needs chemical modification to load the free-hB for it to function. Potential sources of free-hB can be obtained from expired donated blood bag which is one source for this study besides animal blood. HB molecules can be harvested upon isolation and purified from RBC membrane for further chemical modification using suitable polymeric carrier.

1.3 RATIONALE OF THE STUDY

Adverse reactions associated with allogenic transfusion since the beginning. There is 539 reported cases adverse event related to transfusion among the 3.4 million issued blood components in 2004 which is an 19% increase compared to 2003 (Maxwell and Wilsom, 2006). Medicine in current clinical practise has changed in the modern world to reduce adverse transfusion reactions. Alternatives strategies were applied to decrease any adverse reactions such as blood conservation during surgery to minimise blood loss and leukoreduction of donated blood (Sahu and Verma, 2014). As for necessary transfusion, possible adverse reaction phenomenon of blood transfusion has initiated an awareness on the importance of research and development of blood alternatives. Features
of an ideal blood alternative includes universally biocompatibility by eliminating any antigen-antibody reactions, incapable to cause organ toxicity from the breakdown of the artificial blood after usage and small in size to be able to reach small veins and tissues for optimum oxygen delivery.

The use of commercially available bovine hB together with the sourcing of human hB from expired donated blood of less than three days’ post expiry gives the impression of cost effectiveness in minimizing expired blood disposal.

A packed RBC bag has shelf life of 42 days. Once expired, the slowing down of oxygen saturation and the weakening of membrane integrity of red cells may lead to lysis. Nonetheless, the hB should remain complete with all hB protein present even after removal of phospholipid membrane allows the harvesting of the intact molecule within three days after expiring (Willekens et al, 2003). Both bovine hB and purified human hB will be encapsulated within chosen polymer, polyglycidol using guest and host moiety strategy for the imitation of RBC.

1.4 RESEARCH OBJECTIVES

Main objectives
To study nano-encapsulation of hB from expired human blood and bovine for development of red cell substitute.

Specific objectives
1. To isolate of human hB from expired human blood.
2. To evaluate encapsulation efficiency of human and bovine hB into polyglycidol.
3. To evaluate reversible oxygen binding of encapsulated human and bovine hB.
1.5 RESEARCH QUESTION

1. What is the purity hB after of sonication, repeated washing, organic extraction and dialysis on after isolation of human hB?
2. What is the efficiency loading percentage of human hB and bovine into polyglycidol?
3. Will both encapsulated hB able to bind and release oxygen?

1.6 RESEARCH HYPOTHESIS

Null Hypothesis (H₀)

1. There is no difference in absorbance level between pre-encapsulation and post-encapsulated hBs.
2. There is no difference in encapsulation efficiency between encapsulated human and bovine hBs.
3. There is no difference in reversible oxygen cycles between encapsulated hBs and free-hBs.

Alternatives Hypothesis (H₁)

1. There is a difference in absorbance level between pre-encapsulation and post-encapsulated hBs.
2. There is a difference in the encapsulation efficiency between human and bovine hBs.
3. There is a difference in reversible oxygen cycles between encapsulated hBs and free-hBs.
1.7 SIGNIFICANCE OF THE STUDY

This study might be one of the pioneers for blood substitutes research in our country, Malaysia. Any findings from this experiment will add to the literature for developing blood substitutes in the future. The use of polymer such as polyglycidol could be a possible choice to be hB carrier for production of artificial blood. Polyglycidol is a globular aqueous tolerant macromolecule with interior cavity that acts as a ‘host’ to load ‘guest’ molecule, in this study which is the hB molecule using non-covalent chemistry. Polyglycidol offers the capacity of hB loading similar to drug delivery and other applications such as iron oxide nanocomposite for cancer therapy and bio-imaging application ((Moorthy et al., 2016). The preparation steps are accessible to mass manufacturing due to its simplicity. Furthermore, usage of expired human blood will reduce the disposal of expired blood and subsequently improve inventory management.

Blood substitutes with optimum affinity and oxygen release are very useful for treatment, especially when blood is unavailable for first aid. Emergengy. Discoveries from this study may contribute to any improvement of producing artificial blood with great stability and efficient oxygen release in an unfavourable condition of donated blood. Subsequently blood substitutes being an alternative for transfusion of human blood may transform blood transfusion service in Malaysia.
CHAPTER 2
LITERATURE REVIEW

2.1 BLOOD SUBSTITUTE

Blood substitute or any form of alternative blood product ideally must carry the function of delivering oxygen to entire human body. Red cell alternatives were designed primarily to retain all functions of blood and to overcome limitations of transfusion such as limited blood supply, contamination of TTIs, transfusion reactions, and time of cross-matching during emergency. These oxygen carriers hopefully will replace transfusion of humans red blood cells in the future.

Many categories of artificial blood products have been produced since its first begun over a century ago. In the beginning, research activities utilised hB molecules for oxygen delivery but unfortunately caused a fatal side effect of hB-mediated toxicity that led to kidney failure (Alayash, 2017). With the knowledge of kidney toxicity, research works diverted to wrapping the molecule for transportation in the blood system. There are different types of red cell substitutes that have been introduced till current date including stem cell derived RBC, perfluorocarbon (PFC) and hB-based oxygen carrier (HBOC).

PFC is a colourless, non-toxic compound that contains high oxygen and carbon dioxide dissolving capacities as no chemical binding is involved thus easily available to tissues. Characteristics such as low boiling point temperatures, low water and lipid solubility makes PFC ideal to transport oxygen. (Riess, 2006). The ability of controlling size of which is considerable smaller than a normal RBC enables passing through into vessels in difficult areas for treating certain diseases (Fu et al., 2019). They have more capacity to carry oxygen as compared to HBOC, therefore PFC is more favourable for use. Second generation PFC products such as OxyFlour and Oxygent have been rejected
by Federal Drug Administration (FDA) for clinical trial because of the side effects, increased risk of stroke and limited benefit (Moradi et al., 2016).

At the meantime, HBOC has become an option because of its oxygen carrying natural properties but stroma-free hB requires further chemical modification to maintain the stability of the molecule as shown in Figure 2.1.

![Figure 2.1](image)

**Figure 2.1**: Types of chemical modifications done on hB molecules to maintain stability of hB for continuous use of an oxygen carrier. (Adopted from Taguchi et al., 2015).

Development of artificial blood utilised five types of special modifications to improve stroma-free hB stability for efficient therapeutic intervention; cross-linked hB, polymerised hB, conjugated hB and surface-modified hB and liposome encapsulated hB. Cross-linked hB consist of intermolecular cross-linking between α and β sub-unit to improve stability. Polymerisation approach through poly-functional cross linking would produce higher molecular weight than molecular weight of normal hB. One hB molecule can be conjugated by biocompatible polymer to add to its molecular weight to improve stability (Sakai et al., 2002). On the other hand, the hB encapsulated into a lipid membrane or polymeric vesicles to create the natural structure of RBC show better performance than other chemically modified hB (Shi et al., 2009).
Half-life of HBOC in human is between three to four days in normal adults but only 18 hours during haemorrhagic conditions which is significantly shorter than a normal RBC life span of 120 days (Taguchi et al., 2009). However, it will still be useful during emergency transfusion, acute massive bleeding and can temporarily support life while transportation in an ambulance until transfusion with banked blood. HBOC also have superior shelf life up to 320 days in room temperature as compared to normal shelf life of RBC which is only 42 days in two to six degree Celsius (Bian and Chang, 2015).

