CHEONG THENG HO PEGYLATED GOLD NANOPARTICLE 2019 MSC (TRANSFUSION SCIENCE)

PEGYLATED GOLD NANOPARTICLE (PEG AUNP) DEVELOPMENT FOR ENHANCEMENT OF RED CELL ANTIBODY AGGLUTINATION REACTION

BY

CHEONG THENG HO

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

ADVANCED MEDICAL AND DENTAL INSTITUTE

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DISCLAIMER

I hereby declare that this dissertation research entitled **PEGylated Gold Nanoparticle (PEG AuNP) development for enhancement of red cell antibody agglutination reaction** is the result of my own research except as cited in the references. I declare that this research has been sent to Universiti Sains Malaysia (USM) for the degree of Master of Science (Transfusion Science). It is not to be sent to any other universities. With that, this research can be used for consultation and photocopied as a reference.

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CHEONG THENG HO

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LIST OF ABBREVIATIONS

Abbreviation	
AHG	Antihuman globulin
AST	Antibody screening test
dH_2O	Deionised water
DLS	Dynamic light scattering
FWHM	Full width at half-maximum
HCL	Hydrochloric acid
HDFN	Haemolytic disease of the foetus and new-borns
HTR	Haemolytic transfusion reaction
Ig	Immunoglobulin
IS	Immediate spin
LISS	Low ionic strength solution
MV	Molecular weight
NaOH	Sodium hydroxide
NSS	Normal saline solution
PBS	Phosphate buffered saline

PEG	Polyethylene glycol
PEG AuNP	PEGylated gold nanoparticle
RBC	Red blood cell
RPM	Revolutions per minute
SPSS	Statistical Package for the Social Sciences
UV-VIS	Ultraviolet-visible spectroscopy

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ABSTRAK

Latar belakang kajian: Reagen potensi peningkatan seperti LISS dan PEG telah digunakan secara meluas dalam ujian saringan antibodi untuk mempercepatkan reaksi aglutinasi antibodi sel darah merah (SDM). Nanopartikel emas dengan ciri-cirinya yang stabil secara kimia dan mudah diubahsuai, telah terbukti dalam memudahkan reaksi ini. Oleh itu, kajian ini bertujuan untuk menghasilkan satu larutan alternatif dengan menggunakan gabungan PEG dengan nanopartikel emas (PEG AuNP).

Rekabentuk kajian: Sintesis satu langkah melalui konjugasi telah digunakan untuk pembentukan PEG AuNP dengan kehadiran NaOH mengakibatkan pembentukan yang cepat. Larutan yang baru dihasilkan ini telah dicirikan dengan menggunakan spektrofotometer UV-VIS pada suhu bilik di seluruh gelombang spektrum 200nm hingga 700nm. Ujian saringan antibodi bagi tiga sampel telah dilakukan dengan membandingkan reagen potensi peningkatan yang berbeza (LISS, PEG, Gold, dan PEG AuNP), dan antara kepekatan PEG AuNP yang berbeza pada fasa antiglobulin untuk reaksi aglutinasi antibodi SDM.

Keputusan kajian: PEG AuNP telah berjaya disintesis berikutan perubahan warna dari telus kepada ungu dan menunjukkan pada penyerapan maksimum 565nm. Larutan PEG AuNP menunjukkan skor reaksi yang sama dibandingkan dengan LISS dan PEG dalam ujian saringan antibodi. Tidak terdapat perbezaan yang signifikan dalam skor aglutinasi antibodi SDM antara kepekatan PEG AuNP yang berbeza (p = 0.4).

Kesimpulan: PEG AuNP yang baru dihasilkan ini mampu membantu dalam reaksi aglutinasi antibodi SDM dan dengan itu boleh menjadi reagen potensi peningkatan gantian dengan tujuan ini.

PEGYLATED GOLD NANOPARTICLE (PEG AUNP) DEVELOPMENT FOR ENHANCEMENT OF RED CELL ANTIBODY AGGLUTINATION REACTION

ABSTRACT

Background: Enhancing potentiators such as Low Ionic Strength Solution (LISS) and Polyethylene Glycol (PEG) have been widely used to facilitate red blood cell (RBC) antibody agglutination reaction. Gold nanoparticles with its inert and tunable property, have been proven in fastening this reaction. Hence, this study aimed to develop an alternative enhancing potentiator for RBC antibody agglutination by using the combination of PEG and gold nanoparticle (PEG AuNP).

Methods: One step synthesis through conjugation has been used for the development of PEG AuNP in the presence of sodium hydroxide (NaOH) resulted in the fast formation of PEG-coated gold nanoparticle. Characterisation of the newly developed solution was performed using UV-VIS spectrophotometer at room temperature across the spectral wavelength of 200nm until 700nm. Antibody screening test for three samples were performed comparing between enhancing potentiators (LISS, PEG, Gold, and PEG AuNP), and between different PEG AuNP concentrations at antiglobulin phase for RBC antibody agglutination reaction.

Results: PEG AuNP was successfully synthesised as the colour changes from transparent to violet and showed maximum absorbance at 565nm. The PEG AuNP solution showed

similar reaction score as compared to LISS and PEG alone in antibody screening test. There was no significant difference in agglutination score between various concentration of PEG AuNP ($p = 0.4$).

