HAEMOGLOBIN AND PORPHYRIN ENCAPSULATED SELF-ASSEMBLED POLYMERS AS ARTIFICIAL OXYGEN CARRIER

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HAEMOGLOBIN AND PORPHYRIN ENCAPSULATED SELF-ASSEMBLED POLYMERS AS ARTIFICIAL OXYGEN CARRIER

by

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LIST OF SYMBOLS

%	Percentage
α	Alpha
β	Beta
℃	Celcius
M	Molar
λ	Lambda

LIST OF ABBREVIATIONS

RBC	Red blood cell
Hb	Haemoglobin
TPP $Fe^{(III)}$	Tetrakis-4-phenyl porphyrin $iron^{(III)}$
mPEG	Methoxy-polyethylene glycol
ε-CL	ε-caprolactone
HPG	Hyperbranched polyglycidol
ROP	Ring opening polymerisation
CMC	Critical micelle concentration
EE	Encapsulation efficiency
UV-Vis	Ultraviolet-visible light
ml	Milliliter
rpm	Rotation per minute
mg	Milligram
MW	Molecular weight
w/w	Weight over weight
PDI	Polydispersity index
DLS	Dynamic light scattering
FT-IR	Fourier transform infrared
H NMR	Proton nuclear magnetic resonance
C NMR	Carbon nuclear magnetic resonance
ESI-MS	Electrospray ionisation mass spectrometry
GPC	Gel permeation chromatography
SEM	Scanning electron microscope
TEM	Transmission electron microscope
LMW	Low molecular weight
HMW	High molecular weight
M	Molar
nm	Nanometer
µl	microliter
$gmol^{-1}$	Molar mass
pH	Potential of hydrogen

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POLIMER SWAHIMPUN BERKAPSUL HEMOGLOBIN DAN PORPHYRIN SEBAGAI PEMBAWA OKSIGEN TIRUAN

ABSTRAK

Perkhidmatan pemindahan darah mempunyai pelbagai risiko antaranya penyakit bawaan darah, ketidakserasian kumpulan darah dan kekurangan bekalan darah. Antara usaha menangani isu ini, para penyelidik telah meneroka untuk membangunkan pembawa oksigen tiruan menggunakan makromolekul yang menyerupai struktur hemoglobin (Hb) manusia. Matlamat kajian ini adalah untuk menghasilkan polimer swahimpun berkapsul hemoglobin dan porfirin sebagai pembawa oksigen tiruan. Tetrakis-4-fenil porfirin (TPP), telah disintesis melalui tindak balas pirola dan 4-benzaldehid diikuti penambahan ferum besi ($Fe^{(III)}$) bagi membentuk tetrakis-4-fenil porfirin $Fe^{(III)}$ (TPP $Fe^{(III)}$). Blok metoksi-polietilena glikol ($mPEG_{45}$) dan poli(ε-kaprolakton) (mPEG-*b*-PCL) yang membentuk kopolimer dua blok dan teras *para*-nitrofenol dan glisidol yang membentuk poliglisidol hiper bercabang (HPG) telah disintesis via pempolimeran bukaan gelang (ROP). Hb yang diekstrak dan TPP $Fe^{(III)}$ yang disintesis kemudian dikapsul dalam sistem misel yang mempunyai kepekatan misel kritikal (KMK) pada 1.0-2.0 mg/ml dan poliglycidol hiper bercabang yang disediakan dalam molar selari dengan kepekatan Hb dan TPP $Fe^{(III)}$. Purata kecekapan pengkapsulan (KP%) misel-Hb manusia dan misel-TPP $Fe^{(III)}$ adalah 67 ± 5.39% dan 78 ± 1.38% dibandingkan dengan 68 ± 2.22% misel-bovin. Disebaliknya, KP% bagi HPG-Hb manusia dan HPG-TPP $Fe^{(III)}$ adalah 58 ± 6.87% dan 22 ± 7.12% dibandingkan dengan 65 ± 9.11% HPG-Hb bovin. Bagi kajian kestabilan, misel-Hb dan misel-TPP $Fe^{(III)}$ menunjukkan terbitan setengah hayat pada 438,204 ± 10 min dan 317.3 ± 10 min,

manakala, untuk HPG-Hb dan HPG-TPP $Fe^{(III)}$ pada 99.15 ± 10 min dan 57.89 ± 10 min dibandingkan dengan 3.65 ± 10 min Hb bebas dan 3.48 ± 10 min TPP $Fe^{(III)}$ bebas. Hasil kajian pengikatan oksigen boleh balik menunjukkan keupayaan misel-Hb dan TPP $Fe^{(III)}$-misel menahan purata minima 4-7 kitaran pengikatan. HPG-Hb dan HPG-TPP $Fe^{(III)}$ menunjukkan keupayaaan menahan 5-6 kitaran pengikatan jika dibandingkan dengan Hb bebas dan TPP $Fe^{(III)}$ bebas yang hanya berupaya menahan satu kitaran pengikatan sahaja. Kesimpulannya, kedua-dua polimer menunjukkan potensi sebagai pembawa oksigen tiruan yang melindungi teras dalaman bagi membolehkan pengikatan oksigen berlaku.

HAEMOGLOBIN AND PORPHYRIN ENCAPSULATED SELF-ASSEMBLED POLYMERS AS ARTIFICIAL OXYGEN CARRIER

ABSTRACT

Blood transfusion services have a lot of risks such as blood-associated risk, blood incompatibility and shortage of blood supply. To address this issue, researchers explored an artificial oxygen carrier through macromolecules by mimicking the structure of human haemoglobin. The aim of this study is to develop haemoglobin and porphyrin encapsulated self-assembled polymers as artificial oxygen carrier. A porphyrin derivative, tetrakis-4-phenyl porphyrin (TPP) was first synthesised through the reaction of pyrrole and 4-benzaldehyde following the addition of iron, $Fe^{(III)}$ forming tetrakis-4-phenyl porphyrin $Fe^{(III)}$ (TPP $Fe^{(III)}$). Methoxy-polyethylene glycol ($mPEG_{45}$) block and poly (ε-caprolactone) (ε-CL) (mPEG-*b*-PCL) that formed diblock copolymer and *para*-nitrophenol core and glycidol that formed hyperbranched polyglycidol (HPG) was synthesised *via* ring-opening polymerisation (ROP). Extra cted Hb and synthesised TPP $Fe^{(III)}$ were then encapsulated in micelle system with a critical micelle concentration (CMC) at 1.0-2.0 mg/ml and hyperbranched polyglycidol prepared in equal molar to Hb and TPP $Fe^{(III)}$ concentration. The average encapsulation efficiency (EE%) of human Hb-micelle and TPP $Fe^{(III)}$-micelle were 67 ± 5.39% and 78 ± 1.38% as compared to 68 ± 2.22% of bovine Hb-micelle. On the other hand, the EE% of human Hb-HPG and TPP $Fe^{(III)}$-HPG were 58 ± 6.87% and 22 ± 7.12% as compared to 65 ± 9.11% of bovine Hb-HPG. In stability study, the derived half-life of human Hb-micelle and TPP $Fe^{(III)}$-micelle were 438,204 ± 10 min and 317.3 ± 10 min and for human Hb-HPG and TPP $Fe^{(III)}$-HPG were 99.15 ± 10 min and 57.89 ± 10 min as compared to 3.65 ±