Discovery of hB structure in 1960s by Perutz and his colleague triggered strong attention about the functionality of the construction and ways to modify them to be useful in their field. To this presence day, there have been products in clinical testing at the advanced stages for the regulatory approval process. The latest invention HBOC-201, polymerised bovine hB with Polyheme are still in Phase 3 of clinical trials. (Dubé, Pitman and Mackenzie, 2017). The results also showing that HBOC-21 are well tolerated in different clinical settings with wide range of doses (Mackenzie et al., 2017). Some HBOC products have successfully passed the safety trial phase and still in the process of efficacy improvement and the better outcome to minimise side effects. The comparison of these HBOC blood products are compared in Table 2.1 below.
Table 2.1: Comparison of HBOC products (Chen *et al.*, 2009; Buehler *et al.*, 2010; Alayash, 2014; Alayash, 2017).

<table>
<thead>
<tr>
<th>Products</th>
<th>Source</th>
<th>Approach</th>
<th>Company/ Sponsor</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemopure &amp; Oxyglobin</td>
<td>Bovine hB</td>
<td>Polymerized hB</td>
<td>Biopure Corp.</td>
<td>Hemopure approved for usage in south Africa Oxyglobin approve for animal use.</td>
</tr>
<tr>
<td>HemAssist</td>
<td>Human hB</td>
<td>Crosslinked hB</td>
<td>Baxter</td>
<td>Discontinued after Phase III</td>
</tr>
<tr>
<td>Hemospan</td>
<td>Human hB</td>
<td>Conjugated &amp; Surfaced modified hB</td>
<td>Sangart Inc.</td>
<td>Discontinues at early clinical trials</td>
</tr>
<tr>
<td>Optro</td>
<td>Human hB</td>
<td>Crosslinked hB</td>
<td>Somatogen.</td>
<td>Discontinued after Phase II</td>
</tr>
<tr>
<td>Polyheme</td>
<td>Human hB</td>
<td>Crosslinked &amp; Polymerized hB</td>
<td>Northfield Laboratories Inc.</td>
<td>Phase III clinical completed</td>
</tr>
<tr>
<td>Pyrodoxylated HB (PHP)</td>
<td>Human hB</td>
<td>Crosslinked &amp; Conjugated hB</td>
<td>Apex Bioscience Inc.</td>
<td>Discontinued</td>
</tr>
<tr>
<td>Hemolink</td>
<td>Human hB</td>
<td>Crosslinked &amp; Polymerised hB</td>
<td>Hemosol Inc.</td>
<td>Clinical Trial Phase I</td>
</tr>
</tbody>
</table>

An artificial blood must be designed to behave as similar to a natural RBC in the body. So, a red cell substitute ideal characteristic for an artificial blood includes capability of oxygen delivery, extended half-life in circulation, elimination of blood typing and cross-matching, and elimination of any possibilities of transfusion transmitted infections. It must also be readily available, easy access, trouble-free storage environment such as in an ambulance and long shelf life. It is an important factor that the artificial blood can be eliminated entirely from the body and do not accumulate in tissues which can lead to toxicity and would not cause any adverse transfusion reactions (*Yadav et al.*, 2016). Nevertheless, a complete understanding about hB structure is needed to ensure its optimal ability as an RBC mimic.
2.2 HAEMOGLOBIN

Effort to develop a HBOC needs a comprehensive understanding of hB structure before any red cell mimic can be proposed. Each erythrocyte contains up to 300 million hB molecules. Haemoglobin (hB) is a transporter containing four haem groups and four globin subunit chains. The iron in the haem forms an unstable reversible bond with oxygen. An organic compound of the haem, protoporphyrin IX by iron in ferrous state (Fe$^{2+}$) atom will bind the oxygen for transportation to the tissues. Each haem has one iron atom therefore each molecule of hB can bind to four atoms of oxygen. The four haem groups are surrounded by the four subunit globin chains; two α chains and two β globin chains forming a tetrahedral structure as shown in Figure 2.2 below. When an oxygen molecule binds to hB, it forms oxyhaemoglobin and becomes deoxyhaemoglobin upon oxygen release to tissues (Glenn and Armstrong, 2019).

![Figure 2.2: Structure of hB molecule shows two α-chain structures shown in blue and two β-chains shown in green (Adopted from Schechter, 2008). Each subunit has a heam molecule, therefore a unit of hemoglobin has four haems to bind to four oxygen molecules.](image)

In early development at foetal stage the embryo will produce the foetal hB (HBF) which consists of 2α and 2γ chains. As near birth, the hB will slowly transitions to adult hB (HBA) which contains 2α and 2β globin chain (Dunn, Mythen and Grocott, 2016).
Normal adults have majority HBA, and up to 3.5% are HBA2 and <1.0% HBF (Paikari and Sheehan, 2018).

Normal level of hB in women is 11.5 – 16.0 g/dL and 13.5 – 18.0 g/dL in men. Each subunit has similar structure, size and the molecular weight of 16kDa. Therefore, total molecular weight for one quaternary structure of hB is about 64kDa (Thomas and Lumb, 2012).

HB molecules are produced from the bone marrow and released into the circulation are protected by the bi-lipid membrane forming the red blood cell. RBC are biconcave in shape with no organelles nor nucleus to maximise hB load when they enter the circulation. The size of a RBC is about 1.5 to 2.5 μm in thickness and 6.5 – 8.5 μm in diameter with lifespan of 120 days containing 5 nm hB molecule in blood circulation. Biconcave disc shape offers a large surface area to facilitate maximum and fast gas exchange, allows flexibility to deform to allow passage through small capillaries and still maintain structure integrity to function (Glenn and Armstrong, 2019). Damage to the cytoskeleton will affect cell flexibility therefore lead to the release hB molecules freely in the circulation (Welbourn, et al, 2017).

Without the encapsulation of the RBC membrane, free hB in the circulation can potentially cause harmful side effects by being recognised as non-self-pathogen and triggers immunity to be broken down into smaller, toxic compound in the body. An α-haemoglobin stabilizing protein (AHSP) protects erythrocytes from oxidation. Oxidised haem (Fe\(^{3+}\)) which is highly toxic until it is reduced to functional Fe\(^{2+}\) haem when required for binding oxygen (Forget and Bunn, 2013).