Conclusion: The newly developed PEG AuNP is capable to potentiate RBC antibody agglutination and thus can be a substitute solution for this purpose.

CHAPTER 1

INTRODUCTION

1.0 Background of study

Blood transfusion is remarkably safe and plays a vital role in routine clinical management (Seitz & Heiden, 2010). The requirement of blood transfusion varies between the different blood products or components used. For instances, red blood cells (RBCs) transfusion is required in bloos loss, haemorhage, and it is also used to increase tissue perfusion and oxygenation such as in patient with symptomatic anemia, acute sickle cell diorder, or massive blood loss. Fresh frozen plasma is used to reverse the anticoagulant effect and coagulation factor deficiencies. While platelet is indicated in patient with underlying thrombocytopenia and platelet function disorders; and cryoprecipitate is used to treat for fibrinogen deficiency (Sharma *et al.*, 2011).

Pre-transfusion testing is an essential laboratory testing that is required prior to any transfusion. This test is performed by testing both patient's sample and the intended blood components to check for the compatibility between the patient and the blood product intended to be transfused especially for RBC products (Lane, 2017).

In pre-transfusion testing, ABO and Rh(D) grouping will be performed by forward and reverse grouping. In addition, patients' plasma will also be tested for antibody screening

to detect the non-ABO clinically significant antibodies that may cause severe transfusion reactions. This is because, beside ABO grouping which serves as the most important blood group contributed to acute haemolytic transfusion reaction, there are also presence of non-ABO antibodies that are clinical significant (Makroo *et al*., 2014). These alloantibodies may develop following sensitisation through pregnancy or previous blood transfusion and can cause either acute or delayed transfusion reaction (Al-Joudi *et al.*, 2011). Thus, antibody screening is an important steps in pre-transfusion testing that acts as the first guard to ensure a safe transfusion.

Crossmatching between the patient's blood and the donor's RBC will be performed as the last guard in pre-transfusion testing to ensure ABO compatibility between recipients and the blood product that intended for transfusion; and to detect the clinically significant antibodies that may not detected from the antibody screening test. Any agglutination reaction indicated incompatibility between the transfusion recipients and the blood products (Armstrong *et al.*, 2008).

1.1 Problem statement

For antibody screening test, it took about 30 to 60 minutes to detect any clinically significant of unexpected antibodies (Aronson *et al*., 2011). This is a critical part to be concerned as for some serious cases such as acute massive bleeding, time is an important factor to reduce further mortality and morbidity in managing patient (Choktaweesak *et al.*, 2016). Moreover, if there are presence of unexpected antibodies, further test which is antibody identification will be performed. Antibody identification may take several hour to days and thus may jeopardise the patients' life who in need of an urgent blood transfusion.

In addition, there are still some clinically significant antibody that will be missed out during the antibody screening such as those antibodies against low incidence antigens and those that who show dosage effect (Milkin *et al*., 2013). If the antibody is not detected during pre-transfusion testing, it will have a detrimental effect to patient such as adverse transfusion reaction (Resnick *et al.*, 2008).

Hence, in order to address these problems, enhancing reagents or potentiators such as Low Ionic Strength Solution (LISS), albumin, polyethylene glycol (PEG) have been used extensively in blood bank service to facilitate and expedite the antibody screening test. However, these enhancing reagents are costly to be used in most of the blood bank especially in those resource restricted countries.

1.2 Research justification

Enhancing reagent or potentiator such as LISS and PEG are widely used in blood bank service due to capability to shorten the antibody screening test duration from 30 minutes to 15 minutes. Currently, gold nanotechnology is a popular trend that has been extensively being tested in medical sector due to its comprehensive function (Longo *et al*., 2011; Ulbrich *et al*., 2011) for example, it has been reported from Wiwanitkit and his colleagues (2007) that gold can be used to enhance antibody-antigen agglutination reaction and can even facilitate in enhancing protein reaction and detecting weak subgroup system. Therefore, in our study, we aimed to develop an alternative solution using PEGylated gold nanoparticle (PEG AuNP) that may facilitate and expedite the antibody-antigen agglutination process during antibody screening in pre-transfusion testing.

1.3 Research objectives

General:

To develop a solution for enhancement of RBC antibody agglutination reaction using PEG and gold nanoparticle (PEG AuNP).

Specific:

I. To synthesis a new agglutination enhancing solution using PEG and gold nanoparticle.

II. To characterise the newly synthesised PEG AuNP solution.

III. To determine the optimal concentration of PEG AuNP solution for RBC antibody agglutination reaction.

CHAPTER 2

LITERATURE REVIEW

2.0 Antibody screening

There are variety of proteins and carbohydrates based antigens on the membrane of the RBC. Approximately, there are over 600 RBC antigens which are divided into 36 blood group systems nowadays (Rath *et al.*, 2014). The existence of the antigens will determine the identity of one's blood group system such as ABO blood group and Rh(D) blood group.

During antibody screening procedure, patients' serum is used to detect any unexpected antibodies present within it other than naturally occurring anti-A and anti-B. Patient's serum is tested against known commercial O RBC embedded with variety of clinically significant antigens onto it. Normally, two panel cells or three panel cells will be used in antibody screening for detection of the unexpected antibodies (Fung *et al*., 2014).

