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POLYMERIC NANO-ENCAPSULATION OF HAEMOGLOBIN ISOLATED FROM EXPIRED HUMAN REDBLOOD CELLS FOR USE AS BLOOD SUBSTITUTE

BY

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DECLARATION

I hereby declare that this research has been sent to Universiti Sains Malaysia for the degree of 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.

Khadijah binti Mahmud

P-IPM 0041/17

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LIST OF SYMBOLS AND ABBREVIATION

°C	-	degree Celcius
hb	-	haemoglobin
g	-	gram
mL	-	milliliter
µg/L	-	microliter
h	-	hour
%	-	percentage
DMSO	-	Dimethyl sulfoxide
et al.	-	and others
DCM	-	Dichloromethane
RBC	-	Red blood cells
SD	-	Standard deviation
DMEM	-	Dulbecco's Modified Eagle's Media
nm	-	nanometer

HEMOGLOBIN DIASINGKAN DARIPADA SEL DARAH MERAH MANUSIA YANG SUDAH MELEPASI TARIKH LUPUT YANG DIKURUNG DALAM POLIMER UNTUK KEGUNAAN SEBAGAI DARAH ALTERNATIF

ABSTRAK

Sehingga kini, kekurangan darah dalam perkhidmatan transfusi menyebabkan kajian saintifik giat dijalankan untuk mencari alternatif yang dapat memberi fungsi seakan sel darah merah. Dalam kajian ini, kami berhasrat untuk mengekstrak dan menulenkan hemoglobin manusia (hb) daripada sel darah merah yang telah tamat tempoh dengan melalui teknik yang melibatkan cucian berganda, sonikasi, kaedah pengekstrakan organik dan dialisis. Perbandingan analisa spektrum UV-Vis hb dan sel darah merah menunjukan bahawa protein tersebut telah berjaya diasingkan. Kemudian, hb tersebut telah dikapsulkan dengan misel polimer melalui interaksi hidrofobik oleh *poli (etilena glikol)-b-poly (Ɛ-kaprolakton)* (PEG-PCL), iaitu dua blok polimer-bersama larutan penimbal. Kompleks hb-misel kemudiannya dianalisa untuk menentukan jangka separuh hayat, kemampuan mengikat oksigen secara berbalik serta ujian terhadap sel-sel normal. Secara purata, keberkesanan pengkapsulan hb oleh polimer ialah 45%. Hal ini memandangkan kelarutan hb bebas dalam larutan akueus adalah tinggi lantas menghadkan kemasukan hb ke dalam polimer. Namun begitu, 2 unit hb secara puratanya telah berjaya dilingkungi dalam polimer tersebut. Hb-misel telah merekodkan jangka hayat separuh yang lebih tinggi iaitu 112,242 minit berbanding 30 minit yang dicatatkan hb dalam ujian kestabilan bawah pendedahan udara. Sistem hb-misel juga menunjukan kemampuan untuk merekat oksigen secara berbalik. Proses oksigenasi dan nyahoksigenasi mampu direkodkan sehingga purata 6 kitaran sebelum sampel terhidrasi

akibat pengoksigenan, berbanding hanya 1 kitaran purata oleh hb bebas sebagai kawalan. Dalam ujikaji keserasian sel, sampel hb-misel dengan kepekatan hb sebanyak 1.89 $x10^{-5}$ mg/mL dalam dua penyediaan misel 1.0 dan 2.0 mg/mL, manakala hb kawalan ialah sampel hb daripada manusia dan lembu yang berkepekatan 0.1 dan 0.2 mg/mL dan hb berasaskan lembu-misel (kepekatan hb 2.21 $x10^{-5}$mg/mL) telah diuji ke atas sel makrofaj tikus (RAW 264.7) yang telah diinkubasi selama 24 jam sebelum rawatan. Sel terawat kemudian diinkubasi selama 48 jam yang mana kadar pertumbuhan sel dianalisa pada titik masa 4, 8, 24 dan 48 jam. Secara umum, hb bebas merekodkan pengurangan pertumbuhan sel sebanyak 10% dan 18% seawal 4 jam berbanding hb-misel yang tidak menunjukkan kesan-kesan keracunan ke atas sehingga selepas 24 jam. Hal ini menunjukkan hb bebas pada dos yang digunakan mungkin masih mengandungi sisa lipid daripada pecahan membran sel darah merah semasa proses pengekstrakan. Namun, penemuan dalam kajian ini menunjukkan sistem hb-misel dapat berinteraksi secara selamat dengan sel normal untuk penggunaan masa depan yang meyakinkan berbanding dengan hb yang bebas tanpa perlindungan polimer. Penemuan ini menunjukkan bahawa potensi hb yang telah diekstrak dari sel darah merah yang sudah tamat tempoh dan dibalut oleh polimer-bersama berpotensi untuk dikembangkan sebagai alternatif sel darah merah.