RBCs consist of 2,3 diphosphoglycerate (2,3-DPG) that possessed the most efficient control of oxygen in human. 2,3-DPG along with protons, chloride ions and carbon dioxide are modulators of oxygen affinity. Body temperature and blood pH also
greatly affects the affinity of oxygen binding to haem. When hB binds to oxygen molecules, oxygen saturation increasing following a sigmoidal curve shown in Figure 2.3. Therefore, when 2,3-DPG is raised by the binding between of β-globin chain of deoxygenated hB, the change of hB structure also causes a decrease in the oxygen affinity of hB (Mozzarelli et al., 2010). Even though stroma-free hB has high oxygen affinity, instability of free-hB molecule in circulation easily breaks down the hB tertiary structure and form αβ dimer. The α-globin will disassociate with AHSP and bind with any a free β-globin subunit forming a stable αβ dimer which leads to rapid renal excretion and extravasation (Forget and Bunn, 2013). Moreover, high oxygen affinity of free hB tends to undergo autoxidation with methB where oxygen will react with hB molecules instead of bonding leads to interference of oxygen release to tissues (Alam et al., 2014).

Figure 2.3 : The Sigmoid curve of oxyhB will shift to the right and left when factors such as temperature, carbon dioxide, 2,3-BPG and acidity changes. (Adopted from Alam et al., 2014).

Usage of free hB for therapeutic purposes showing more harm by than benefits are shown by clinical trials done in early 20th century in the naval fleet (Chen et al, 2009). The side effects triggered the discussion of creating a carrier that could carry hB inside with suitable chemical modification needed so that problems of using free-hB can be solved.
2.3 POLYMER

A polymer is a giant molecule that is composed of both synthetic or natural small molecules called monomers. Polymers are classified into three main structures: linear polymer, side-branched polymer and cross-linked structures. As shown in Figure 2.4 shown below, each architecture has their own uniqueness and its functionality (Gosecki et al., 2016). Linear polymer is a long chain of continuous carbon-carbon bond back to back whereas branched polymer has multiple secondary chain hanging by the primary backbone. Cross-linked polymers are usually moulded as branched or linear and then cross-linked by forming covalent bond between polymers except their tip ends (Dworak et al., 2013).

Figure 2.4: Polymer consist of three main classification of polymer architecture which is the linear, the branched and crosslinked. (Adopted from Romani et al., 2002).

Theoretically, polymers must to be chemically stable to maintain their properties during processing into product, during storage and usage. Recent interests to use a branched polymer such as polyglycidol as a carrier. Clinical trials produce positive results for the potential application in the biomedical such as drug delivery of curcumin using branched polymer for wound dressing treatment (Perumal et al., 2017). Therefore, of developing a well-defined globular and macromolecule structure to mimic RBC is possible.
2.4 POLYGLYCIDOL

Polyglycidol production from controlled anionic polymerisation glycidol toward hyperbranched polyglycidol started ago more than a decade ago since 1999, many captivating sides have been discovered. One of those discovered is slow monomer addition combining with ring opening multi-branching can be used to prepare well-defined, compound polymer architecture (Sunder et al., 1999).

The polyglycidol is formed by multiple monomer form covalent bonds with each other forming hyperbranched polymers such as dendritic structured polyglycidol. A glycidol is monomer that consists of two reactive group, hydroxyl and epoxide with a reactive side group -CH$_2$OH as shown in Figure 2.5. Therefore, ring opening polymerisation (ROP) of monomer glycidol shown is Figure 2.6 always leads to branched polymer. The propagation step of nucleophilic attack on the endocylic CH$_2$ group and CH$_2$-O band results in adding of a subunit that consist the primary hydroxyl side group. However anionic polymerisation causes intramolecular chain transfer of the primary chain leads to possible branching. The primary and secondary chain may have active hydroxyl group inside the branches along the chains raise the chances of hyperbranching within interior architecture of the polymer (Gosecki et al., 2016) shown in Figure 2.7.

![Figure 2.5: Single glycidol structure with reactive side group -CH$_2$OH (Adopted from Gosecki et al., 2016).](image-url)
Figure 2.6: Ring opening of a single glycidol (Adopted from Darensbourg and Yeung, 2014).

Figure 2.7: Ring opening of multiple monomer produces hyperbranched structure. (Adopted from Weiss, 2012). Primary and secondary chain with hydroxyl group inside the branches along the chains causes hyperbranching within interior architecture of the polymer.

To date, soluble hyperbranched polyglycidol has been used as a starting material to produce a nanocapsules carries with similar micellar properties. Several works report on using polyglycidol polymer as a drug delivery system utilising selective core-shell architectures such a star polymer (Frey and Haag, 2002) shown in Figure 2.8 and dendritic or unimolecular polymer (Luo et al., 2011) shown in Figure 2.9.
Hyperbranched polymer has also provided a hopeful substitution of material for as carriers for several medicinal application and nanomedicine technologies. This polymer favourable to be utilised is due to flexible globular structure and biocompabilities. Advantages of using hyperbranched polymer that they non-immunogenic and are well tolerated as the accumulation of polymer are minimum in vital organs after application. Other advantages including low toxicity and resistant to protein absorption. (Abbina et al., 2017). Examples of ideal characteristic of hyperbranched polymer polyglycidol are shown in Figure 2.10.
Water soluble hyperbranched polymer has been widely considered for diverse biomedical application including delivery of drugs, dialysis and macromecular therapeutics application. Polyglycidol is a hydrophilic polyethers with similar structure to the well-studied and polymer called poly(ethylene glycidol) but with better biocompatibility characteristic (Kainthan et al., 2006), which is widely applied in the medical application, pharmaceutical industry for drug conjugation delivery system and protein resistant to blood contacting surfaces (Wilms, Stiriba and Frey, 2010). Other examples application of hyperbranches polymer currently in used in the medical field are shown in Figure 2.11. Therefore, using hyperbranched polymer, polyglycidol for an ideal alternative material for oxygen carrier for a host-guest system.
This host-guest non-covalent interactive system is very beneficial for loading of protein molecules such as hB as a oxygen carrier. Unlike covalent bond that shares electron between molecule, molecules are interacting which each other by electromagnetic forces with no electron shared. One of the trial factors is developing a vesicle is the size to reside a 64kDa, 5nm diameter hB protein for RBC substitute. Ability of encapsulation of hB using combination dendritic polymer and linear polymer may have to have great potential for RBC substitute. (Satoh, 2009)
Random hyperbranched dendritic polymers shown in Figure 2.12 have similar structure and physical properties to dendrimers in Figure 2.9. Comparing to dendrimers, they are inexpensive and often requires little time to synthesis as it only requires one-step synthesis (Wilms, Stiriba and Frey, 2010). Low concentration of aggregation by glycidol hyperbranching can maintain its shape and structure as it is functioning in the circulation (Darensbourg and Yeung, 2014). According the research conducted by Khadim et al (2019), measurement of a dendritic structure synthesized by random hyperbranching work out diameter of 40-90 nm. Porphyrin cored aggregation with spherical structures shows a diameter of 20 – 40 nm (Weiss, 2012). This shows that dendritic structures can replace the red cell membrane that is able to contain molecules as a significant smaller size RBC.