10 min of free Hb and 3.48 ± 10 min of free TPP $Fe^{(III)}$. Results in oxygen binding reversibility study had demonstrated the capability of Hb-micelle and TPP $Fe^{(III)}$-micelle to endure an average minimum of 4-7 binding cycles. Meanwhile, Hb-HPG and TPP $Fe^{(III)}$-HPG were able to endure an average minimum of 5-6 binding cycles. In comparison, free Hb and free TPP $Fe^{(III)}$ were only capable of enduring an average of one binding cycle only. In conclusion, both polymers showed potential as an artificial oxygen carrier, providing protection towards the active site and thus allowing oxygen binding to take place.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Blood is crucial for the sustenance of human systemic and physiological functions. Human whole blood consists of red blood cells, white blood cells, platelets, and plasma, making up to about 70 ml/kg of body weight. These elements delegate various function including regulation of body temperature, waste excretion, immune and coagulation system. However, the principal function of blood is the transport of oxygen and carbon dioxide gases to and from the lungs and tissue respiration. However, the principal function of blood is the transport of oxygen and carbon dioxide gases to and from the lungs and tissue respiration. This task is carried by red blood cell which is the most abundant formed elements within whole blood (Ashton, 2013). Within red blood cell, resides haemoglobin, a metallo-protein responsible for the binding of respiratory gases. Haemoglobin (Hb) consists of four polypeptide globin chain, known as 2α and 2β with each having a porphyrin ring structure called haem (ferrous protoporphyrin IX). For the binding of oxygen to happen, haem group will need to first bind covalently to the central ferrous iron atom ($Fe^{(II)}$). With four globin chain, each haemoglobin has the potential to bind four oxygen molecules per time (Jia, Duan and Li, 2016).

Blood transfusion services have been the key life-saving measures in transplant and cardiovascular surgeries, traumatic injury and blood disorder (Palmer, A.F, Intaglietta, 2014; Webster et al., 2017). This practice became a norm during the 17^{th} century and the significant discovery of ABO blood group by the Landsteiner in the 19^{th} century has allowed deeper understanding on safe blood transfusion and compatibility (Fastag, Varon and Sternbach, 2013; Tan and Graham, 2013).

However, it was soon realized that blood transfusion would be potentially accompanied by side effects and risks such as transfusion transmitted disease, allergic reactions, transfusion-related acute lung disease and haemolytic reactions towards the recipient in certain circumstances (Palmer, A.F, Intaglietta, 2014). In addition, the demand for blood supply is not proportionate to the amount blood storage in blood bank and it became scarcer with low awareness among people. According to World Health Organization (2018), 118.4 units blood donation were collected globally and almost half of them were from high income countries. In Malaysia, the number of blood transfusions were 36.8% higher compared to blood donation from the year 2000 to 2014 (Lim et al., 2018). In this era of 21st century, more challenges are threatening the current state of blood transfusion such as increase in population growth, emergence of infectious disease, natural disasters and population aging (Moradi, Jahanian-Najafabadi and Roudkenar, 2016).

Steps to overcome these challenges have been undertaken by researchers and blood substitute exploration is shown to be the most ideal solution. Generally, a blood substitute has the capability to mimic the functions of natural red blood cell. Most of the studies focus on the development of functional alternative with oxygen-carrying capability (Chang, 2012; Habib, Cohn, 2014). Blood substitutes can be categorised into two namely haemoglobin-based blood substitute (HBOC) and perfluorocarbon-based blood substitute (PFOC). Haemoglobin-based blood substitute utilise haemoglobin from various sources before chemically modified the tetrameric protein through different chemical means such as polymerisation, conjugation, cross-linked and encapsulation. On the other hand, perfluorocarbon-based blood substitute consists of synthetic and biologically inert compound in the form of emulsion that allow storage of dissolved oxygen (Tao and Ghoroghchian, 2014). The biological

inertness of PFCs were displayed through the linear oxygen binding/transport profile where it loosely binds oxygen between its molecule allowing a higher off-loading of oxygen compared to Hb (Sen Gupta, 2017).

Application of polymers as one of the components for the development of blood substitute has gained a wide interest among researchers. This polyester group has shown promising potential due to their advantageous properties such as ease of design, non-toxic, good biocompatibility and biodegradability (D'souza and Shegokar, 2016). Aside from that, polymer has shown to be applicable in many fields such as pharmaceutical, medical, tissue engineering, food industry and many more (Woodruff and Hutmacher, 2010; D'souza and Shegokar, 2016). Polymers can be divided into three different categories namely linear polymer, branched polymer and cross-linked polymer. The unique features of polymer known as block copolymer offer a potential use as Hb nanocarrier in the development of blood substitute (Samaddar, Deep and Kim, 2018). Combination of two or more block copolymers of which its possible architecture includes hydrophobic core interior and hydrophilic exterior. Depending on the synthesis and preparatory methods, different types of morphologies such as cylindrical, lamellae, spherical can be designed. These morphologies can be induced through their self-assembly behaviour when exposed in a particular environment (Feng et al., 2017).

This study aimed to explore synthetic polymeric micelle consisting of diblock copolymer and hyper branched polymer known as polyglycidol as a synthetic oxygen carrier. Diblock copolymer, polyethylene glycol-block-poly (ε-caprolactone) (PEG-b-PCL and hyperbranched polyglycidol had been shown to be compatible in clinical application such as targeted drug delivery system (Deng et al., 2014; Alami-Milani et al., 2018; Brandt et al., 2019).