POLYMERIC NANO-ENCAPSULATION OF HAEMOGLOBIN ISOLATED FROM EXPIRED HUMAN RED BLOOD CELLS FOR USE AS BLOOD SUBSTITUTE

ABSTRACT

To date, blood scarcity in transfusion service demand vigorous scientific works looking for possible red blood cells alternatives. In this study, we aim to isolate and purify human haemoglobin (hb) from expired red cells by multiple washing, sonication, organic extraction and dialysis method. Analysis of the UV-Vis spectrum comparing hb and red blood cells (RBC) showed that the protein has been isolated successfully. Subsequently, the hb was encapsulated by polymeric micelle upon hydrophobic interaction (micellisation) of the methoxy poly (ethylene glycol)-*b*-poly (Ɛ-caprolactone) (PEG-PCL), a diblock copolymer in an aqueous buffer. The hb-micelle complex was then further analysed for half-life, oxygen reversibility, and cell-viability assay. Average encapsulation efficiency of the hb within the polymeric micelle achieved 45%. This is due to the high solubility of free hb in aqueous solution limiting the entry of hb into the polymer. However, the result indicated that the average of two units hb were successfully encapsulated by each polymer micelle. The hb-micelle recorded a higher half-life of 112,242 mins as compared to 30 mins of the free hb alone during oxygen stability study. The hb-micelle system also showed the ability to bind oxygen reversibly. Oxygenation and deoxygenation processes recorded average of six cycles before the sample was evaporated due to oxygenation, in contrast with only one cycle at average for free hb as a control. In cell viability study, the hb-micelle sample with concentrations of 1.89 $x10^{-5}$mg/mL in two preparations of micelles at 1.0 and 2.0 mg/mL

while as a control, free hb sample from human and free bovine at 0.1 mg/mL and 0.2 mg/mL concentrations and also bovine hb-micelle (hb concentration at 2.21×10^{-5} mg/mL) have been tested on murine macrophage cells (RAW 264.7), incubated for 24 hours before treatments. Treated cells were then incubated further for 48 hours where the cell viability analysed at time points of 4, 8, 24 and 48 h. Generally, the free hb recorded reduction in cell growth approximately by 10% and 18% as early as 4h compared to hb-micelle which showed no toxic effects on the cells until 24 hours onwards. This result probably due to the free hb from human used in the test may have contained lipid debris from the membrane lysis during extraction process. However, it showed that hb-micelle system can safely interact with normal cells for potential future use compared to free hb without encapsulation by polymer. These findings indicated the potential of the hb 'harvested' from expired human red cells encapsulated by the diblock copolymer for development as red blood cells substitute.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Blood is an essential component circulating in human body and plays several crucial physiological roles. However, the main task of blood is delivery of oxygen gas from respiratory system throughout the body before taking up carbon dioxide gas back to the lungs for exhalation. Human blood comprises of two main parts; plasma and formed elements. Plasma contains mostly water, hormones and nutrients whereby ninety percent of formed elements are red blood cells (RBC) and the remaining are white blood cells and platelets (Bhadange *et al.*, 2015).

The task of delivering of oxygen and carbon dioxide up-taking in human body lies upon RBC. Current blood transfusion practice uses emergency O blood group in cases such as trauma and road accident as well as in unknown blood or rare blood group. However, transfusion of pack RBC raise several issues such as the risk of transfusion transmitted infection such as HIV, transfusion reaction, limited viability, short shelf-life and others (Goodnough, Shander and Brecher, 2003).

Current trend on annual blood demand indicates the need to attend more elderly group, blood disorders and malignancy patients and other medical treatment. Besides adverse transfusion reaction, blood donation awareness amongst public in a country like Malaysia is still relatively low causing blood scarcity especially during festive seasons (Cohn & Cushing, 2009). This

calls for an effort to venture into development of blood substitute to possibly compensate the demand-supply gap.

Global research on blood substitute and artificial oxygen carrier have been going on for the past decades in the quest of finding the best candidate that suits biocompatibility, optimum oxygen delivery and other important characteristics. Short-term aim foresees application of artificial blood to complement post-haemorrhagic fluid therapy such as colloids or crystalloids during trauma event for instance. It may give better solution than the two, because not only it can increases blood volume and restore cardiac output during massive bleeding, but it also enhances of tissue oxygenation especially in poorly perfused area (Creteur & Vincent, 2009).

Several approaches have been taken to design blood substitute which includes natural model (biotic) and synthetic (biomimetic) model (Castro and Briceno, 2010). The former is categorised into haemoglobin-based oxygen carrier (HBOC) and stem cell-derived RBC while the later have perfluorocarbon (PFC) and polymer-based oxygen carrier. This study will explore the development of synthetic oxygen carriers using polymeric material known as diblock copolymer micelle. Such approach has been proven to be successful in certain drug delivery application utilising host-guest molecules interaction model. For example, the encapsulation by poly(ethylene glycol)-block-poly(Ɛ-caprolactone) (PEG-b-PCL) micelles of Docetaxel has increases the drug solubility and stability (Makadia & Siegel, 2011). The polymer will be formulated with natural hb before reversible oxygen binding profiling and cell viable testing are furthered.

1.2 PROBLEM STATEMENT

According to World Health Organisation (2017), almost 112.5 million blood units were collected in the 180 countries collected in year 2016 over the world. Global donor to population ratio is only 2.5% while annual blood demand increasing by 6-8%. In Malaysia, annual donation is increasing only by 2-3% leading to 15-20 thousand shortage units per year . The trend will escalate further if more donor selection restrictions are imposed in near future such as during disease outbreak or natural disaster. The ageing population will also influence with blood transfusion demands added with limitation of donor pools. Due to several issues with human blood transfusion such as the afore mentioned HIV or other TTI like hepatitis, compatibility due to blood group antigens and availability in remote or restricted area, research on blood substitute could possibly be the key answer to the problems. Most of the works pertaining development of artificial blood products focus on few key ideal features of an ideal substitute including reversible oxygen binding capacity, half-life (stability) in plasma and biocompatibility.

Some blood substitutes such as perfluorocarbon and other hb based oxygen carrier (HBOC) have gone through several clinical trial phases. But most of the products were withdrawn form clinical application expect in countries like Mexico and South Africa but limited to certain medical procedures. HBOC needs chemical modification to package the hb for its use. This is due to the fact that free hb may cause renal toxicity, extreme oxygen affinity, compromise structural integrity and limited half-life (Napolitano, 2009). One of the potential sources to obtain hb is from expired blood bag. Upon isolation from its membrane, the hb will be harvested and purified for further chemical modification using polymer.