Although a single RBC contains 300 million hB molecules, it is possible to create a smaller RBC version with lesser number of hB molecule. As an hB is only 5 nm, with diameter produced by dendritic hyperbranching, it possible to load one unit of hB between two branched subunits. Therefore, it is ideally to load at least four units of hB into a dendritic structured polyglycidol. Hence, the usage of hyperbranched polymer is supported as size can be controlled for maximum hB loading while still be small enough for the purpose of delivering oxygen to reach small capillaries at the most optimum level.
CHAPTER 3
MATERIALS AND METHODOLOGY

3.1 MATERIALS

3.1.1 Apparatus and Equipment.

All apparatus and equipment used in this study are listed in the table 3.1

Table 3.1 : The list of apparatus used in the study

<table>
<thead>
<tr>
<th>Apparatus/ Equipment</th>
<th>Brands</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV/Vis Spectrometer</td>
<td>Perkin Elmar, Malaysia</td>
</tr>
<tr>
<td>Latex examination disposable gloves</td>
<td>Fisher Scientific, US</td>
</tr>
<tr>
<td>Disposable pipette</td>
<td>Fisher Scientific, US</td>
</tr>
<tr>
<td>Electronic weighing balance</td>
<td>Mettler Toledo, US</td>
</tr>
<tr>
<td>Beaker</td>
<td>Duran, Germany</td>
</tr>
<tr>
<td>Disposable syringe (10cc/mL)</td>
<td>Terumo, Philippines</td>
</tr>
<tr>
<td>Cuvette</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Pipette tips 200 µL Yellow</td>
<td>Thermo Scientifics, US</td>
</tr>
<tr>
<td>Pipette tips 1000 µL Blue</td>
<td>Thermo Scientifics, US</td>
</tr>
<tr>
<td>Pipette tip box</td>
<td>Eppendorf Scientific, US</td>
</tr>
<tr>
<td>Micropipette</td>
<td>Eppendorf Scientific, US</td>
</tr>
<tr>
<td>Measuring Cylinder (50 mL and 100 mL)</td>
<td>Favorit, Malaysia</td>
</tr>
<tr>
<td>Falcon Tube (15 mL and 50 mL)</td>
<td>Becton, Dickinson, USA</td>
</tr>
<tr>
<td>Parafilm</td>
<td>Bemis, USA</td>
</tr>
<tr>
<td>Ultra sonicator</td>
<td>Mujigae, Korea</td>
</tr>
<tr>
<td>Magnetic Stirrer ST0707V2</td>
<td>Favorit, Malaysia</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>Dialysis Tubing</td>
<td>Sigma- Aldrich Co., USA</td>
</tr>
</tbody>
</table>

3.1.2 Chemicals and Reagents

All chemicals and reagents used in this study are listed in Table 3.2

Table 3.2 : The list of chemicals and reagents used in this study

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Brands</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl (Normal saline)</td>
<td>Bio Basic Canada inc., Canada</td>
</tr>
<tr>
<td>Ultrapure water</td>
<td>Thermo Scientifics, US</td>
</tr>
<tr>
<td>Dried hB (bovine)</td>
<td>Sigma-Aldrich Co., USA</td>
</tr>
<tr>
<td>Dichloromethane (DCM)</td>
<td>Systerm, Malaysia</td>
</tr>
</tbody>
</table>
3.2 PREPARATION AND ISOLATION OF HUMAN HB

3.2.1 Collection of Expired Human Red Blood Cells

One Packed cell of human RBC post expiry was collected from Blood Bank Advanced Diagnostic Lab (ADL), in Advanced medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM) in a cooled box to the laboratory and immediately kept in the fridge with temperature of 2 to 6°C maximum of three days. This study has been approved by ‘Jawatankuasa Etika Penyelidikan (Manusia) of USM (USM/JePEM/16110533).

3.2.2 Isolation of human hB

Expired human red cells extracted from the blood bag was washed in falcon tube (50 mL) with equal amount of normal saline and then separated by centrifugation at 1000g for 20 minutes. The first wash supernatant was disposed and washing of cells was repeated again two more times. The human RBC solution was transferred into a crushed ice filled 500 mL beaker. Crushed ice was used to maintain temperature between two to six degree Celsius. Then, the RBC solution was agitated with sonication to induce haemolysis for 20 mins. The supernatant now consists of lysed blood. Then, it was mixed with dichloromethane (DCM) and continued shaking for three mins followed by centrifugation at 1900g for 30 minutes.

The supernatant containing hB product solution was kept and the precipitate layer containing leftover RBC membrane was discarded. The hB supernatant was washed repeatedly by mixing with DCM. Lastly, excess DCM was left stirring overnight at room temperature for self evaporation.
3.2.3 Purification of Human hB.

Isolated hB solution was added into a pre-chosen dialysis tube of 14,000 Dalton (kDa) molecular weight cut-off (MWCO). The whole tube was flooded with pure water, using the principle of the osmosis to remove low molecular weight ions metabolites and small excess by-products of lipids.

3.2.4 Determination of Human hB Concentration

Concentration extracted HB sample was valued based on the difference of initial UV-Vis absorbance reading of the expired RBC and the final dialysed pure hB absorbance reading of UV-Vis spectrophotometry.

3.3 PREPARATION OF BOVINE HB

Commercial prepared dried bovine hB (0.204 gm) was suspended in 10 mL saline to make up to the concentration of 1.37x10^{-5} M.

3.4 PREPARATION OF POLYGLYCOL

HB : Polyglycidol ratio of 4 : 1 is estimated. A total of 1.182 g of polyglycidol is diluted in 1ml saline for encapsulating human hB solution of 1.59x10^{-4} M concentration. Where as 0.102 g of polyglycidol is diluted in 1ml of saline for encapsulating bovine hB solution of 1.37x10^{-5} M concentration. The polymer was previously synthesised and characterised by our group and ready for use without further purification.

3.5 ENCAPSULATION OF BOVINE HOBS AND HUMAN HB WITHIN POLYGLYCIDOL

Encapsulation procedure of both hB within polyglycidol was carried out by simple homogenisation technique as purified human hB solution (4 ml) and bovine hB solution
(4 ml) was added dropwise into the stirring 1 ml polyglycidol solution each at room temperature. The mixture was then continuously mixed until it is fully homogenous. Then the mixture is left for one hour to stabilised forming polyglycidol-hB complex. UV-Vis spectrophotometry absorbance reading was used calcuate the concentrations using the linear equation obtained from before. Encapsulation efficiency was determined from the difference between the pre-encapsulation concentration and post-encapsulation concentration and then converted to percentage. The maximum λ absorption of hB which is the Soret band is at the wavelength between 406 nm until 409 nm.