1.2 Problem statement

According to the World Health Organization (WHO) 118.4 million blood donations were recorded globally, with a marked difference between low, middle- and high-income countries. The percentages of blood donation in high income countries, account for 32.6 donation per 1000 people. The South-East Asia contributed only 16.7%. Despite the demand for blood is rising 6-8% annually, the global ratio of donor to population is only 2.5%. In other words, there is an inadequacy in the amount of donated blood per people in South East Asia, comparing to the WHO requirement of at least 10 blood units per 1000 people (WHO, 2016).

Despite being an advanced developing nation status, Malaysia is facing a lack of blood supply, in which the blood donation rate from 2.0 to 2.25%. According to Noh et al. (2019) shift in socio-demographic, population aging and attitude of local population influence the demand-supply chain of blood products. For example, it was noted that the state of Terengganu recorded the lowest number of donation rate and the refusal to donate blood to those of another religion being the main issue that prevents the practice. Additionally, the issues arise due to natural blood donation such as the risk of transmission of infectious disease, blood incompatibility and availability to rural areas has cause researchers to pursue an alternative to overcome these problems (Xu et al., 2015).

Blood substitute is found to be an ideal solution to the rising issues. Characteristics of an ideal blood substitute include universal group, sufficient half-life, optimum uptake and delivery of oxygen and absence of unwarranted side effects (Chang, 2010). Countless studies on blood substitute (i.e., HBOC and PFOC) haves been performed in the past decades and despite most of the products were capable to carry the principal function of red blood cell, there were also life-threatening side

effects reported (Castro and Briceno, 2010; Palmer, A.F, Intaglietta, 2014). Free haemoglobin, albeit, having high affinity to bind with oxygen is unable to maintain its tetrameric structure, leading to dimerization which can cause renal failure, renal toxicity and an extreme oxygen affinity (Elmer, Alam and Wilcox, 2012). Therefore, chemical modification is required to sustain the structure of free haemoglobin. Free haemoglobin can be obtained from a few potential sources namely expired human blood. Haemoglobin from expired blood can be extracted and purified before further modified to obtain a better stability regulated oxygen affinity, making it a suitable potential oxygen carrier.

1.3 Rationale of study

Due to the several issues such risks of allogenic blood transfusion and transfusion transmitted infections particularly in advanced nation such as Malaysia, it is about time for local scientists to embark on blood substitute research. This vision enables exploration of potential blood product alternatives especially red blood cell with universal group that limit the issue of incompatibility. The haemoglobin can be sourced from expired human blood and bovine blood are unlimitedly available for future needs. Nonetheless, the haemoglobin needs chemical modification to stabilise and prepare the tetrameric protein to endure reversible oxygen cycles.

1.4 Research objectives

General objective:

To prepare haemoglobin and porphyrin loaded self-assembled polymers as artificial oxygen carrier.

Specific objectives:

a) To extract haemoglobin from expired red blood cell (RBC)

b) To synthesise and characterise tetrakis-4-phenyl porphyrin $Fe^{(III)}$

c) To synthesise and characterise diblock copolymer, methoxy-polyethylene glycol-block-poly (ε-caprolactone) ($mPEG_{45}$-*b*-PCL_{30}) and hyperbranched polyglycidol

d) To investigate the encapsulation efficiency, stability (half-life) and oxygen binding reversibility of haemoglobin and porphyrin within self-assembled polymers

1.5 Research hypotheses

Null hypothesis (H_0)

a) There is no difference in encapsulation efficiency between haemoglobin and porphyrin encapsulated self-assembled polymers against control Hb.

b) There is no difference in stability (half-life) and oxygen reversibility between haemoglobin and porphyrin encapsulated self-assembled polymers against free Hb and porphyrin.

Alternative hypothesis (H_1)

a) There is a difference in encapsulation efficiency between haemoglobin and porphyrin encapsulated self-assembled polymers against control Hb.

b) There is a difference in stability (half-life) and oxygen reversibility between haemoglobin and porphyrin encapsulated self-assembled polymers against free Hb and porphyrin.

1.6 Significance of the study

It is an established fact that haemoglobin-based carriers can be sourced from either human blood or animal blood (famously bovine). The World Health Organization (WHO) (2016) recorded about 33% of blood units discarded annually.

This research hopes to make use of expired blood units and turn them into a more beneficial prospect by minimizing blood wastage and improve the blood bank inventory management. Further, any findings in this research will contribute to the addition of new knowledge and improvement in the blood substitute development. Successful development of artificial oxygen carriers will help greatly in emergency as it can be used directly without cross-matching, kept at room temperature and only require the use of saline prior to administration into the body. However, more work is needed especially animal studies, preceded with clinical studies and if the product pass through, the blood transfusion services in Malaysia can be revolutionize at minimal in being alternative or complementary to human blood in certain cases.

CHAPTER 2

LITERATURE REVIEW

2.1 History of blood transfusion

Blood has been since antiquity a mysterious liquid that held an interest in the circle of researchers. From mythology to scientific findings, there had been many recorded works of literature on blood transfusion. In Greek mythology, it was believed that individual quality and vitality could be passed on through the act of blood-drinking and blood exchange. Not only that, but a 'humorism' theory was postulated by Hippocrates and Galen which listed blood as one of the four elements that can affect an individual's health and individuality. The 16th century recorded the first blood transfusion of three healthy boys to Pope Innocent VII which resulted in death to all of them (Learoyd, 2012a).

Following the 17th century, the era displays significant discovery by a prominent scholar that contributed to the understanding of blood transfusion. William Harvey was well known to be the discoverer of the pulmonary and systemic circulation. However, prior to Harvey, there are other scholars that contributed to the postulation on the mechanism of blood circulation such as Razi, Ahwazi, Haly Abbas, Ibn Nafis, and Michael Servetus. Among these scholars, Ibn Nafis postulated the most accurate description of the pulmonary blood circulation. He described the circulation of blood as such that the blood needs to flow from the right ventricle through the pulmonary artery to the lung to be oxygenated and returned to the left chamber of the heart through the pulmonary vein (Azizi, Nayernouri and Azizi, 2008).

The pioneer of blood transfusion was denoted to Richard Lower, who, in 1666, performed the first successful animal to animal blood transfusion. In the following year, Jean Baptiste reported the successful animal to human blood transfusion and in

the same year Richard Lower performed animal to human transfusion which resulted in the death of the patient. Due to the issue surrounding the death of patient post-transfusion, this practice was later banned and abandoned for the next 15 decades (Fastag, Varon and Sternbach, 2013).