Antibody screening is performed at three phases namely immediate spin (IS), incubation at 37 ℃, and at antihuman globulin (AHG) phase. Immediate spin phase is performed at room temperature to detect IgM or "cold" antibodies, and it is done without the need of any incubation (Fung *et al*., 2014). After IS phase, enhancement reagent will be added and the test will be incubated at 37℃ in order to create an enhanced environment for

antigen-antibody reaction to occur. After incubation phase, any positive reaction in term of agglutination in AHG phase will be considered as clinical significant antibodies presence in the patient's blood. Profoundly, AHG phase is to provide enhancement environment for antigen-antibody reaction for second stage of RBC antibody agglutination reaction (Pathak *et al.*, 2011).

2.1 Clinically significant antibodies

Nowadays, all the blood transfusion services' centre confront the problem that procrastinating of blood for urgent transfusions (Chowdhry *et al.*, 2014). This is mostly due to the antibody screening test (AST) that gained a positive result. Once positive result in AST yielded, antibody identification must be performed prior to any transfusion activity (Green *et al.*, 2015).

The RBC antibodies can be divided into either clinically significant or non-clinically significant antibodies. Clinically significant antibody is normally reactive at AHG phase while antibody that react below 37 ℃ is commonly not clinically significant and may not elicit any clinical symptoms (Pathak *et al.*, 2011).

These clinically significant RBC antibodies have known to cause haemolytic transfusion reaction (HTR) as well as haemolytic disease of the foetus and new-borns (HDFN) (Chowdhry *et al.*, 2014). The example of common non-ABO clinically significant blood group systems include Rh, Kell, Kidd, Duffy, MNS, and P (Armstrong *et al.*, 2008). A summarised table that depicting the clinically significant antibodies to the blood group antigen of majority are tabulated below (Table 2.1) (Christopher *et al*., 2004):

Table 2.1 Clinically significant antibodies with respect to its blood group systems.

Common Clinically significant	Clinically Significant if Reactive at 37 ℃
A and B	A_1
Diego	H
Duffy	Le^a
H in O_h	Lutheran
Kell	M, N
Kidd	P_1
P, PP_1P^k	
Rh	
S, s, U	
Vel	

2.2 Antigen-antibody reaction

The AST involves the detection of unexpected antibody agglutination between the RBC antigen and antibody interactions (Milkin *et al*., 2013). The RBC antibody agglutination process can be divided into two stages namely first stage of non-visible sensitisation and second stage of visible agglutination. First stage of non-visible sensitisation involves the binding of the RBC antibodies binding to its antigens on the RBC' membrane. The second stage of visible agglutination, is a stage whereby the antibody sensitising RBC will collide each other randomly to cause a crosslink among antibody coated RBC (Fernandes *et al.*, 2011).

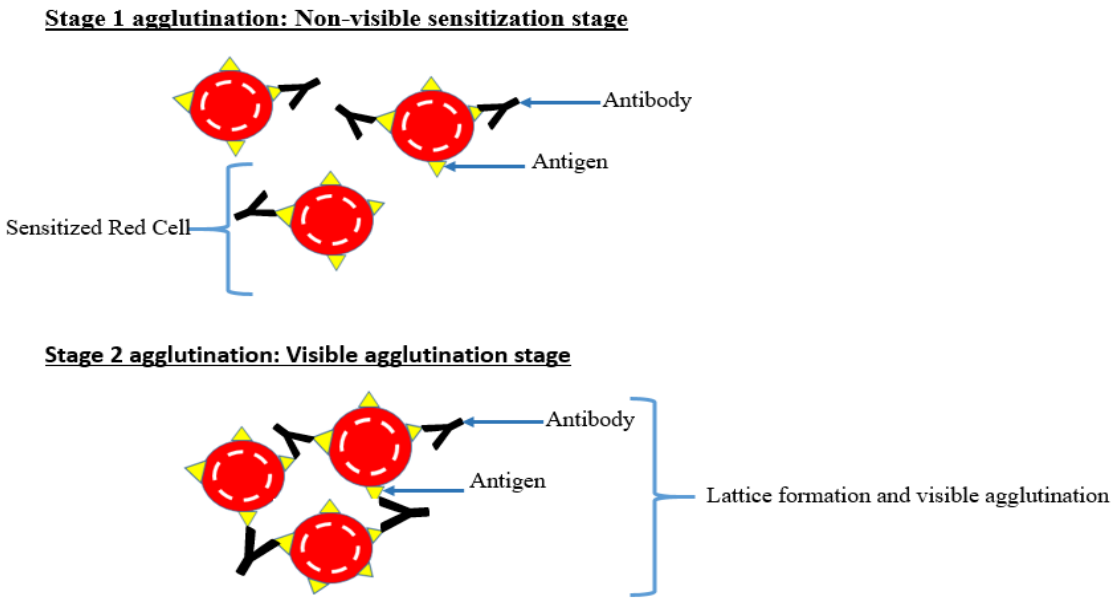

Figure 2.1 Representation of the RBC antibody agglutination reaction. Blood group antigens and antibodies formed a lattice bridge of RBC and caused agglutination.

2.3 Factor affecting antigen-antibody agglutination reaction

There are many factors that can influence the reaction between antigen and antibody in laboratory testing and can be categorised as factors that affect the sensitisation or agglutination stage.