Encapsulation efficiency % = \frac{Post-encapsulation concentration}{Pre-encapsulation concentration} \times 100

### 3.6 REVERSIBLE OXYGEN BINDING STUDY

The principle is to study the binding and releasing oxygen and how many reversible cycles before total oxidation of encapsulated hBs and free-hB molecules. The saline prepared encapsulated polymer-hB complex prepared was positioned for 15 mins under continuous bubbling of nitrogen gas. Gas was administered by using a balloon and needles. The complex solution was transferred to glass cuvette sealed and air removed. The initial reading of UV-Vis spectrum was measured and recorded at Soret band of between 406 nm and 409 nm with Q band between 525 nm and 565 nm. Then, the hB-polymer complex solution still in the cuvette was placed under continuous oxygen bubbling to initiate oxygenation for 15 mins. After 15 mins, the solution is measured again and λ absorption of the oxygenated complex was recorded at 410 nm and 414 nm, Q bands at 505 nm and 545 nm. The complex solution is administered again with continuous bubbling of nitrogen gas for another 15 mins and then measured again at maximum λ absorption around 406 nm to 409 nm, Q bands 525 nm and 565 nm which
is further left than the reading of HBO₂. This complete one reversible cycle of oxygen binding. The proses was conducted repeatedly until total oxidation of hB molecule.

### 3.7 STATISTICAL ANALYSIS

The statistical analysis used was Statistic Package for Social Science (SPSS) version 22.0. For non-parametric data analysis in encapsulation study, analysis of the median differences in each group were calculated and analysed using Wilcoxon Singed-Rank Test. Mann-Whitney Test was used to compare among two different group between human hB and bovine hB interns of differences of absorbance and percentage of encapsulation efficiency. Results of reversible oxygen binding study was subjected to descriptive using line graph and comparison among non-encapsulated and capsulated hB and comparison between both hB groups using with Mann-Whitney Test. All statistical analysis results are expressed as median ± IQR. The association between the dependent and independent variables were considered significant if the p-value was less than 0.05 (p≤0.05).
CHAPTER 4
RESULTS AND DISCUSSION

4.1 ISOLATION OF HUMAN HB

In this study of developing haemoglobin-based oxygen carrier, the tetrameric globulin of human hB molecule was extracted from expired donated human blood carefully without denaturing it by using mechanical manipulation. The general goal is to modify the purified free-hB using polyglycidol by providing a new carrier for the hB molecule to reside in. In other words, the particular polymer chosen will replace the membrane of RBC to safeguard stability of the hB. The expired RBC used were within three days which is up to 45 days old. Lysis rate of RBC increases as red cells membrane structural integrity deteriorates as times passed towards expiry at 42 days.

RBC phospholipid bilayer membrane are weakened towards 42 days and the sonication process increases the breakdown the membrane without damaging the hB molecule Previous studies conducted by Willekwens and his collueges shows that RBC loses about 20% oh hB molecules during the latter half of its 120 days lifespan but most RBCs still contain intact hB molecules with all hB components and properties intact (Willekens et al., 2003: Willekens et al., 2009). In vitro, breaking down of bi-lipid membrane induced by sonication is in an even fashion compared to lysis induced by hypotonic solution that causes swelling and bursting of RBC membrane with the results of multiple by-products of different sizes and possible presence of ghost cells (Kostić et al., 2014).

Blood samples underwent procedures including multiple saline washing by centrifugation, usage of organic solvent and dialysis system for isolations. High speed centrifugation separates the heavier and denser hB molecules to the bottom as precipitate while hemolysate and other wastes will remain in the supernatant of the saline solution.
(Gardiner et al., 2016). However, it won’t be enough to remove all debris and remaining phospholipid by-products. A simple purification process using dialysis method to remove traces of phospholipid by-products is enough instead of using high-performance liquid chromatography (HPLC) bearing in mind that the study only involved of ex-vivo analysis. The dialysis method to removed excess by-products by using a cellulose tubing with a molecular weight cut off at size 14kDA. In principle, the final product of saline washing with hB was dialysed against pure water allowing the fragment of impurities to flow through the membrane leaving larger molecule such as hB molecules 64kDA in the tube for collection. Purification of process could be confirmed by using gel electrophoresis (Sakai et al, 1993). This step was absent in this study because as hB is the only molecule that has bigger size than 14kDA, which is the cut off weight of the dialysis cellulose tubing, thus keeping intact hB molecules in the dialysis tubes. Successful purification from hB isolation is determined by UV-Vis spectroscopy and the comparison of different spectra are demonstrated in Figure 4.1.

![Figure 4.1](image)

Figure 4.1 : UV-Vis spectrum of pre- (red) and post-dialysised hB (blue) molecule against expired RBC (green)
The absorbance graph of RBC shown at the green line is wavelength 417, 542 and 577 nm whereas the hB detected after dialysis shown with the blue line is at wavelength 407, 539 and 576 nm. The first peak which is the Soret band indicative of the macrocyclic porphyrin structure of the haem group. While two slight peaks further down the called Q bands are the results of oxygen binding between gas molecule and the porphyrin. The broad and right shifted Soret brand produced by RBC is caused by RBC phospholipid membrane. This membrane has overlapped the electron transition of the conjugated haem structure on to other molecular energy positions Hence the broad and low absorbance and right shift of the RBC green band at wavelength 417 nm. After sonofication, the membrane are broken down to release the hB molecules shown by the red Soret band with a left shift indicates succesfull lysis but still with the interference of excess bilipid product. The final narrow and sharp blue Soret band peak after dialysed free-hB shown with blue with the wavelength at 407 nm compare to 417 nm indicates succesfull lysis and isolation of human hB molecules (Mohammed and Vauthey, 2008).

4.2 ESTABLISHMENT OF BEER-LAMBERTS LAW GRAPH.

The final concentration of isolated and purified hB is double of its initial 12.5 g/dl concentration to give 25.0g/dl. Serial dilution of hB concentrations of free-hB were done then measured using UV-Vis spectroscopy. Data from collected from UV-Vis was plotted to give the Beer-Lambert plot as demonstrate in Figure 4.2 below. Beer-Lambert graph of manufactured bovine hB concentrate also established using the same dilution technique. Xenogeneic hB sources from animals which is the bovine hB and allogenic human stroma hB free solution have been developed in the past since 1900s to overcome the drawbacks associated with blood transfusion that uses natural hB molecule. Among the prime products discussed are Oxyglobin and Hemapure that utilized polymerised
bovine hB while Hemolink utilised cross-linked human hB. Both human hB and bovine hB tetramer structures are similar which composed of four subunits with the same tertiary folding and they each contain eight alpha helices with a few substitutions of amino acid valine with methine and deletion of histidine which cause a lower affinity of oxygen. Since there are plentiful sources of bovine blood, bovine hB is highly chosen to be studied against human hB especially oxygen binding properties which is one of the reason bovine hB was chosen for this study.

![Beer-Lambert's Law of Bovine and Human HB](image)

Figure 4.2: Establishment of Linear Regression Line of human hB ($y = 2178x + 0.4608, R^2 = 0.9427$) and bovine hB ($y = 289201x + 0.0452, R^2 = 0.9987$). This establish the relatioship of concentration and absorbance that they are directly proportional.