The interest in blood transfusion was rekindled in the 19th century when James Bundell, an obstetrician performed the first successful human to human blood transfusion, saving a post-partum haemorrhagic mother. His successful attempt, however, was the result of multiple blood transfusions both between inter-species and humans. Despite the successful blood transfusion, there were issues such as blood clotting and severe side effects accompanying this procedure (Learoyd, 2012b). The practice of safe blood transfusion came to light after the discovery of ABO blood groups by Karl Landsteiner in the 1900s. Through his discovery, it was found that when blood from different blood groups was mixed, the antibodies present in the serum will cause agglutination. This resolves the reason behind the blood clotting associated with blood transfusion (Tan and Graham, 2013).

The importance of blood transfusion became more apparent during World War I as it became the centre for the resuscitation of life. During this period, two individuals contributed to the advancement of blood transfusion namely Lawrence Bruce Robertson and Oswald Hope Robertson. The former performed direct transfusion of blood without prior screening while the latter introduced the usage and safety of the transfusion bottle which acts as a storage for blood (Stansbury and Hess, 2009). The medical advancement during world war I had led to the establishment of blood banks with the application of blood transfusion as life-saving measures becoming more recognized.

2.2 Issues in blood transfusion

In every medical practice, there are bound to be hazards and risks accompanying it. This goes without saying when it comes to the practice of blood transfusion. While blood transfusion may be safe in general, there are unavoidable disadvantages and risks associated with it. It was described from the historical perspective that blood transfusion hazards can be both infectious and non-infectious (Alter and Klein, 2008). Infectious hazards include transfusion-associated hepatitis, transfusion-transmitted HIV, zoonotic infection and bacterial contamination whilst non-infectious hazards include haemolytic transfusion reaction, reactions associated with leukocyte and leukocyte antibodies, graft-versus-host disease and transfusion-related acute lung injury (Vagholkar et al., 2015). Furthermore, massive transfusion may lead to hypothermia, coagulopathy, electrolyte abnormalities, and acid-base disturbance. Inherent risks associated with system failure and human errors as well as inappropriate, delayed or unnecessary transfusion added more to blood transfusion hazard (Clevenger et al., 2014).

Blood also has several disadvantages. Within the body, the lifespan of the red blood cell is 120 days before it was recycled (Ashton, 2013), however, upon extracted from the body, the lifespan of the red blood cell shortens to about 42 days, even with the added preservatives (D'Alessandro et al., 2010). When the blood was not used within the timeframe, it will only end up being discarded, which is a waste in the blood bank. Longer storage of blood had been associated with adverse effects to humans such as the increased risk of the lung in injury in sepsis patients, in the bladder cancer patient and in a post-operative patient (Manlhiot et al., 2012; Janz et al., 2013; Chappidi et al., 2017). Haemoglobin concentration was also recorded to decrease with longer storage time (Rydén et al., 2019). In addition to the short half-

life, every transfusion requires cross-matching which may not be convenient in the face of an emergency. Temperature is also essential in maintaining the morphology of the red blood cell. Following extraction from the body, blood was kept at a low temperature to slow degradation and thus preserved the blood longer. It was found that in elevated temperature, red blood cells experienced haemolysis due to the imbalance between intracellular and extracellular fluid (Jaferzadeh et al., 2019). All in all, these disadvantages had led to the need for an alternative to cater to the problem. This is where blood substitutes came to picture.

2.3 Blood substitute

The term ‘blood substitute’ was denoted as such due to their similar functionality to that of real blood. The construction of a blood substitute is hoped to overcome the limitation associated with blood transfusion. For the past decades, research on blood substitutes had been continually improved to ensure the function was maximized. Artificial oxygen carrier can be divided into two main categories namely perfluorocarbon-based blood substitute (HBOC) and haemoglobin-based blood substitute (HBOC).

Perfluoro-carbon (PFC) is described as a synthetic and biologically inert fluorinated hydrocarbon due to its hydrophobic and lipophobic properties. Unlike RBC which binds with oxygen, PFC dissolves oxygen through Van Der Waals interaction where oxygen loosely binds between PFC monomer whereby this oxygen dissolution formed a linear oxygen-binding profile which also allows the release of oxygen more easily. Not only that, but this compound also exists in either a linear or cyclic hydrocarbon backbone and the former form had better affinity as an oxygen carrier. Research on PFOC had reached until the third generation.

To date, there are a few HBOC that had proceeded to advanced stage of preliminary and clinical trials for regulatory approval process. HBOC that had reached phase III clinical trials consists of HemAssist, Hemopure (HBOC-201), Oxyglobin (HBOC-301), Polyheme and Hemoximer or pyridoxilated haemoglobin-polyethylene conjugate (PHP). HemAssist in phase III clinical trial was tested in surgical and trauma patients with the former displayed side effects such as blood sparring, pancreatitis and myocardial infection but the latter displayed high mortality rate that leads to termination of the product. Hemopure completed phase III clinical trial and had been approved for use in South Africa for treatment in adult anemic patients and for acute anemia in Russia. Oxyglobin was approved for veterinary use and was only permitted by Food and Drug Agency (FDA) in emergency case if there is no other option. This product, however, was not approved for use in United States (US) and was only permitted in life-threatening anemic patient in clinical trials and in FDA expanded access program (EAP). In Polyheme phase III clinical trial, treatment for anemic patients resulted in lower total Hb in circulation and higher mortality rate. The adverse effects of this product in clinical trial led to unfavourable review by FDA, and thus, ceasing its production. Hemoximer or PHP was tested as nitric oxide (NO) scavenger in NO dependant distributive shock in clinical trial phase III but failed due to high mortality rate and was thus discontinued.

Haemoglobin-based blood substitutes (HBOC) can be further divided into two types namely acellular and cellular products (Jia et al., 2016). HBOC utilize haemoglobin extracted from expired blood. Sources of blood were usually from bovine or human, though recently blood from other animals such as worm, crocodile, and camel was also experimented (Jani et al., 2017; Roamcharern et al., 2019; Shokry et al., 2014)Pioneer blood substitute used stromal-free haemoglobin and while it was

proven to be able to bind and deliver oxygen, the side effects following after were equally fatal due to the dissociation of haemoglobin (Elmer et al., 2012). Various strategies were then employed such as cross-linking, polymerisation, conjugation and encapsulation in order to retain the Hb structure while optimizing its function (Moradi et al., 2016).