2.3.1 Factors affecting sensitisation stage (Stage 1)

During non-visible sensitisation, serum-to-cell ratio plays an important role to increase chances of the antigen-antibody collision in order to achieve higher probability of sensitisation event to occur. Serum contains antibody, and thus by increasing the serum amount in the test, it may increase the binding of those antibodies to the RBC antigen (Kathy and Paula, 2009).

In addition, different antibody will react effectively at different temperature (Kathy and Paula, 2009). For example, cold type RBC antibodies such as anti-Le^a, anti-Le^b, anti-I, anti-P_1, anti-M and anti-N are stable and reactive at lower temperatures, mainly at or below ambient room temperature about 20-24 ℃, whereas other clinically significant antibodies are IgG antibodies in nature such as anti-D, anti-K, anti-Jk^a, anti-Jk^b, anti-Fy^a, anti-Fy^b, anti-S, and anti-s are react best at temperature of 37 ℃ (Kathy and Paula, 2009). Thus, by incubating the testing sample in respective temperature, it can further enhance the first stage of agglutination reaction (Encarnação *et al.*, 2017).

Furthermore, RBC antibody reacts most effectively with the physiological condition of pH nearly to neutral, seven. Out of this pH range, there will be inhibition of the antigen-antibody activity (Kathy and Paula, 2009). This is due to the reason that, extreme pH will cause alteration of the antibody conformation and aftermath incompatible to the antigen determinants (Reverberi and Reverberi, 2007). According to Martin and his colleagues (2014), phosphate buffered saline (PBS) is recommended to be used in RBC washing as compared to normal saline solution (NSS) because NSS pH tend to have more fluctuation as the storage time increases. Furthermore, PBS pH can be maintained at longer period of time and more stable without any significant fluctuation (Martin *et al*., 2014).

Moreover, ionic strength is another contributing factor that may affect the RBC antigen-antibody agglutination reaction *in* vitro. Under isotonic circumstances in saline solution, sodium ions and chloride ions tend to bind to the opposite charged groups on both antigen and antibody molecules, and decreasing their affinity to attract each other to cause sensitisation (Kathy and Paula, 2009). Thus, the reaction of RBC antigen-antibody reaction is hindered. According to Reverberi and her colleague (2007), there will be

increase in the antibody titre by lowering ionic strength in the *in vitro* environment. This is due to the lessen hindering effect and increased in the uptake of antibody to corresponding antigens (Kathy and Paula, 2009).

2.3.2 Factor affecting lattice formation stage (Stage 2)

Once the RBC is sensitised with antibody, random collision is needed between the sensitised RBC to form a crosslink relationship in visible agglutination stage or lattice formation stage *in vitro*.

In nature, RBC membrane has a net of negatively charged ions due to the presence of sialic acid onto it in physiological saline solution environment (Figure 2.2) (Fernandes *et al.*, 2011). This net of negatively charge will get attracted by positively charged ions in the solvent system. The equilibrium of the positively charge masked RBC will repulse each other in the physiological saline environment and this will keep RBC from being in the vicinity of one another (Brecher, 2005). This phenomenon is called zeta potential, an electrostatic potential between the RBC in the dispersed system (Fernandes *et al.*, 2011).

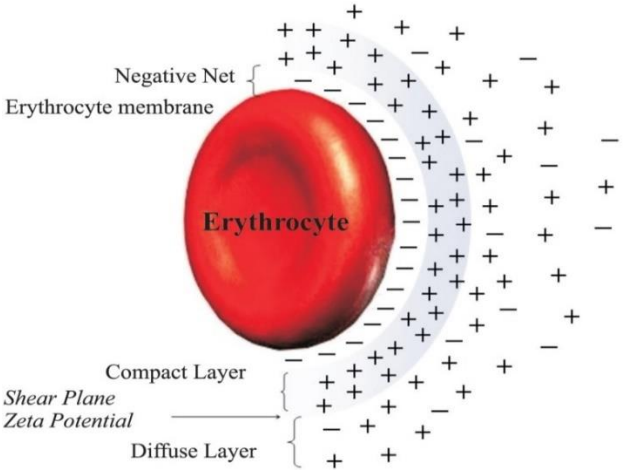

Figure 2.2 Zeta potential effect on the second stage of agglutination. Figure adapted from Fernandes his colleagues (2011).

Therefore, it is important to reduce the zeta potential so that there will be higher probability of positively charged antibodies to overcome the repulsion force and get closer to the negatively charged sialylated RBC membrane and eventually promoting the visible agglutination reaction (Caruccio and Wise, 2013).

Different antibodies have different morphologies in their nature and tend to have different aptitudes with respect to agglutination. Size of the immunoglobulin plays role in different binding ability (Kathy and Paula, 2009). In term of agglutination, IgM antibodies tend to have more binding efficient ability than IgG (Fernandes *et al.*, 2011). This is due to the reason that IgM is pentameric large molecule with 970 kDa and by having five Y shaped units with ten antigen binding sites which can facilitate the agglutination between cells. Whereas IgG is comparatively smaller in size with 150 kDa and having merely one Y shaped units equipped with two antigen binding sites causing them difficult to span the distance between neighbouring cells produced by the zeta potential (Carl and David, 2014). Thus, the binding efficiency of IgG to its antigen is comparatively lower for visible agglutination reaction (Kathy and Paula, 2009). Hence, antihuman globulin (AHG), a polyclonal anti-IgG reagent is used in all laboratory and blood bank service to address the hurdle of IgG visible agglutination by forming a stable lattice bridge and caused agglutination when anti-IgG attaches to the IgG sensitised RBC (Pathak *et al.*, 2011).