The linear relationship in the Figure 4.2 illustrates that both plots obey the Beer-Lamberts law which allows to establishment the linear equation of relationship between absorbance and concentration of human and bovine. They are directly proportional means that as the independent variable which is the concentration of hB solution increases, the dependent variable which is the absorbance increases as well. Derivation of the linear
equations are crucial and were used for determination of hB concentration in the next analyses.

Linear Equation Line for human hB : \( y = 2.2178x + 0.4608 \) (\( R^2 = 0.9427 \))

Linear Equation Line for bovine hB : \( y = 289201x + 0.0452 \) (\( R^2 = 0.9987 \))

4.3 ENCAPSULATION EFFICIENCY STUDY

Size of a hB molecule is only 5 nm in diameter, therefore it needs to be wrapped in a larger carrier for it to be able to function as an artificial red cell. For this purpose, the water soluble polymer polyglycidol was suggested to ensure tolerable space within its compartment for the hB guest molecule to be in. It is predicted that of four hB units are likely to enter in between the branches of polyglycidol structure.

An ideal encapsulation includes water soluble and ale to sustain its core interior hydrophobicity. The structure of polyglycidol used in this study with the presence of dendritic box was utilised to entrap the hB molecules through non-covalent binding forces. The water solubility property of polyglycidol ensures easy entrapment of hB but up to certain extent it may also allow the release of hB molecule. The presence of terminal hydroxyl (OH) of the polymer is to ensure solubility in water. Its interior linear units from one end to another made up the approximate 24 nm diameter while globular an architecture of the branching units offer spaces for the hB guest molecule to reside in.

The encapsulation process by mixing human and bovine hB solution to polyglycidol solution until homogenous ensure loading of hB into the polymer in the first place. The aqueous tolerance of hB will be non-covalently encapsulated within the hydrophobic dendritic box of the polymer. Considering the dendritic structure of polyglycidol, strategy of maximising encapsulation efficiency is by applying 1:4 polyglycidol: hB ratio. The UV-Vis spectrum of encapsulated hB and free-hB of both human hB and bovine hB are shown in Figure 4.3.
The Soret band of encapsulated hB-polymer complex for human hB and bovine hB display similar fashion as of intact RBC with at a lower absorbance, broader and a slight right shift of Soret band. These patterns indicating successful encapsulation into polyglycidol with stabilised hB molecule following pattern of hB molecules in the red cell membrane. After homogenous, the solution is to settle down for one hour. The stroma free hB molecule itself is not stable and forms non-covalent bond to the polyglycidol, centrifugation was not used for separation as the centrifugal force may release the hB protein out the polyglycidol shell. As there was no additional method to separate the free and the encapsulated hBs, extra precaution was taken when sampling of the encapsulated was being extracted. This is because the molecular weight encapsulated hB are heavier than free-hBs, therefore encapsulated hBs will settle at the bottom of the solution and the free-hB will remain at the top of the solution. So sampling of the solution for encapsulation was taken at the at the bottom to ensure only the encapsulated hBs are being tested. The pre-encapsulation and post-encapsulation concentrations of both hB were calculated using the absorbance value from Uv-Vis data and utilizing the respected Beer-
Lambert linear equation prepared earlier. The differences of the two were calculated to give results to the percentage of encapsulation efficiency as displayed in Table 4.1.

Table 4.1: The encapsulation efficiency percentage (EE%) of encapsulated hB (n=3), calculated by implying absorbance data UV-Vis into Beer-Lambert’s Linear Regression Line.

<table>
<thead>
<tr>
<th>N</th>
<th>Pre-Encapsulation Absorbance (A)</th>
<th>Pre-Encapsulation Concentration (M)</th>
<th>Post-Encapsulation Absorbance (A)</th>
<th>Post-Encapsulation Concentration (M)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 4.4204</td>
<td>1.513x10^{-5}</td>
<td>4.0014</td>
<td>1.368x10^{-5}</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td>2 4.3515</td>
<td>1.489x10^{-5}</td>
<td>3.7380</td>
<td>1.277x10^{-5}</td>
<td>85.9</td>
</tr>
<tr>
<td></td>
<td>3 4.3561</td>
<td>1.491x10^{-5}</td>
<td>3.8030</td>
<td>1.299x10^{-5}</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td><strong>Average = 88.1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Bovine HB**

|    | Human HB                        |                                   |                                  |                                     |        |
|    | 1 4.4235                        | 1.754x10^{-4}                     | 4.3501                           | 1.754x10^{-5}                      | 98.3   |
|    | 2 4.4235                        | 1.787x10^{-4}                     | 4.4168                           | 1.784x10^{-4}                      | 99.8   |
|    | 3 4.3588                        | 1.756x10^{-4}                     | 4.3183                           | 1.739x10^{-4}                      | 99.0   |
|    | **Average = 99.0**              |                                   |                                  |                                     |        |

In Table 4.2, Wilcoxon-Signed Test analysis of each group shows there is no significant difference (p > 0.05) of absorbance before and after encapsulation among the three replicates in bovine hB (p-value = 0.109) and human hB (p-value = 0.109). These suggest that the unencapsulated hB difference for each group did not differ among themselves. The difference of concentration and absorbance between pre-encapsulated and post encapsulated which is possible failed encapsulated hB does not affect the efficiency encapsulation. By using Mann-Whitney Test as shown in Table 4.3, analysis shows that there is no significant difference (p > 0.05) in absorbance and encapsulation efficiency with p-value of 0.100 among groups between bovine hB and human hB. This
demonstrates that human hB and bovine hB possess the same behaviour encapsulating into polyglycidol.

Table 4.2: Difference in UV-Vis peak absorbance and calculated concentration between pre-encapsulation and post-encapsulation (n=3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-Encapsulation</th>
<th>Post-Encapsulation</th>
<th>Z-statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine hB</td>
<td>Absorbance, (A)</td>
<td>4.361 (-)</td>
<td>3.8030 (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration, (M)</td>
<td>1.492 X 10^{-5}(-)</td>
<td>1.299 X10^{-5}(-)</td>
<td>1.604</td>
</tr>
<tr>
<td>Human hB</td>
<td>Absorbance, (A)</td>
<td>4.4235(-)</td>
<td>4.3500(-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration, (M)</td>
<td>1.787 X10^{-4}(-)</td>
<td>1.754 X 10^{-4}(-)</td>
<td>1.604</td>
</tr>
</tbody>
</table>

*(-)=IQR was not applicable as the sample size is too small.
*a=Wilcoxon Signed-Rank Test

Table 4.3: Absorbance difference and encapsulation efficiency percentage comparing between group of post encapsulation bovine hB and post-encapsulation human hB.