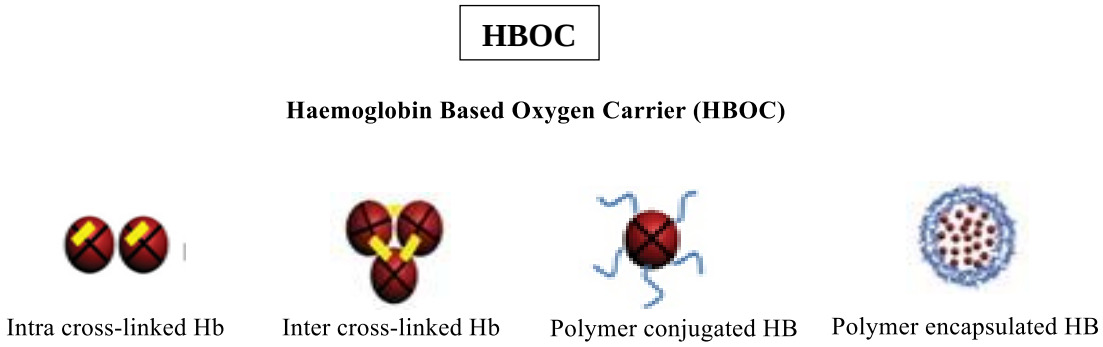

Figure 2.1 Diagram depicted various modification to prevent spontaneous Hb chain dissociations (Adopted from (Jia et al., 2016))

2.4 Red blood cell (RBC) and Haemoglobin (Hb)

Red blood cells (RBC), also known as erythrocyte account for the highest percentages within blood cells in the body. Morphologically, it is biconcave in shape with 6-8µm in diameter and approximately 2µm in width (Ashton, 2013). One of the characteristics of RBC is their flexibility to manoeuvre within tight and small blood vessels. This flexibility is attributed to the lack of organelle and energy-dependent assembly and flexible spectrin-based cytoskeleton that make up the membrane of RBC. The structure provides RBC with the ability to pick up large amounts of oxygen and maneuver through the small capillaries despite the difference in their size (Lux IV, 2016).

The life span of RBC from its development to senescence is approximately 120 days. (Kaestner and Bogdanova, 2014). Production of RBC is triggered by the lack of oxygen in circulation, which in turn is detected by the kidneys, thus secreting a

hormone called erythropoietin. This release of hormone signalled the bone marrow, which is the main site of development to generate more RBC through the process called erythropoiesis (Ashton, 2013). In brief, haemopoietic stem cells differentiate into myeloid stem cell which in turn differentiate into progenitor cells called erythroblast. In this form, erythroblasts possess organelle like normal cells. However, as it matures, the organelle is slowly lost until it forms reticulocyte. Once reticulocyte matured and haemoglobin is formed, this reticulocyte is released into circulation and term as matured erythrocyte (Palis, 2014).

After 120 days, 'markers of senescence' will be signaled to initiate the removal of RBC (de Back et al., 2014). A series of physical and metabolic damage were endured by RBC throughout their life span, particularly reactive oxygen series (ROS). Constant encounter with ROS caused Hb to endure auto-oxidation daily. Despite the enzyme within RBC to counter against this species, the content continually depleted as it ages (Lutz and Bogdanova, 2013; Mohanty, Nagababu and Rifkind, 2014). Changes in the RBC membrane triggered the process of eryptosis, in which mononuclear phagocyte recognized, catabolized and removed the aged RBC from the circulation. This extravascular clearance by reticulo-endothelial system is carried out by the spleen as it contains a unique structure not found elsewhere as well as liver and lymph nodes (Kaufman, 2018).

As mentioned before, the principal function of red blood cells lies in their oxygen-binding capability. This capability is shouldered by an iron-containing metalloprotein termed as haemoglobin. Each erythrocyte contains about 300 million Hb molecule (Sen Gupta, 2017). In attempt to design an effective HBOC, understanding of the Hb structure is essential before red cell mimic can be proposed. Haemoglobin (Hb) exists in the form of 2α and 2β polypeptide chain, each globin unit

with 141 and 146 amino acids respectively (Yuan et al., 2015). Each sub-unit contains a haem group (ferrous protopoprhyrin IX) which binds with central ferrous iron atom to allow oxygen binding (Ashton, 2013). Two conformational Hb structures, T (tense) and R (relaxed) state co-exist in equilibrium in response to oxygen saturation whereby the former is the deoxygenated state while the latter is the oxygenated state (Safo et al., 2011). The molecular mass of quaternary structure of Hb is approximately 64,000 Dalton (Da) (Tsuchida et al., 2009).

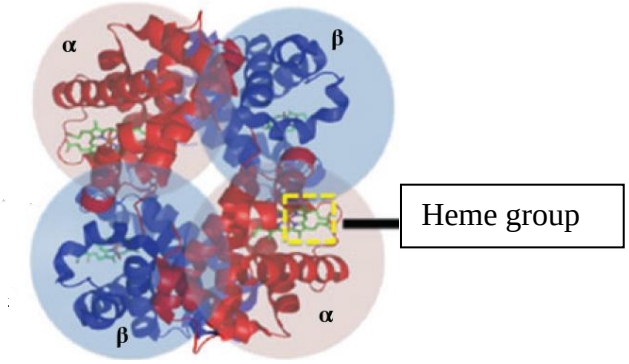

Figure 2.2 The diagram represents haemoglobin with red and blue colour, each denoting respective α and β subunits (Adopted from (Sen Gupta, 2017))

Endogenous heterotrophic effectors such as carbon dioxide (CO_2), pH, chloride ion (Cl^-), hydrogen ion (H^+), 2,3-diphosphoglycerate is important in modulating the oxygen affinity and cooperative behaviour of Hb. Bohr effect is a result of the changes in pH, influencing the shifting of oxygen binding curve to left and right. The Bohr effect is also influenced by the presence of 2,3-diphosphoglycerate. The 2,3-diphosphoglycerate binds to haemoglobin readily in its deoxygenated state, changing the conformational Hb structure hence lowering its affinity for oxygen. In doing so, it can maintain an adequate oxygen dissociation. This cooperative oxygen binding can be represented by the sigmoidal oxygen binding curve (Safo et al., 2011; Yuan et al., 2015). Usage of free Hb for therapeutic purposes had been shown to display fatal side effects. Development of a suitable carrier to

contain the Hb in its tetrameric structure through chemical manipulation is essential in ensuring the problem associated with free Hb is solved.