1.3 RATIONALE OF THE STUDY

Raising issues associated with allogeneic blood transfusion and blood procurement specifically in advanced developing country like Malaysia have triggered a move towards embarking research and development for potential blood alternatives. Ideally the product must be universally compatible with any recipient with additional of other key features mentioned earlier. The use of expired blood as hb source seems to be cost effective to start with. Post 42 day of shelf-life, oxygen saturation in the bag is lowering so does the weakening of RBC phospholipid membrane integrity that may lead to lysis of the cells. Nonetheless, the hb should remain intact even upon membrane lysis which allows harvesting of the haemoprotein within 3 days of post expiry (up to day 45). The challenges are to isolate the hb efficiently and purified it before further preparatory steps. One of the effective method to induce membrane lysis for isolation of hb is sonication (Cruz *et al.*, 2014). The degree of membrane rupture is more efficient and uniform as compared with other approaches like hypotonic solution which produces lipid by-products of different sizes, making purification process harder (Andrade et al., 2004). Nonetheless, multiple washings, organic extraction and dialysis methods are anticipated to be adequate to purify hb resulted from sonication induced lysis. The hb will be subsequently encapsulated within methoxy poly (ethylene glycol)-*b*-poly (Ɛ-caprolactone) (PEG-PCL) micelle using host and guest moiety for haemoprotein mimicry. This approach hopefully could offer better oxygen binding reversibility, stability and biocompatibility, added to its blood group universality blood group that is storable at room temperature with potentially longer shelf-life.

1.4 RESEARCH OBJECTIVES

Main objective

To study polymeric nano-encapsulation of hb isolated from expired blood for development of artificial RBC.

Specific Objectives

1. To investigate isolability of hb from expired RBC.
2. To analyse encapsulation efficiency of hb with polymer micelle.
3. To assess oxygen binding reversibility and stability of the encapsulated hb.
4. To determine cytocompatibility level of the encapsulated hb.

1.5 RESEARCH QUESTIONS

1. What is the effect on sonication of isolation and purification of hb?
2. What is the loading percentage of hb molecule within the polymeric micelle?
3. Will the hb encapsulated micelle bind oxygen reversibly?
4. How long can the hb encapsulated micelle remain in its active state under continuous air exposure?
5. What is the cytocompatibility level of the hb encapsulated micelle on normal cell?

1.6 RESEARCH HYPOTHESIS

Null Hypothesis (H_0)

There is no difference on encapsulation efficiency, oxygen reversibility, stability and cytocompatibility of hb encapsulated polymeric micelle against free hb.

Alternative Hypothesis (H_1)

There is difference on encapsulation efficiency, oxygen reversibility, stability and cytocompatibility of hb encapsulated polymeric micelle against free hb.

1.7 SIGNIFICANCE OF THE STUDY

This study will be the pioneering work for blood substitute research in Malaysia. Any findings will help to add new knowledge for development of future blood substitute. The use of polymer like polymeric micelle is a potential candidate to be the next artificial oxygen carrier. It offers capacity to load the oxygen carrying protein similar to other application such as loading of poorly soluble drug. The preparation steps are scalable to mass production due to its purification simplicity. Moreover, the use of expired blood in this study will minimise blood wastage and subsequently improve the efficiency of blood bank inventory management. Besides, the findings from this study may contribute to successful development of artificial blood in dealing with emergency cases as not ónly the artificial blood won't require cross-matching, it can also be storable in room temperature in the ambulance and is easily prepared by dilution with saline prior to its administration. However, more works are needed especially animal study preceded by clinical studies and should the product pass through, we may revolutionise blood transfusion service in Malaysia at the minimal of being an alternative or complementary to human blood in certain cases.

CHAPTER TWO

LITERATURE REVIEW

2.1 BLOOD SUBSTITUTE

Blood substitute or as denoted in several other terms such as oxygen carrier, oxygen therapeutic and artificial blood refer to alternative blood products that are potentially capable to deliver oxygen and several other functions in human body. Research activities in blood substitute had begun in the 1960s where iron-rich hb complexes were used to deliver oxygen to tissues but they triggered some side effects such as kidney toxicity (Moradi *et al.*, 2016). In early 1950s, the research works had focused on several approaches that include the use of hb particles due to its competency to carry adequate oxygen and compatibility to act as physiological oxygen carrier (Alam *et al.*, 2014). There are at least four strategies of blood substitute that are known to date including perfluorocarbons (PFC), haemoglobin-based oxygen carrier (HBOC), polymer-based oxygen carrier and stem cell derived RBC.

PFC is a clear colourless, inert and promising of nontoxic properties with low boiling point temperatures. It is insoluble in water and alcohol and present in straight and cyclic form with shown that straight form poses better ability to carry oxygen than cyclic form. The size of PFC is much smaller compare to RBC which enables them to pass through vessel that RBC cannot pass in treating certain diseases (Moradi *et al.*, 2016). Some of the PFC products such as Fluosol and Oxygent have managed to enter advanced clinical trial phases but none were approved for clinical use due to limited benefits and their related research and development works have been stagnant or discontinued (Schmidt *et al.*, 2016).