<table>
<thead>
<tr>
<th></th>
<th>Human (n=3)</th>
<th>Bovine (n=3)</th>
<th>Z-statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance Difference,(A)</td>
<td>0.0405(-)</td>
<td>0.5531(-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Encapsulation Efficiency,(%)</td>
<td>99.0%(-)</td>
<td>87.5(-)</td>
<td>1.964</td>
<td>0.100b</td>
</tr>
</tbody>
</table>

*(-)=IQR was not applicable as the sample size is too small.
*b=Mann-Whitney Test

Nature of hydroxyl structure of polyglycidol are easily modified may block the entrance of hB molecule but application of slight force such as slow stirring during homogenisation would increase the chances of encapsulation. Regardless, the difference of concentration and absorbance between pre-encapsulated and post encapsulated which
is indicating possible failed encapsulated hB molecules together with the results of average high encapsulation efficiency percentage of 88.1% (bovine) and 99.0% (human) suggesting that possible of three hB units are captured within the polyglycidol as shown in Figure 4.4. As one unit hB molecules able to bind four oxygen molecules, three molecules within the polyglycidol are able to carry 12 haem/globin sub-units binding with 12 molecules of oxygen.

Figure 4.4: Representative image of possible encapsulation of at least three hB molecules encapsulated into polyglycidol. Each molecule is trapped between two branches of polyglycidol non-covalently.

It is anticipated that hB molecule, a hydrophobic guest would load with a better efficiency into hyperbranched polymer. Higher concentration of glycidol resulting bigger and wider hyperbranching to be able to carry more hB molecules would be a motivating approach. Nevertheless, this polyglycidol system demonstrates its ability to form an entrapment system in a host-guest partnership. Next part is on the reversible oxygen binding of the encapsulated hB-polyglycidol system.
4.4 REVERSIBILITY OXYGENATION STUDY.

The samples were first activated through bubbling nitrogen gas within a closed system. The encapsulated initial UV-Vis reading of hB Soret band recorded at absorbance of 4.3844 (1.77 X 10^{-4} M) for human hB and 4.2027 (1.44 X 10^{-5} M) for bovine hB between wavelength 406 nm and 412 nm. At this state, the haem subunits were activated which is the ferrous (Fe^{2+}) state and are ready to bind oxygen molecules. This study started by administering oxygen gas to the polymer encapsulated hB continuously. As oxygen is binding, absorbance change to 2.2426 (8.03 X 10^{-5} M) for human hB and 2.2422 (7.60 X 10^{-6} M) for bovine hB shows the changes of structure of the hB iron porphyrin ring with oxygen molecules bound. Subsequently, nitrogen gas was administered to the six samples again by vigorous bubbling to release the oxygen and increasing the absorbance peaks back towards the initial absorbance reading. This marks one cycle of complete oxygen binding. The UV-vis spectra demonstrating the absorbance shift upon deoxygenation and oxygenation are shown in Figure 4.5.

![Reversibility Oxygen Binding Study of Polyglycidol-hb](image)

Figure 4.5 : Representation of one complete cycle of oxygenation and deoxygenation shift pattern for encapsulated bovine hB (dotted) and encapsulated human hB (line).
The finding shows that hB-polyglycidol system is capable reversibly bind and release oxygen even with slight deoxygenation with both hB shown by UV-Vis spectroscopy. The deoxygenation peak (orange) remains at absorbance 2.2892 (8.24 X 10^{-5} M) and 2.3068 (7.82 X 10^{-6} M) which are nearer to oxygenated peak (green) instead of initial activated peak (blue). These shows an incomplete deoxygenation thus only slight increase in the absorbance. These solutions hardly deoxygenated regardless of the volume of nitrogen bubbled through the solution after approximately average of five binding cycles before complete oxidation.

We anticipated that the administration of nitrogen to release oxygen could raise the absorbance back to activated absorbance to complete one cycle. The formation of oxygenated iron in hB within 15 minutes of oxygen gas exposure was shown by the drop of absorbance peak. However, the slow release of oxygen by nitrogen gas to reactivate the ferrous (Fe^{2+}) state seen with only slight increase deoxygenation line could be due to its nature of iron that is unfavourable to nitrogen.

Oxygen binding and releasing of the hB molecule are influenced by several external factors such as environment, pH and temperature. Pressure in the lungs are very strong to help bind oxygen into millions of hB molecules in a single RBC. Partial pressure of nitrogen gas was not optimum enough to dislodge oxygen from hB hence only a slight increase of absorbance suggesting 15 minutes of nitrogen exposure was insufficient. Furthermore, atmospheric pressure of the environment in the laboratory in this study could not mimic the physiological pressure in the lung where hB binds and release oxygen into the system. These results suggest for longer exposure and a higher pressure administration of nitrogen gas and in an enclosed system might produce better reversible oxygen results.
Non-covalent interaction between molecules are weak in nature and that suggests easy dislocation of hB molecules within its host. Therefore, the weak bond of guest molecule, hB and the hydrophobic interior of polyglycidol posted a risk of the encapsulated molecules escaping from the carrier to remain freely in the solvent. Furthermore, hB molecules encapsulated non-covalently may not necessarily resided in the central position but could be in various location within the polyglycidol structure including the peripheral sites. The hB molecules located near the periphery could have a higher risk of contact with the solvent which causes higher affinity of oxygen resulting small shift of absorbance towards activated initial activated state. This theory suggesting the possibility of better results with a covalent bond system using the same polymer but with iron which chemical compound of the hB as the core.

A normal life cycle of natural RBC throughout its lifespan of 120 days would experience about 160,000 reversible oxygen binding cycles. Therefore, endurance is an important factor to consider when developing a substitute for blood to undertake as many cycles as possible. A stable system should maintain hB stability until the active state undergoes irreversible degradation. The results of reversible cycle were recorded and shown in Table 4.4. For human and bovine non-encapsulated hBs, they went through average of one complete cycle where as the encapsulated hBs were able to show oxygen reversibility cycles with the average of five cycles for encapsulated bovine hB and six cycles of encapsulated human hB despite its relatively good stability.
Table 4.4: Average reversible oxygen binding cycles recorded of non-encapsulated and encapsulated polyglycidol-hB complex (n=3).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Average Reversible Cycles</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

In Table 4.5, statistic shows that the reversible oxygen cycles of both non-encapsulated hBs shows no significant difference (p > 0.05) as compared to encapsulated hB for both human hB (p-value=0.1) and bovine hB (p-value=0.1) groups. Nevertheless, the encapsulated hBs demonstrated better capability to bind oxygen reversibly with evidence of five to six cycles compared to free-hB which only recoded average of only one reversible cycle.
Table 4.5: Comparison of average reversible cycles of free-hB and encapsulated human hB and bovine hB using Mann-Whitney Test analysis.

<table>
<thead>
<tr>
<th>HB Groups</th>
<th>Median Reversible Oxygen Cycles</th>
<th>Z-statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-encapsulated hB (n=3)</td>
<td>Encapsulated hB (n=3)</td>
<td></td>
</tr>
<tr>
<td>Bovine hB</td>
<td>1(-)</td>
<td>6(-)</td>
<td>2.121</td>
</tr>
<tr>
<td>Human hB</td>
<td>1(-)</td>
<td>6(-)</td>
<td>2.087</td>
</tr>
</tbody>
</table>

<sup>*</sup>Median (IQR) was not applicable as the sample size is too small.