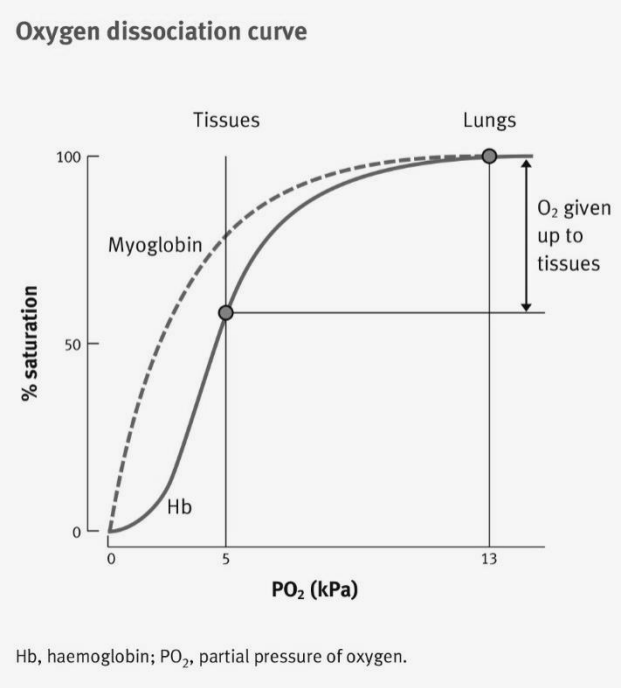

Figure 2.3 Schematic representations of sigmoidal oxygen dissociation curve (Adopted from (Ashton, 2013))

2.5 Synthetic porphyrin system

Natural Hb active site, the 'haem' consists of porphyrin tetrapyrrole ring surrounding an iron atom. This structure undergoes rapid auto-oxidation upon exposure at low temperature, however, that is not the case within the body temperature. The reason behind this lies in the presence of bulky polypeptide which prevents the collision between two haems (Safo et al., 2011). Based on this, researchers attempt to develop $Fe^{(II)}$-based porphyrin system as an oxygen carrier. 'Picket-fence Fe^{2+}' porphyrin had been shown to be reversible oxygen binding ability similar to that of natural Hb but it underwent auto-oxidation upon exposure to aqueous media (Hartle, Prell and Pluth, 2016).

A hydrophobic environment was then created through the embedment with liposome vesicle to overcome the issue (Jonathan Leor et al., 2017). Two other

porphyrin-based systems that were developed involve the use of human serum albumin (HSA) and cyclodextran (HemoCD) (Rozinek, Thomas and Brancaleon, 2016; Kitagishi et al., 2017). Both systems exhibited excellent oxygen-carrying capacity like natural Hb and was further enhanced with polyethylene glycol (PEG) to extend their circulation time and reduced oxidation (Karasugi, Kitagishi and Kano, 2012; Kitagishi, Kawasaki and Kano, 2015).

In order to construct a haem protein model, it is necessary to develop a polymer around the iron porphyrin core, creating a hydrophobic environment for the binding pocket. Through steric hindrance, the active binding site can be kept dry and free from water molecules, avoiding auto-oxidation. Synthetic approach will be another interesting strategy for development of blood substitute, aside from the use of natural system. The application of polymer for haemoglobin mimics can offer a different view towards different choices for alternatives on blood-related therapy.

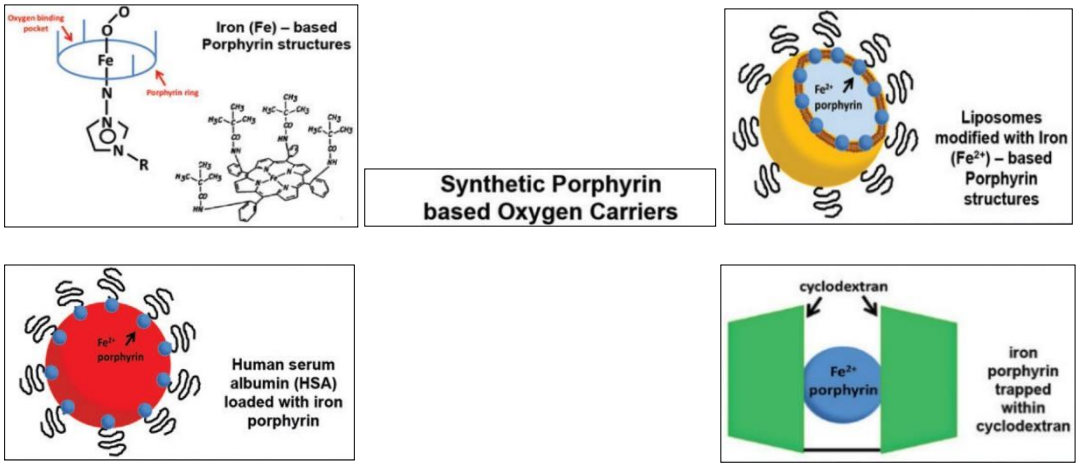

Figure 2.4 Schematic diagram on synthetic porphyrin-based system (Adopted from (Sen Gupta,2017)

2.6 Introduction to polymers

Polymers have become indispensable substances in various fields including drug delivery system, biomedical engineering, medical, pharmaceutical and many more (Woodruff and Hutmacher, 2010; D'souza and Shegokar, 2016). The distinctive

features of polymers can be classified into three categories namely linear polymer, branched polymer and cross-linked polymer with each bearing their own uniqueness. Linear polymers consist of long chain without side linkages while branched polymers has monomers linked to their backbone chain and finally cross-linked polymers has network of polymer chain attached to one another excluding their tip end (Samaddar, Deep and Kim, 2018).