Meanwhile, HBOC has become remarkable candidate because of its natural properties to carry oxygen and hold no blood group antigen. Upon removal of RBC membrane, the stroma of free hb requires further chemical modifications such as illustrated in Figure 2.1. There are mainly four types of chemical modification approaches to prepare hb; surface-modified hb, cross-linked hb, polymerised hb and hb liposomal capsule. Formulation of stroma-free hb from purified human or bovine blood cells needs to be accompanied with additional chemical modification step to increase its stability for efficient therapeutic intervention. Some blood substitute consists an intermolecular crosslinking between α and β subunits using a site-specific cross-linker to achieve better stability. Conjugation to polymer such as polyethylene glycidol is also a helpful method to add the effective molecular weight of hb. Besides, polymerisation approach would generate hb of molecular weight greater than the normal haemoglobin size via poly-functional cross-linking. Alternatively, the hb can be encapsulated within microsomes or liposomes as well as other guest carrier macromolecules in order to establish the natural structural properties of natural RBC (Jia *et al.*, 2016).

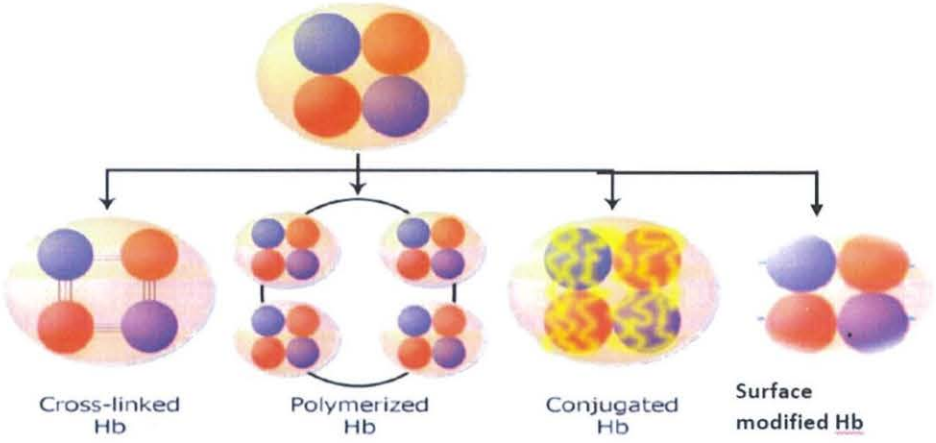

Figure 2.1: The diagram shows four major types of modification of HBOC structure to prevent the breakage of Hb chains. (Adopted from Moradi *et al.*, 2016)

The half-life of HBOC is between 18 to 24 hours, which is considered relatively shorter than the 120 days of normal RBC but useful in dealing with emergency transfusion such as fluid replacement therapy during acute massive bleeding (Napolitano, 2009). Also, HBOC products have superior shelf-life, up to 1-2 years as compared to the 42-days of RBC and some are storable in room temperature. (Keyhanian *et al.*, 2014).

Some artificial blood substitutes have accomplished their safety trial at the present and currently improving the efficiency of the product. After the discovery of myoglobin and hb structure by Perutz and his friend in 1960's, many scientists concerns revolve around the functionality of the molecular structure and how to modify it for use in their field (de Chadarevian, 2018). To date, there are four HBOC products that have been in advanced stage for preliminary and clinical testing of the regulatory approval processes. The comparison of those haemoglobin-based artificial blood products (Henkel-Hanke & Oleck, 2007; Napolitano, 2009) *Hemopure & Oxyglobin, Polyheme* and *Hemolink* are as listed in Table 2.1.

Table 2.1: Comparison of HBOC products

Products	*Hemopure & Oxyglobin*	*Polyheme*	*Hemolink*
Source	Bovine hb	Human hb	Human hb
Approach	Polymerised hb	Polymerised hb	Polymerised hb
Company	Biopure Corporation	Northfield Laboratories Inc.	Hemosol Corporation
Status	Hemopure in phase III clinical trial (completed), Oxyglobin licence for use in South Africa	Phase III clinical trial (inprogress)	Discontinue after Phase III

There are several ideal characteristics of blood substitute such as its ability to carrying oxygen and carbon dioxide, cost effective, not necessary for cross matching and blood typing, reduce of impurity and infectious agents, readily available, easy storage environments, long half-life in the body, can be excreted completely from the body and lack of toxicity as well as not triggering other adverse reactions (Castro & Briceno, 2010). However, before blood substitute can be formulated, design strategy to ensure its optimum capacity as RBC mimic lies on a comprehensive understanding of hb structure prior to any modification approaches.

2.2 HAEMOGLOBIN

Works to produce haemoglobin-based oxygen carrier (HBOC) demand a comprehensive understanding of hb structure before any RBC mimic can be designed. Haemoglobin is an iron-containing oxygen transporter comprising of four polypeptide globin chains (Figure 2.2). The haem group in the hb refer to complex of iron (Fe) with protoporphyrin IX as the non-protein group responsible in binding oxygen and other gaseous and non-gaseous ligand (Alam *et al.*, 2014). Each erythrocyte or known as RBC contain up to 300 million molecules of hb. In normal adult, most of the hb is in the form of haemoglobin A (hb A) consisting of two a- and two b-globin chains. Normal level of hb in men is 13.5–18.0 g/dL and 11.5– 16.0 g/dL in women. This hb has an approximately a molecular mass of 64 kDa with a dense quaternary structure. (Thomas & Lumb, 2017).

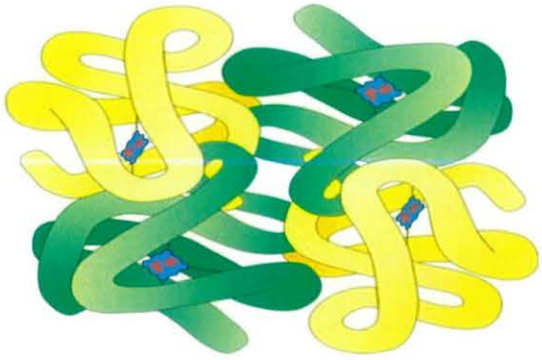

Figure 2.2: The diagram represents fundamental structure of hb A with green and yellow colour denote the structure of α-globin chains and β-globin chains respectively. Each subunit of globin chain consists of a haem–iron complex (blue). (Adopted from Thomas & Lumb, 2017).