Ratio between antibody and antigen should be equivalent for agglutination reaction to occur. Thus, the amount of antibody and antigen should be in optimal condition in the agglutination zone of equivalence. There are three zones in immunoagglutination reaction namely Prozone, Zone of Equivalence, and Postzone. Prozone is defined when the serum antibody concentration is higher compared to the concentration of antigen presence (Tankeshwar, 2010). During this zone, most probably antibodies will only bind to RBC

univalently instead of multivalently and cause no crosslink between RBC and thus giving a false-negative result. While postzone is the opposite of prozone, and resulted in the excessive of antigen over the antibody presented in the serum which also likely to cause a false-negative result (Figure 2.3) (Tankeshwar, 2010). Therefore, range of two to five percent of red cell suspension in saline in blood bank setting result in the optimal concentration for antigen concentrations to let the reaction fall into the equivalent zone to cause agglutination reaction (Kathy and Paula, 2009).

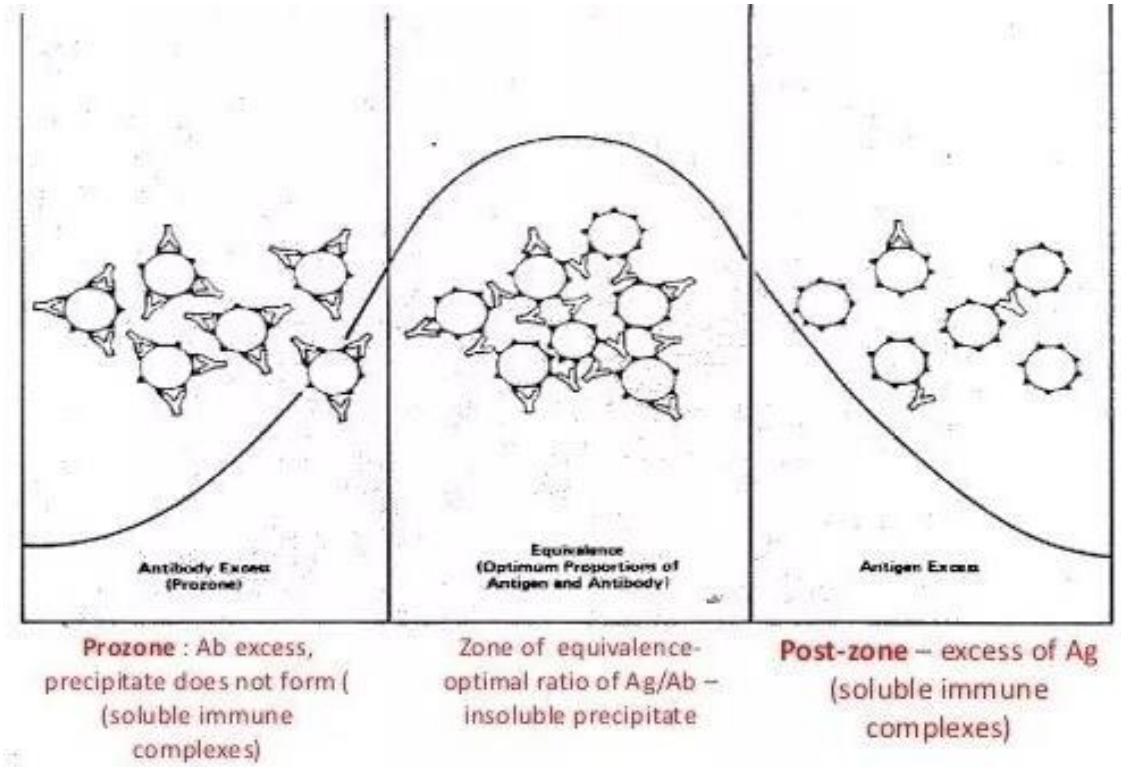

Figure 2.3 Effect of agglutination observed in the zone of equivalence. Figure adapted from Tankeshwar (2010).

Last but not least, centrifugation plays a role for the visible effectiveness of antigen-antibody agglutination reaction. Appropriate time and speed of centrifugation aid in the formation of lattice bridge within close range of RBC in the test environment (Kathy and Paula, 2009).

2.4 Antibody potentiators or enhancement media

The RBC antibody agglutination reaction can be enhanced in the presence of antibody potentiators. These potentiators can be used to further expedite *in vitro* agglutination (Kathy and Paula, 2009). There are three types of common potentiators that are normally used in blood bank such as low-ionic strength solution (LISS), polyethylene glycol (PEG), and proteolytic enzymes.