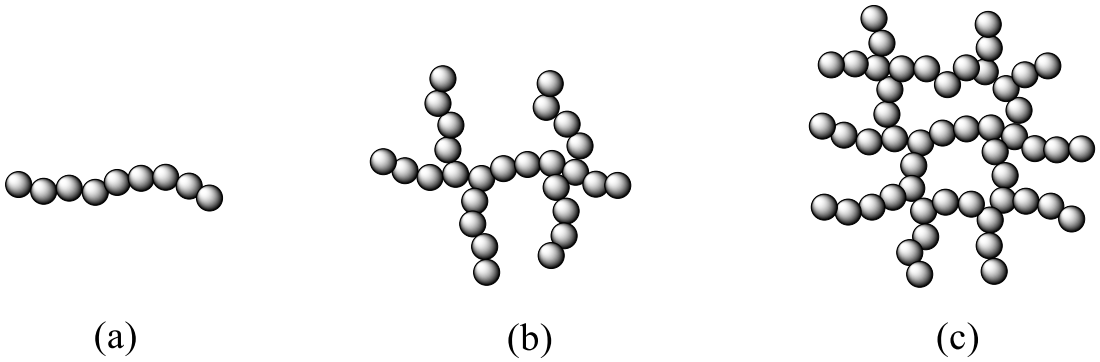

Figure 2.5 Three main classes of polymer (a) linear polymer (b) branched polymer (c) cross-linked polymer (Adopted from Cook and Bibic, 2019)

Recent advances in the past decades had been poured on the synthesis of polymers to obtain a well-defined and globular macromolecule structure. Block copolymers with its plethora of unique features and highly branched polymers known as dendritic polymers had gained immense interest in the recent years. For example, micelle synthesized from block copolymers had been shown tailorable to different morphologies provided certain parameters were followed (Feng et al., 2017). On the other hand, dendritic polymers with its definite multiple branched structure can be tailored for desired purpose (Abbina et al., 2017). Literatures on both polymers had described their potential in the drug delivery system through the host-guest molecule interaction (Deng et al., 2014; Brandt et al., 2019).

2.7 Synthesis of block copolymers

Synthesis of block copolymers can be achieved through interaction between two or more different monomers in linear arrangement. For diblock and triblock copolymers, two or three homopolymers, AB are linked from end to end (Samaddar, Deep and Kim, 2018). Copolymerisation of A and B monomer blocks that are different in structure at large A to B ratio will form polymers with amphiphilic nature. The linear formation of segmented block copolymers begins with the activation of reactive sites at A block before polymerisation of B block began (Brendel and Schacher, 2018). The polymerisation is possible via anionic, radical and cationic technique (Li et al., 2018). In order to select an appropriate synthesis method, factors such as mechanism of polymerisation, copolymer structure, desired molecular weight and monodispersity is taken for consideration.

(a) (b)

Figure 2.6 Examples of block copolymers (a) AB diblock linear copolymers (b) ABC triblock linear copolymers (Adopted from (Samaddar et al., 2018))

An aqueous tolerant micelle requires at least two block copolymers. This two-nature micelle is suitable as RBC mimic. The copolymerisation synthesis exerts less control over chain length and molecular mass of the final product during the reaction. The use of free radical living polymerisation would give better control via addition polymerisation. Other examples of living polymerisation techniques are ring opening polymerisation (ROP) and atomic free opening polymerisation (ATRP). Ring opening polymerisation (ROP) involves incorporation of functional group into the polymer backbone and the process offers ease of synthesis. Certain synthesis combined both

the free radical living polymerisation and ring opening polymerisation, increasing the efficiency of polymer synthesis.

2.8 Micelle

Micellization of block copolymers in solution constitute block with two different water-solubility namely the hydrophilic block (polar segment) and hydrophobic block (non-polar segment). The contrasting behaviour of these blocks known as amphiphilic exist in the surfactant of soap and detergent (Mai and Eisenberg, 2012). Amphiphilic block copolymer self-assembles to form the hydrophobic core and hydrophilic shell through non-covalent forces such as hydrogen bonding, van der Waals, electrostatic forces and hydrophobic forces (Zhulina and Borisov, 2012). Ergo, hydrated shell with minimal water exposure makes micelle suitable for entrapping hydrophobic substance such as drug (Alizadeh et al., 2015).

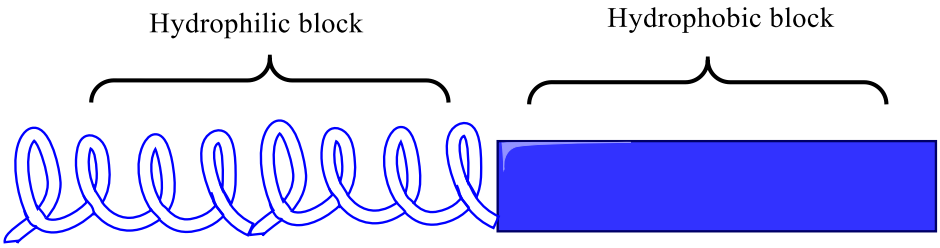

Figure 2.7 Diblock copolymer with hydrophilic and hydrophobic block (Adopted from Zhao 2012)

Manipulation of molecular parameters allow the assemblies of various morphologies such as spherical, lamellae, cylindrical and aggregates with smaller core or smaller shell than their counterparts are known as star micelles and crew-cut micelles respectively (Prazeres et al., 2012; Feng et al., 2017). Other factors that determine the size and shape of micelle includes the length and shape of hydrophobic and hydrophilic chain, solvent system, temperature and most importantly the critical micelle concentration (CMC) (Mai and Eisenberg, 2012).

Critical micelle concentration can be defined as a range of concentration in which micelle aggregates formed through hydrophobic effect (Topel et al., 2013; Hussein and Youssry, 2018). Below CMC, self-assembly could not occur due to insufficient number of chains, thus most conformation at this concentration is in the form of unimers. Above CMC, single chains and micelle exist in equilibrium. At this conformation, the contact between hydrophobic segment and solvent is limited as the micelle in bulk solution is thermodynamically stable (Owen, Chan and Shoichet, 2012). Establishment of CMC for block copolymers cannot be easily achieved as the concentration may be too small due to polydispersity composition and molecular weight of the copolymers. Nevertheless, the stability of block copolymers in aqueous solution can prevent sever dilution (sinking) condition when injected into the human body (Hussein and Youssry, 2018).