The biconcave shape of RBC membrane that surround the hb have a large surface area for rapid gas exchange, a flexible structure to flow through the capillary, and can maintain the structure in addition to give internal environment for hb to function. Free hb that is not encapsulated by this membrane can potentially cause harmful effects by stimulating the phagocytosis process through specific receptors on macrophage (Gordon-Smith, 2013). Apart from that, RBC consists many 2,3-diphosphoglycerate (2,3-DPG) molecules that employs the most efficient control of oxygen affinity in human. 2,3-DPG raises the distribution of oxygen from hb by binding between β-globin chains of deoxygenated hb, hence, the protein structure modified and successively diminishing the oxygen affinity of the hb (Thomas & Lumb, 2017).

As mentioned before, raw hb cannot be used because it can trigger immune response as it would break down into smaller, toxic compounds in the body. Free hb also has issue with the stability of the hb molecule which leads to extravasation and fast renal excretion apart from having high oxygen affinity of hb in suspension which restricts with oxygen uptake by the tissues (Alam *et al.*, 2014). The argument in creating a HBOC is to have suitable chemical manipulation so that

those problems can be resolved. Theoretically, these manipulations should produce a greater ability compound to carry oxygen than the normal RBC. There is prediction that the first of these products will be accessible latest in two years duration (Moradi *et al.*, 2016).

2.3 POLYMER

Polymers are distinctively classified into three main architectures; linear polymer, branched polymer and cross-linked polymer (Figure 2.3). Each conformation offers uniqueness (Qiu & Bae, 2006). For instance, linear polymer has long chains lacking linkage whereas branched polymer has a number of linked monomer hanging by their long backbone chains. Cross-linked polymer form a network chains that attached adjacent to one another except their tip end (Liu *et al.*, 2010).

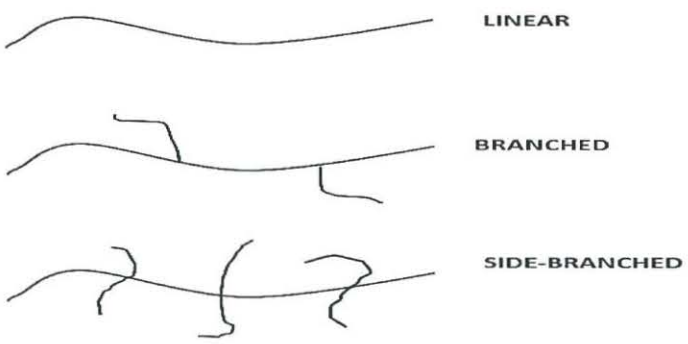

Figure 2.3: Three main classes of polymer architecture (Adopted from Qiu & Bae, 2006).

Great and extensive efforts to synthesis polymers with well define globular and macromolecule structure have been going on for the past three decades. One of the recent interests is to develop supramolecular assembly aggregate known as micelle from block copolymer that is tailorable to have specific surface or interior chemistry. Few reports indicate the potential biomedical applications such as drug delivery via host-guest molecule interaction (Xiong *et al.*, 2013; Zhao, 2012).

2.4 MICELLE

Formation of micelle needs the grouping of either monomer or polymer to take shape as block copoplymer, for example, amphiphilic diblock copolymer. This block copolymer formed when two monomers clustered together. Amphiphilic diblock copolymer as illustrated in Figure 2.4 have segments with polar (hydrophilic part) and non-polar (hydrophobic part) block.

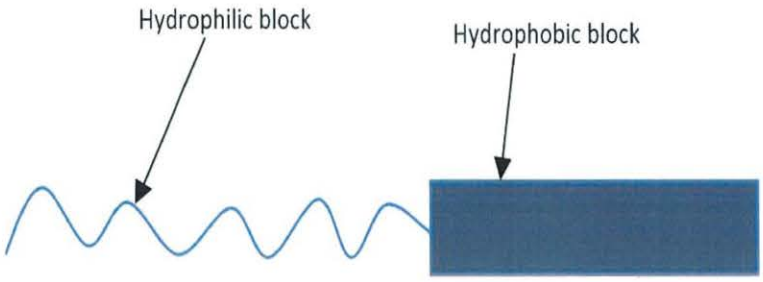

Figure 2.4: Diblock copolymer with hydrophilic and hydrophobic segments (Adopted from Zhao, 2012)

The most popular amphiphilic copolymer is di- or tri-block copolymers that have contrasting behaviours in solvent. The term amphiphilic refers to its surfactant feature, where the copolymers are capable to aggregate in solution to form micelle, vesicle or rod. Such aggregation is possible via self-assembly as the result of non-covalent forces involved (Jhaveri & Torchilin, 2014). The interaction forces determine reversibility of the aggregation in solution upon equilibrium (Figure 2.5)