2.4.1 Low-ionic strength solution (LISS)

LISS is the most commonly used as an antibody potentiator for agglutination reaction detection. LISS comprises of 20% lower concentration of sodium chloride as compared to ordinal isotonic saline solution (Fernandes *et al*., 2011). LISS offers several advantages in term of time reducing for incubation from 30 to 15 minutes, improvement in agglutination reaction, and decrease in zeta potential between RBC, and enhancing the antibody uptake by the antigen (Choktaweesak *et al*., 2016). In physiologic saline environment, sodium ions and chloride ions tend to bind to the opposite charged groups on both antigen and antibody molecules, and thus decreasing their affinity to attract each other in the antigen-antibody complex formation (Kathy and Paula, 2009). Thus, LISS caters a lower number of sodium and chloride ions in the solution which subsequently reduces the ionic cloud formation encircled the RBC and promotes stronger association between the antigen-antibody complexes, and ultimately reduced the incubation time. Profoundly, ionic strength that is decreased by LISS can reduce the thickness of the cations net charge or ionic layers, result in decreasing of zeta potential and enhance the antigen-antibody agglutination reaction (Fernandes *et al*., 2011). However, according to

Garg and his colleagues (2017), LISS reported to have less efficiency towards certain antibody detection such as Kell's antibodies (Anti-K).

2.4.2 Polyethylene Glycol (PEG)

PEG is another enhancement media that can be used in blood bank test. PEG is a type of polymer that has a linear chain and is water soluble in nature with molecular formula of HO-(CH_2CH_2O)n - CH_2CH_2OH. The PEG's OH group can bind with other molecules or surfaces covalently in order to facilitate the stabilisation of the compound (Fernandes *et al*., 2011). Therefore, PEG will eliminate the water from the RBC membrane, reduce the distance between RBC, and concentrate the antibodies to allow a greater odd of random collision between the antigen and antibody in the test environment (Fernandes *et al*., 2011). In addition, Wang and his colleagues (2004) showed PEG able to facilitate antigen-antibody complexes, and aids in signals' amplification which enhances the sensitivity of the system. Besides that, PEG is better in detecting anti-K, anti-E, anti-c, and anti Jk^a as compared to LISS (Reverberi and Reverberi, 2007; Okutsu *et al*., 2011). According to Nance and Garratty (1997), the optimal concentration used was reported to be at 20% w/v with the PEG molecular weight (MV) of 4000. However, as a precautionary step while using as potentiator, centrifugation after incubation phase is unnecessary as centrifugation can cause aggregates formation of PEG and make it difficult to resuspend after washing procedures, and subsequently can give false positive reaction. Thus, if PEG is used, AHG phase is performed following incubation phase to eliminate the possibility of getting a false positive result (Kathy and Paula, 2009).

2.4.3 Proteolytic enzymes

There are many proteolytic enzymes normally used in immunohematology laboratory or blood bank in term of agglutination reactions such as papain, ficin, bromelin, trypsin, dispase, chymotrypsin, and neuraminidase which are commonly available in the market. Within a variety of proteolytic enzymes, bromelin and papain are more commonly used (Armstrong *et al*., 2008). These proteolytic enzymes alter the sialylated RBC' membrane by eliminating the anion charges on the surface and therefore reducing the zeta potential and subsequently result in agglutination (Figure 2.4) (Fernandes *et al.*, 2011). The reaction of enzymes with respect to RBC antigens are showed in with respect to its target antigen as summarised in the Table 2.2 (Armstrong *et al*., 2008):

Table 2.2 Enzyme addition with respect to enhancement or denaturation of antigens (modified from Armstrong *et al*., 2008).

Antigen enhanced by enzyme addtion	Antigen showed no changes/ denatured by enzyme addition
Le^a	M
Le^b	N
I	S
P_1	Fy^a
Rh	Fy^b
	K
	Xg^a

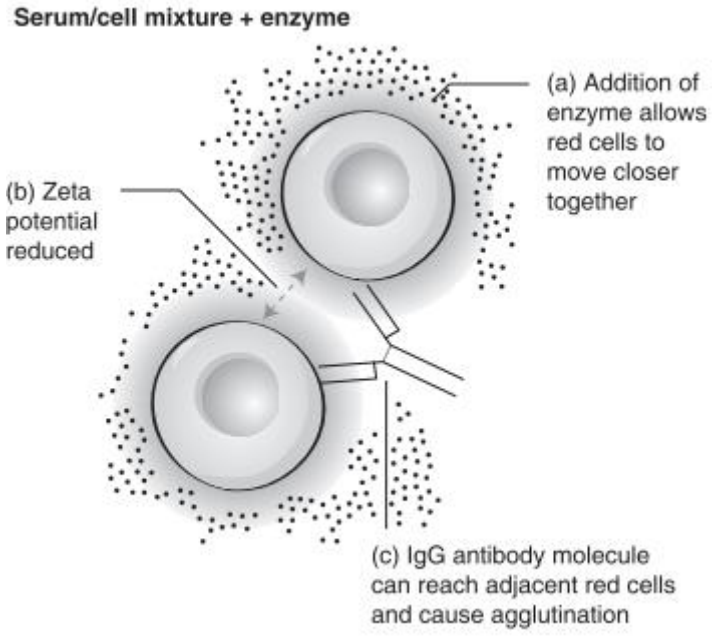

Figure 2.4 Action of enzyme on sensitised red blood cells. Figure adapted from Armstrong and his team (2008).