<sup>b</sup>Mann-Whitney Test

Environmental factors that influences binding and releasing oxygen such as pH at 7.0 and temperature at 37°C using waterbath were able to control in the laboratory which was evidenced by the five to six cycles. According to Yao and team, pH has an effect on structure and function of an RBC to bind and to release oxygen (Yao et al, 2003). Therefore, possible reason of incomplete duxygenation is due to the incompatible pH for hB encapsulated in polyglycidol as polyglycidol is not lipid-based compared to RBC membrane. Uncontrollable factors such as vigorous bubbling causes the solvent to tend to evaporate leads to the end of reversibly cycles.

Moreover, when the oxygen partial pressure is higher, only the free stroma hB has an affinity for oxygen. So, the free hB molecules has faster degradation rate and leads to limiting its oxygen binding abilities. Therefore, stabilising the hB using polyglycidol in this study as a membrane substitute optimises any facilitation of oxygen binding to occur without any obstacles.

As this study uses a balloon for gas administration on the polyglycidol-hB solution, the pressure is not consistent. Fluctuation of vigorous gas bubbling done manually might have triggered the hB release from polyglycidol host no matter how
minimal due to the water solubility preventing the release of oxygen when was visible directly to solution outside causing faster oxidation of encapsulated hB resulting the halt of reversible cycles.

From the findings above, the absence of carrier after losing the phospholipid bilayer of a red cell exposes to faster degradation, limiting the oxygen reversibility capacity. Thus, it is vital to stabilise the hB unit using appropriate strategies such as carbon monoxide (CO). Functionality of CO comes in handy because of its high affinity for transition metal. It can bind to hem 210-250 times than oxygen molecules. They also function as suppressing the release of oxygen from other hem site (Quaye, 2015). Therefore, administration of CO before lysis could highly improve hB molecules stability by prevention oxidation of free-hB to reach optimum stability and then only includes the variety of material for encapsulation strategies such as polyglycidol of micelle system as carriers for possibility of reversible oxygen binding study.

A study done by Ratanasopa and his colleagues explains that initial binding of nitrate to the hB molecule is favoured activating to functional Fe$^{2+}$ which results in easy oxygen binding. The ferrous state is then reduced by excess nitrate thus yielding FeIII-NO2 which concludes that presence of high concentration nitrate produces methaemoglobin which has very low affinity to oxygen and nitrate as final product. As the cycles continues, the gradient formation of metHB increases until the total loss of uptake of oxygen leading to the stop of the reversible cycles (Ratanasopa et al., 2015).

Using Mann-Whitney Test, the oxygen reversibility cycles recorded shows no significant difference (p > 0.05) between both encapsulated human hB and encapsulated bovine hB (P-value=0.0700) show in Table 4.6. This demonstrates that human hB and bovine hB possess the same behaviour encapsulated in polyglycidol.
Table 4.6: Comparing reversible oxygen cycles among hB group of encapsulated human hB and encapsulated bovine hB.

<table>
<thead>
<tr>
<th></th>
<th>Median (IQR)</th>
<th>Z-statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Encapsulated Human hB (n=3)</td>
<td>6(-)</td>
<td>0.696</td>
<td>0.700b</td>
</tr>
<tr>
<td>Encapsulated Bovine hB (n=3)</td>
<td>6(-)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*(-) = IQR was not applicable as the sample size is too small.
*b = Mann-Whitney Test

Even though this study shows polyglycidol-hB system has limited oxygen reversible capability for now, but it shown that there is a possibility of polyglycidol to as a hB carrier loaded with bovine hB.
CHAPTER 5
CONCLUSION, LIMITATION, RECOMMENDATION AND FUTURE STUDY

5.1 CONCLUSION AND LIMITATION OF THE STUDY

Issues of increasing risk of infection, blood incompatibility, and shortages of volunteer donors have triggered the interest on blood substitutes. Products based on hB molecules are among the most popular approach to develop red cell substitute. The isolation and purification processes are critical to prevent degradation of hB molecule which contamination could cause organ toxicity. Repeated washing, vigorous centrifugation, sonication and dialysis process were carried out to make certain the hB is purely isolated. Gel electrophoresis could have been carried out to confirm the purity of only intact hB with presence of one band. However, the steps taken was adequate to produce pure hB for this study in vitro.

Strategy and technique to maximise encapsulation of the bovine hB into polyglycidol need improvement as compare to human hB to ensure for more efficient encapsulation of hB units between dendritic branches. The polymer system offered hydrophilic exterior for solubilisation in aqueous environment while hydrophilic interior for the guest molecule, the hB unit to reside in via non-covalent chemistry. Glycidol as monomer can be manipulated by size having in longer or wider chain. More vigorous stirring and mixing during encapsulation may also increase encapsulation efficiencies. An improvement of the system utilising the chemical compound of the hB, the iron covalently with polyglycidol could carry oxygen the more firmly within its interior compartment.

In the encapsulation process, this study is limited to slow and minimal mixing during homogenous process because of the fragility of hB molecule as no modification such as carbon monoxide was used to improve hB stability.
In the reversible oxygen binding study, the ability for the selected hB-polyglycidol is to reversibly bind and release oxygen specifies the potential of this system as red cell substitute. However, this study was limited to only three replicates due to insufficient expired human donated blood available. Moreover, 15 minutes for each gas exposure interval was applied during oxygenation and deoxygenation was insufficient was findings show incomplete deoxygenation.

The experiment conducted comparing both human hB and bovine hB showed the similarity of both hB, the isolability of the hB and the capability for encapsulation as well as its reversible oxygen binding and stability. This research shows the possible usage of polyglycidol being use as a carrier that mimics RBC phospholipid membrane loaded with bovine hB comparing to human hB extracted from expired blood. Thus, the findings in this study add to the depth of knowledge for any upcoming study related to the progress of blood substitute development in this country.

5.2 RECOMMENDATION AND FUTURE STUDY

Limitation present in this study required further improvement. Better representation of data if possible with gel electrophoresis to confirm successful isolation and purification of human hB. Establishment of carbonylated hB with carbon monoxide which has a higher binding affinity than oxygen will increase hB stability and can be easily recorded by UV-Vis spectrometry. This prevent denaturation of hB during the process of extraction from RBC solution. Therefore it will further enhance the isolation process and the purity of intact hB. Increased stability of carbonylated hB may also enable the possibility of more vigorous mixing or using centrifugation force during homogenous process. If this happens, it may maximise encapsulation efficiency by preventing hB release from the carrier. In the reversible oxygen binding study, alternation of gases at
longer exposure especially the introduction of nitrogen gas could improve precision of the result. Recommendation for closed system in handling the sample also to prevent evaporation during gas bubbling. Time intervals of gas flow could be prolonged for better binding and releasing of oxygen.
REFERENCES