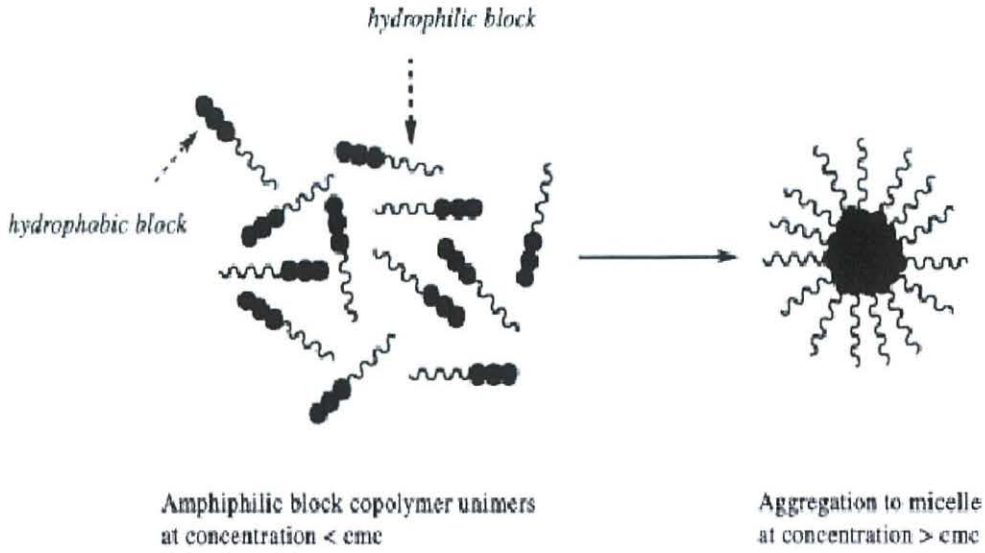

Figure 2.5: Formation of micelle by copolymer self-aggregation at equilibrium. (Adopted from Latere Dwan'Isa *et al.*, 2007)

Several factors will determine the size and shape of micelle including the length of the segments of hydrophilic and hydrophobic, solvent system, temperature and most importantly polymer concentration in solution known as critical micelle concentration (CMC). CMC can be defined as a phase of a concentration for a surfactant aggregates and form micelle. CMC of any copolymer that drop within the CMC region in solution will direct micelle formation from the initial linear shape to micelle. If the concentration is much higher, the self-aggregated version of the polymer will change into the form of vesicle, rod and worm (Owen *et al.*, 2012) Several works on micelle reported possibility of utilizing it as drug carrier system (Jhaveri & Torchilin, 2014). It is very intriguing to learn that micelles are capable to host drug molecule for efficient bio-distribution and bio-compatibility such as poly(ethylene glycol) monomethyl ether-SS-poly(D-leucine-L-leucine) (mPEG-SS-Pleu) copolymer, carrying doxorubicin as reported by Ren *et al.*, (2011) This host-guest interaction system is very useful to load other type of molecule such as hb. One of the challenge is to tailor the carrier size reside the 64kDa

tertiary haemoprotein for development of RBC substitute. One potential block copolymer that is potentially worth exploring for hb loading is a diblock copolymer that is made up of two well-known polymer blocks; polyethylene glycol (PEG) and poly (caprolactone) (PCL). When the two blocks are joint copolymer, it offers huge prospect for development of material to prepare hb to act as blood substitute at the most optimum level.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 MATERIALS

3.1.1 Apparatus and Equipment

All apparatus and equipment used in this study are listed in Table 3.1.

Table 3.1: The list of apparatus used in the study

Apparatus /Equipment	Brands
UV/VIS Spectrometer AK36735	Perkin Elmer, Malaysia
Stereomicroscope	Olympus Corporation, Japan
Ultra sonicator	Mujigae, Korea
Vortex Lab Dancer V	Yellowline, Germany
Magnetic stirrer ST0707V2	Favorit, Malaysia
Centrifuge	Eppendorf, Germany
Latex examination disposable gloves	Fisher Scientific, US
Disposable pipette	Fisher Scientific, US
Electronic weighing balance	Mettler Toledo, US
Measuring cylinder (25 mL and 50 mL)	Favorit, Malaysia
Beaker	Duran, Germany
Falcon tube (15 mL and 50 mL)	Becton Dickinson, USA
96-well tissue culture testplate	Fisher Scientific, US
Disposable syringe (10 cc/mL)	Terumo, Philippines
Syringe filter (0.45 µL)	Thermo Scientific, US
Dialysis tubing	Sigma-Aldrich Co., USA
Cuvette	Sigma-Aldrich Co., USA
Pipette tips 200µl yellow	Thermo Scientific, US
Pipette tips 1000µl blue	Thermo Scientific, US
Pipette tip box	Eppendorf, Germany
Micropipette	Eppendorf, Germany
Microcentrifuge tubes	Thermo Scientific, US
Magnetic stir bar	Thermo Scientific, US
Parafilm	Bemis, USA

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 the study

Chemicals	Brands
0.9% NaCl (Normal saline)	Bio Basic Canada Inc., Canada
Ultrapure water	Thermo Scientific, US
Dichloromethane (DCM)	Systerm, Malaysia
Dried erythrocyte (bovine)	Sigma-Aldrich Co., USA
Presto Blue	Thermo Fisher Scientific, US
Phosphate-buffered saline (PBS)	Bio Basic Canada Inc., Canada
Methanol (MeOH)	Systerm, Malaysia

3.2 METHODOLOGY

3.2.1 Collection of Expired Human Red Blood Cells

The expired human packed RBC blood bag which within 3 days of post expiry (at 45 day) was collected from the Blood Bank, Advanced Diagnostic Lab (ADL), in Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM). The blood bag was then transported from the ADL to the laboratory in a cool box and stored immediately in chiller until use. This study had been approved by 'Jawatankuasa Etika Penyelidikan (Manusia)' of USM (USM/Jepem/16110533).