2.5 Nanotechnology

Recently, there are many ongoing intensive researches on the field of nanotechnology from a wide range of application in term of physical, chemical, biological, and engineering sciences. Nanotechnology results in a promising and revolutionary in the field of medical application by interconnecting with biological molecules. Nanotechnology or sometime called as nanoscience which interchangeable denoted that the particle study that can be grouped as a certain number nanometers in size or having a nanoscale of less than 100nm in measurement (Tiwari and Tiwari, 2014). Besides, many proteins are in approximately 10 nanometers in size which can be specifically designed according to its nanoscale incorporated into the system of biology (Eustis and El-Sayed, 2006). An exponentially increased in nanoparticle publications with the nanomaterials of gold and silver since 1990 (Eustis and El-Sayed, 2006). The development of nanoparticles

has attracted many researchers to enhance and improvise the synthesis of nanoparticles for biological and medicine application (Eustis and El-Sayed, 2006).

2.5.1 Gold Nanoparticles

Gold nanoparticles (AuNP) have been broadly used in wide range of biomedical fields owing to their high biocompatibility, optical electronic properties, good conductivity, having large surface area, and not hassle to be synthesised (Khan *et al*., 2014). Importantly, gold nanoparticles has low toxicity and has high chemically inertness properties which are safer to be used in *in vitro* testing without affecting the chemical equilibrium in the system (Giljohann *et al.*, 2010). Besides that, gold nanoparticles have tunable properties chemically and physically which can be interacted with different biological macromolecules and organic molecules for instance antibodies and oligonucleotides (Khan *et al*., 2014). According to Jazayeri and his colleagues (2016), AuNPs with high electron densities have increase the cellular uptake while interacting with the cell membranes. In addition, gold nanoparticles can form a strong linkage exist between nanoparticle-protein interactions, immunogenecity and cytotoxicity while interact with human serum (Ajdari *et al.*, 2017). Interestingly, there was a study done by Wiwanitkit and his workmates (2007) found that gold nanoparticles can enhance protein agglutination and enhance weak blood group detection. Recently, there are many strategies to modify the gold nanoparticle surface conjugated with ligands. For instance, adding polyethylene glycol (PEG) to improvise its bioreactivity for better targeting and cellular uptake for biomedical application (Jazayeri *et al*., 2016). Yet there is limited study to investigate the relationship between the used of gold nanoparticles and blood group system.

CHAPTER 3

METHODOLOGY

3.0 Study design

This was an experimental study to elucidate the development of RBC antibody agglutination enhancement reaction using PEG and gold nanoparticle (PEG AuNP) for antibody screening.

3.1 Study venue

This study was mainly conducted in Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM) in Bertam, Penang in which:

AMDI:

- Animal Research Complex
- Transfusion Medicine Unit, Advanced Diagnostic Laboratory

3.2 Study duration

This study was carried out from 14th January 2019 to 31 May 2019.

3.3 Study variables

3.3.1 Dependent variable

- Agglutination scoring/ grading

3.3.2 Independent variable

- Type of enhancing potentiators
- Volume/ concentration of PEG AuNP

3.4 Sample size

The estimated sample size in this study was calculated from objective 3 (to determine the optimal concentration of PEG AuNP solution for RBC antibody agglutination reaction. Comparable study by Wiwanitkit and his colleagues (2007) which used only two samples in the study. Therefore, α (two-tailed) = 0.05 (level of significance for probability to reject the null hypothesis which indicated Type I error rate. β= 0.80 which accept null hypothesis and about 20% of error is made on difference of variable under study or literally type II error rate.

r = 0.999 (expected correlation coefficient)

Standard normal deviate for $\alpha = Z_{\alpha} = 1.960$ while standard normal deviate for $\beta = Z_{\beta} = -0.842$c = 0.5* In [(1+r)/ (1-r)] = 3.80. Thus, the total number required for the sample size was 3 samples.