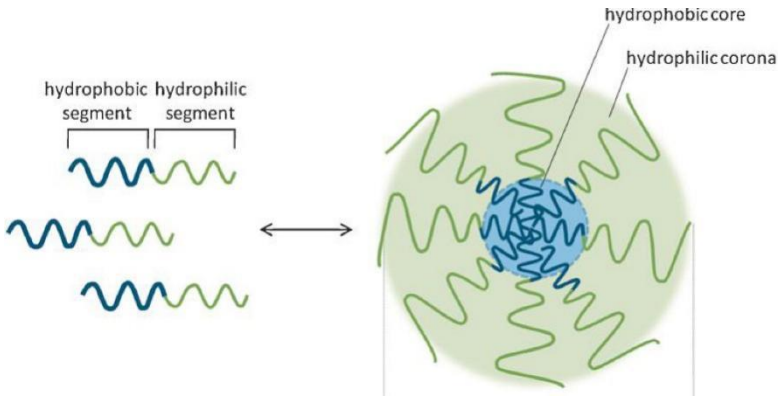

Figure 2.8 Formation of micelle through self-aggregation at equilibrium (Adopted from (Owen et al., 2012))

Polymeric micelle of amphiphilic nature had long perked the interest of researchers for its application in drug delivery system (Zaheer aet al., 2014). The interior compartment offers cavity for encapsulation of any guest material especially insoluble molecules (Lu and Park, 2013). At the same time, the exterior shell provides not only protection of guest molecule from harsh environment but also has better solubility and structural integrity (Gong et al., 2012). Properties of polymeric micelle

such as its nanometric structure, ranging between 10 to 100 nm, tailor made functionality, stability and different option for polymeric synthesis were reasons for its selection as drug carrier (Miyata, Christie and Kataoka, 2011).

It is important to note that stability of micelle is essential in ensuring the deliverance of guest molecule. The drug-core interaction contributes to the stability of the micelle through an additional hydrophobic interaction (Yokoyama, 2014). However, high concentration of guest molecule could disrupt the equilibrium through the conjugation with polymer chains resulting in precipitation (Lu and Park, 2013). Therefore, it is crucial not to overload the guest molecule within the micelle to avoid precipitation. Utilization of micelle for encapsulation of haemoglobin and iron porphyrin unit is ideal for RBC mimic.

For polymeric micelle to achieve not only a stable structure but also an optimum function, selection of appropriate polymers is necessary. Polyethylene glycol (PEG) and poly (ε-caprolactone) (PCL) are two polymers that had gained attention for its various application (Woodruff and Hutmacher, 2010; D'souza and Shegokar, 2016). PEG, also known as Macrogols is made up of repeated units of ethylene glycol. Biocompatibility and hydrophilicity which helps formed a hydrated capacity, absence of steric hindrance and high structure flexibility were properties of PEG that attracts it as polymer of choice. The structure of PEG exists in two forms namely brush or mushroom conformation. Within the body, metabolization of PEG depends on its molecular weight, whereby higher MW warrants easier renal clearance (D'souza and Shegokar, 2016). PCL, on the other hand, is also a biocompatible and hydrophobic polymer approved by Food and Drug Agency (FDA). It is not feasible, however, to apply PCL as a stand-alone polymer due to its lipophilic feature. Thus, combination

with PEG, a hydrophobic polymer can stabilize hydrophobic-hydrophilic ratio of molecule (Brandt et al., 2019).

2.9 Hyperbranched polymer

Hyperbranched polymer (HP) was described as a highly branched polymer with dendritic structure. The early development of HP was established by Jacob Berzelius through the synthesis of tartaric acid and glycerol forming resin. Following, the term ‘hyperbranched polymer’ was initially used by Kim and Webster after successfully synthesized dendritic macromolecule through one-step polymerisation (Zheng et al., 2015). This type of polymer had been characterized by a broad range and irregular architecture with high polydispersity index. It had no entanglement and shows low viscosity but high solubility in contrast with the usual linear polymer (Daniel, Stiriba and Holger, 2010; Zheng et al., 2015). Generally, the architecture of hyperbranched polymer gives rise to three types of branching unit namely the dendritic unit, linear unit and terminal unit. The degree of branching (DB) is one of the parameters determining the structure of hyperbranched polymer, with the value centered between linear polymer and dendrimers (Daniel, Stiriba and Holger, 2010). A study conducted in Sheffield University successfully synthesised iron-porphyrin cored hyperbranched polymer or ‘plastic blood’. Unlike HBOC, risk of transfusion reaction can be mitigated as this synthetic model is free from natural or biological components (Tyman 2006). The potential portrayed by hyperbranched polymer offers a hopeful substitution of material in biomedical application.

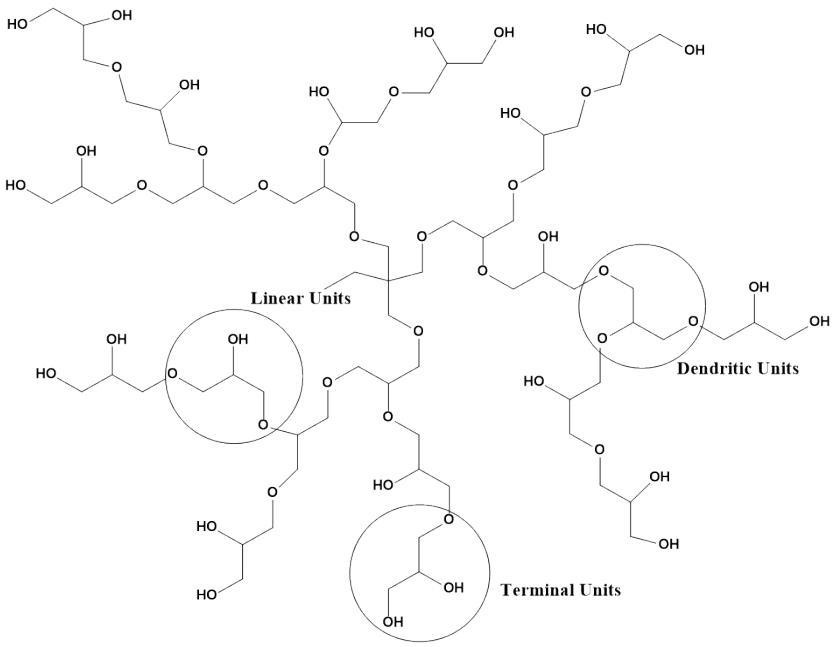

Figure 2.9 Branching units of AB2 hyperbranched polymer.

2.10 Hyperbranched polyglycidol

Polyglycidol is an example of hyperbranched polymer which possessed about half of dendritic structure (Abbina et al., 2017). Polymerisation of glycidol, a reactive oxirane monomer began as early as 1960s followed with various experimentation to produce hyperbranched polyether. In 1999, cationic ring opening polymerisation of glycidol was performed through slow monomer addition resulting in production of hyperbranched polyglycidol (Daniel, Stiriba and Holger, 2010). Glycidol is made up of epoxide group and hydroxyl group. During synthesis, the former involved in propagation and the latter in chain transfer reaction. The blocking and unblocking of hydroxyl group using selected solvent influence the shape of the produced polyglycidol. Due to the reactive hydroxyl group, polymerisation of glycidol often lead to branching of polymer.