3.2.2 Isolation of Haemoglobin

20 mL of expired human RBC from the blood bag was washed with equivalent amount of normal saline in a falcon tube (50 mL) by centrifugation (1000g, 20 min). The supernatant was disposed, and the washing was repeated twice. Subsequently, the RBC solution in the tube was transferred in to a 500 mL beaker filled with crushed ice to maintain the temperature at 8 ^{o}C before sonicated for 20 min to induce haemolysis. The suspension of lysed cell was then mixed with dichloromethane (DCM) before shaken for 3 min and centrifuged (1900g, 30 min). The bottom organic layer was discarded while the top layer containing hb solution was collected and mixed again with DCM for repeat washing. Then, the excess DCM was let to evaporate at room temperature upon overnight stirring (Himbert, Alsop and Rheinstädter, 2018).

3.2.3 Purification of Haemoglobin

The isolated hb solution from the falcon tube was added to a dialysis tube of 14,000 Dalton (kDa) molecular weight cut-off (MWCO) and submerged into pure water to remove small lipid by-products, ions and metabolites with low molecular weight based on the osmosis principle.

3.2.4 Determination of Hb Concentration

Concentration of the purified hb sample was estimated based on the difference of initial UV-Vis spectrophotometry reading of the RBC and the final isolated and dialysed hb.

3.2.5 Preparation of PEG-PCL Diblock Copolymers for Encapsulation of Hb

i. PEG-PCL Diblock Copolymers

The polymer was previously synthesised and characterised by our group and ready for further use. No additional characterisation is required.

ii. Determination of PEG-PCL Critical Micelle Concentration (CMC) Value

13.6 g of monopotassium phosphate (KH_2PO_4) was dissolved in ultrapure water (1000 mL) and adjusted with HCL (0.1M) to prepare a pH 7.4 solution of phosphate buffer solution (PBS). After that, 60 mg of PEG-PCL is dissolved in 10mL of the prepared phosphate buffer solution and vigorously stirred for 30 min. To determine the critical micelle concentration (CMC) value, several concentrations were diluted down from stock solution and analysed using 5 runs of particle size analyser, each lasting 1 min. The light was scattered at an angle of 90° (Owen *et al.*, 2012).

3.2.6 Encapsulation of Hb within PEG-PCL micelle

Encapsulation of hb within PEG-PCL micelle was carried out by 'co-precipitation' technique. 20 mg of the polymer was dissolved in 10 mL phosphate buffer solution to form micelle at CMC value of 2mg/mL. 10 mL (12.5g/dL) of hb was emulsified in organic solution, methanol (MeOH) and added drop-wise to the stirring PEG-PCL micelle solution over 30 minutes. Alternatively, the emulsion was added into aqueous solution of the polymer and re-emulsified by homogeniser for 3 min (at 200 bar or lower). The mixture was then continuously stirred

overnight until the organic solvent was evaporated yielding PEG-PCL micelle-hb complex. UV-Vis spectrophotometry reading was utilised to indicate successful encapsulation. Encapsulation efficiency was derived from the difference between initial and final absorbance (reflective of concentration) and convert to percentage (a). The maximum λ absorption of hb (Soret band) was between 406-409 nm (Hanson & Ballantyne, 2010).

$$\text{Encapsulation efficiency} = \text{initial absorbance} - \text{final absorbance} \times 100 \quad \text{(a)}$$

3.2.7 Reversible Oxygen Binding Study

The hb encapsulated micelle prepared in PBS buffer (0.1M, pH 7.4) was placed under continuous nitrogen gas flowing for 15 min. The solution was transferred into Suba-sealed glass cuvette and initial reading UV-Vis spectrum was measured and recorded at maximum λ absorption of hb at approximately 406 to 409 nm and Q bands around 525 nm and 565 nm. Then, the sample in cuvette was placed under oxygen flow for 15 min to initiate oxygenation. The sample was analysed under UV-Vis spectroscopy and λ absorption of the oxygen-adduct species was recorded at approximately 410-414 nm, Q bands at 505 nm and 545 nm. Next, the sample was deoxygenated by flowing nitrogen gas for 15 min and measured under UV-Vis spectroscopy at maximum λ absorption around 406nm, Q bands at 525 nm and 565 nm which had further up-field than the UV-Vis reading of HbO_2. This completes one reversible oxygen binding cycle.

3.2.8 Stability Study (half-life, $t_{1/2}$ analysis)

The hb encapsulated micelle prepared in PBS buffer (0.1M, pH 7.4) earlier was transferred into Suba-sealed glass cuvette and flushed with continuous nitrogen flowing. The initial UV-Vis spectrum reading was recorded (measure maximum λ absorption at approximately 410-414nm, Q bands: 525nm and 565nm). The sample in cuvette was introduced to oxygen flow for 15 min for oxygenation. Then, the sample was analysed using UV-Vis spectroscopy time-scanning at fixed wavelength (410nm-414nm) was set and the sample was left continuously stirring under atmospheric air. The reading was taken every 15-30 seconds for 1-2 hour or until the time-scanning curve reaches plateau (at 406 nm) indicating complete deoxygenation.

3.2.9 *In-vitro* Compatibility Assay on Normal Cells

i. Cell Viability Study

Macrophage-like cells (RAW 264.7) as model for normal cell lines would be plated at 5 x 10^3 cells per well in a 96-well plate. The cell taken from passage number 10 and as it was an attached cell, scrapper was used to unattached before sub culture in the well plate. The cells will be cultured in DMEM medium containing 10% foetal bovine serum (FBS) and will be incubated at 5% carbon dioxide (CO_2) atmosphere for 24 h. The polymer complexes of 1 mg/mL and 2 mg/mL (micelle) incubated with the cells at 37°C for 4, 8, 24 and 48 h along with control and untreated cells. The number of cell viability was determined by using Presto Blue cell viability assay technique where the reagent give blue color and rapidly taken up by cells when added to the media. The well plate was incubated for 20 minutes after adding the reagent. The colour would quickly reduce by metabolically active cells. The UV absorbance change

detected the red-fluorescent dye converted from the original blue colour induced by the reducing environment. The procedure was repeated with a new plate for free hb from human and commercialised bovine hb. Three replicates would be independently measured (mean ± SD). The plate was protected from long term exposure to light and contamination as it would reduce the sensitivity of the reagent, as this can reduce sensitivity. All techniques done under sterile condition to avoid contamination.