3.5 Study Flowchart

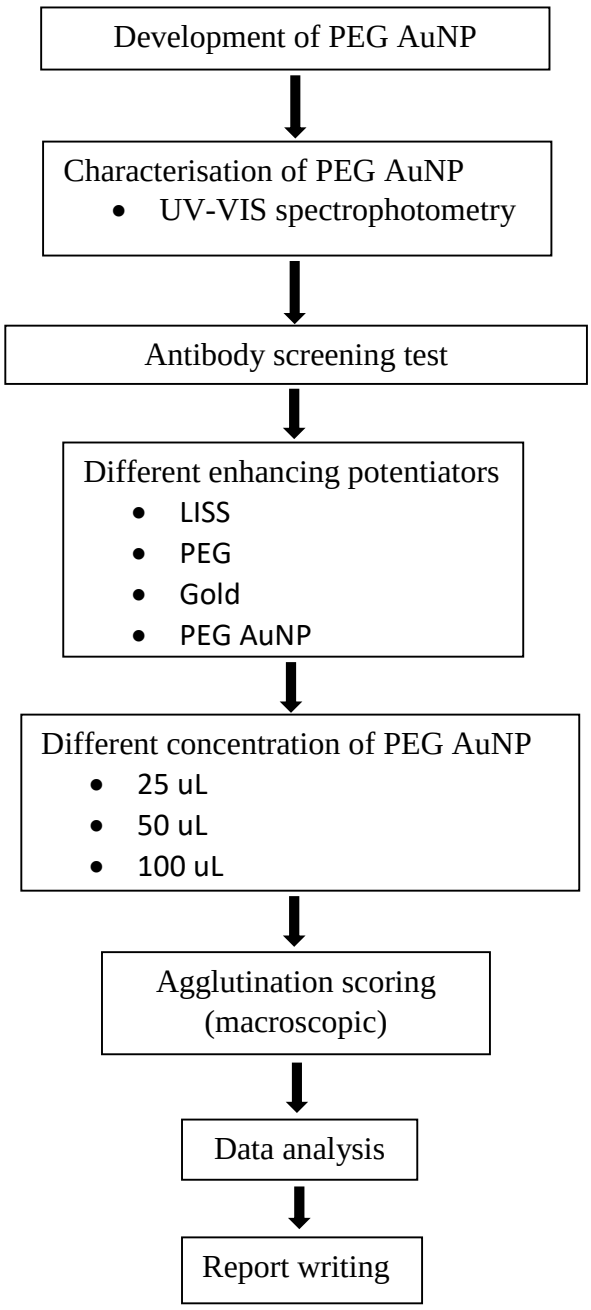

Figure 3.1 Flowchart of the study

3.6 Chemicals, Materials, and Apparatus

Before synthesis, glassware was washed, rinsed with distilled water and put into the dryer or oven overnight at 80 to 100 ℃. For solvent preparation, deionised water (dH_2O), and 1% of sodium chloride solution were prepared. Polyethylene glycol (PEG) with the molecular weight of 4600, Chloroauric acid trihydrate ($HAuCl_4.\ 3H_2O$) or gold (lll) chloride hydrate, were purchased from Sigma Aldrich (USA) company. All the chemicals and equipment used throughout the experiment were tabulated in Table 3.1 and Table 3.2 respectively as below:

Table 3.1 Type of chemical used with respect to its company and country of origin respectively.

Chemical	Company	Country of Origin
Polyethylene glycol (PEG)	Sigma Aldrich	USA
Chloroauric acid trihydrate ($HAuCl_4.\ 3H_2O$)	Sigma Aldrich	USA
Panoscreen I and II	Bio-Rad	USA
Gamma H-Hance (LISS)	Immucor	USA
Diaclon Coombs Serum (AHG)	Bio-Rad	USA
Seruracell^{TM}	Immulab Pty Ltd	Australia

Table 3.2 Equipment used with respect to its manufacturer, model, and country of origin respectively.

Equipment	Manufacturer	Model	Country of Origin
Hotplate	LMS Harmony	HTS-1003	Japan
pH meter	Eutech	pH 700	Singapore
Hotplate with temperature and speed monitors	Fisher Scientific	Isotemp	USA
Sonicator/ Sonic bath	Jeiotech	UC-10	Korea
UV-VIS spectrometer	PerkinElmer	UV Lambda 25	USA
Centrifuge (sample separation)	Hettich Zentrifugen	Rotofix 32 A	Germany
Incubator	DiaMed-ID	ID-incubator 37 S I	Switzerland
Centrifuge (AST)	DiaMed	Diacent-12	Switzerland

3.7 Enhancing potentiators preparation

3.7.1 Preparation of PEG solution

PEG stock solution was prepared based on monthly basis and the method of preparation was used based on Nance and Garratty (1997) and Stiufiuc and his colleagues (2013). Firstly, 1.24g of PEG was dissolved in deionised water and added to the final volume of 10mL of deionised water in a 50mL beaker. The mixture was then transferred to 25mL of bottle and heated up on the hotplate (LMS Harmony, HTS-1003, Japan) to ensure it was homogeneously dissolved (Figure 3.2). Then, the mixture was cooled at room temperature and adjusted to pH 7 to 7.4 and kept in 4 ℃ for storage. Prior for usage, the stock solution was equilibrate to room temperature before testing is carried out.

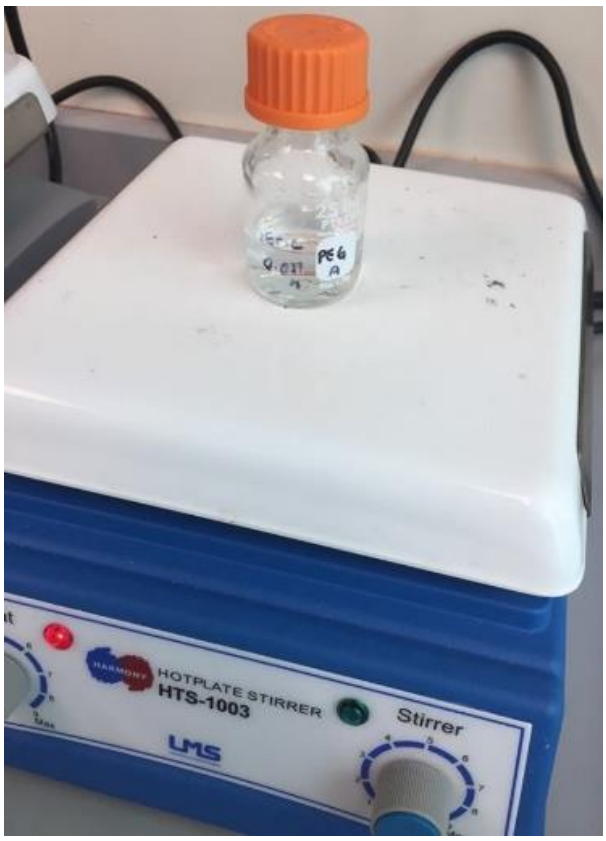

Figure 3.2 PEG preparation.