3.2.10 Statistical Analysis

Results are expressed as mean ± SD. Results of oxygen reversibility was subjected to descriptive and qualitative analysis with no statistical analysis.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 ISOLATION OF HAEMOGLOBIN

In this study of developing haemoglobin-based blood substitute, the hb was harvested from expired human blood upon chemical and mechanical manipulation to isolate the tetrameric globin without denaturing it. The general aim was to produce red blood cell alternative by modifying the processed hb using polymer, specifically to provide new environment for the protein to reside within. In other words, the selected polymer will mimic the membrane of RBC aiming to ensure stability of the hb. The expired blood bag was collected within 3 days post expiry. Theoretically, the lysis of RBC is proportionate over time, hence the structural integrity of the red cell declined towards its 42 days expiry. As the membrane is weakening, the process of sonication will further induce the breakdown of the phospholipid bilayer releasing the hb without any structural damage. Sonication proved to be inducing membrane lysis in a more uniform fashion as compared to other methods such as lysis induced within hypotonic solution that results to ranges of by-product sizes because of the cell membrane 'burst' upon extreme swelling (Cruz *et al.*, 2014). Subsequently, the hb solution was subjected to isolation procedures involving multiple washings using saline via centrifugal force, organic solvent extraction and dialysis method. High speed centrifugation within optimum time is generally useful to separate cellular mass of different density in solution medium (Cvjetkovic *et al.*, 2014). Thus, the denser and heavier hb would settle in the bottom layer while the hemolysate of other smaller waste chunks remain in the upper layer of the saline solution. However, it won't be sufficient to remove other non-polar debris mainly phospholipid by-products.

Therefore, removal of phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SPH) traces via organic extraction technique utilised dichloromethane (DCM), yielding hb solution in saline in the upper layer while the bottom layer lipids waste was disposed of. Even though the purification process to remove the lipid traces using high performance liquid chromatography (HPLC) technique would be more efficient, the simpler organic extraction method is sufficient considering that the study only encompassed of ex-vivo analysis. The final dialysis technique was meant to remove other smaller and water-soluble by-products using a cellulose tubing with molecular weight cut off (MWCO) size of 14kDa. In principle, the hb sample solution was dialysed against pure water allowing the solutes to pass through the membrane leaving the larger 64kDA hb in the tube for collection. Successful isolation of the hb from the RBC is determined by UV-vis spectroscopy and the comparative spectra are as illustrated in Figure 4.1.

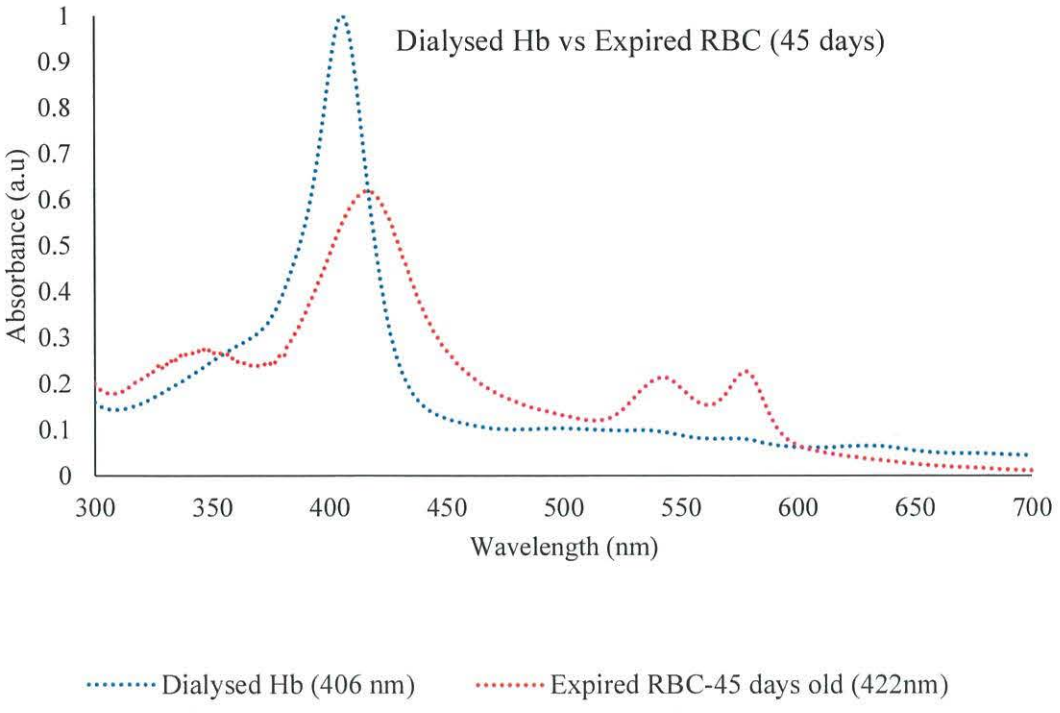

Figure 4.1: Comparison UV-Vis spectra between expired RBC and dialysed Hb