SYNTHESIS AND CHARACTERISATION OF PEGYLATED GOLD NANOPARTICLES FOR ENHANCEMENT OF AGGLUTINATION REACTION IN ANTIBODY SCREENING TEST

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by

NORDIYANA BINTI ISHAK

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LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
AHG	Anti-Human Globulin
AMDI	Advanced Medical and Dental Institute
AST	Antibody Screening Testing
Au	Gold
AuNPs	Gold Nanoparticles
$AuCl_4^-$	Tetrachloroaurate ion
BET	Brunauer, Emmett and Teller
BSA	Bovine Serum Albumin
DIC	Disseminated Intravascular Coagulation
DLS	Dynamic Light Scattering
EDX	Energy-Dispersive X-ray spectroscopy
EFTEM	Energy-Filtered Transmission Electron Microscopy
FDA	Food and Drug Administration
FESEM	Field Emission Scanning Electron Microscope
FFP	Fresh Frozen Plasma
FT-IR	Fourier-transform infrared spectroscopy
GSH	Group, Screen & Hold
$HAuCl_4$	Gold (III) chloride hydrate or tetrachloroauric acid
HCl	Hydrochloric acid
HDFN	Haemolytic Disease of the Foetus and Newborn
HSJ	Hospital Seberang Jaya
HTR	haemolytic transfusion teactions
IAT	Indirect anti-globulin test

INFORMM	Institute for Research in Molecular Medicine
ISBT	International Society of Blood Transfusion
JEPeM-USM	Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia
KBr	Potassium bromide
LISS	Low-Ionic Strength Solution
MPCs	Monolayer Covered Clusters
MREC	National Medical Research & Ethics Committee
Mw	Molecular weight
NaOH	Sodium hydroxide
NIR	Near-Infrared Region
NPs	Nanoparticles
PBS	Phosphate-Buffered Saline
PEG	Polyethylene glycol
PEG-AuNPs	PEGylated Gold Nanoparticles
PEO	Polyethylene Oxide
PL	Photoluminescence
POE	Polyoxyethylene
PSA	Particle Size Analysis
RBC	Red Blood Cells / Sel darah merah
ROC	Receiver Operating Characteristic
RT	Room Temperature
SCD	Sickle Cell Disease
SEM	Scanning Electron Microscope
SERS	Surface-Enhanced Raman Spectroscopy
SPR	Surface Plasmon Resonance
SPSS	Statistical Package for the Social Sciences

TEM	Transmission Electron Microscopy
USM	Universiti Sains Malaysia
UV-Vis	Ultraviolet–Visible spectroscopy
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-ray Powder Diffraction

LIST OF APPENDICES

SINTESIS DAN PENCIRIAN TERPEGIL PARTIKEL NANO EMAS BAGI PENINGKATAN TINDAK BALAS AGLUTINASI DALAM UJIAN SARINGAN ANTIBODI

ABSTRAK

Pengaglutinatan antara antigen dan antibodi sel darah merah (RBC) merupakan tindak balas yang sangat penting dalam ujian saringan antibodi (AST) untuk mengenal pasti kehadiran tidak dijangka antibodi yang signifikan secara klinikal dalam plasma penerima. Walau bagaimanapun, dengan kaedah semasa yang menggunakan larutan garam ionik rendah (LISS) sebagai medium peningkatan untuk mengesan antibodi, terdapat beberapa kelemahan seperti ketidakupayaan mengenalpasti sesetengah antibodi yang mempunyai klinikal signifikan dalam plasma penerima yang boleh menyebabkan reaksi tindakbalas transfusi. Oleh itu, kaedah baru menggunakan polietilena glikol berat molekular 4000 (PEG4000) berkonjugat dengan partikel nano emas (AuNPs) telah dibangunkan untuk meningkatkan tindak balas pengaglutinatan antara antigen dan antibodi RBC semasa ujian AST dijalankan. Setelah PEG-AuNPs disintesis, larutan ini diukur dan dikenalpasti sifat optiknya dengan menggunakan spektroskopi Ultraungu-nampak (UV-VIS), perincian morfologi oleh mikroskopi elektron transmisi tenaga turas (EFTEM) dan mikroskopi elektron penskanan pancaran medan (FESEM), analisa saiz zarah dan keupayaan zeta oleh cahaya dinamik sebar-balik (DLS) serta struktur dan komposisi bahan pengikat oleh spektroskopi transformasian Fourier inframerah (FT-IR). Perbandingan tindakbalas pengaglutinatan RBC antara larutan PEG-AuNPs dan LISS menggunakan kaedah tiub dalam ujian AST menggunakan 120 sampel yang terdiri daripada 60 sampel negatif dan 60 sampel positif mengandungi antibodi dijalankan. Warna akhir larutan PEG-

AuNPs berubah menjadi merah delima menunjukkan pembentukan AuNPs bersalut PEG4000 berjaya dilakukan. Larutan PEG-AuNPs menunjukkan satu puncak penyerapan maksimum pada gelombang 529 nm, dan ia sepadan dengan penyerapan permukaan resonans plasmon AuNPs. Imej EFTEM PEG-AuNPs menunjukkan purata koloid berbentuk sfera diameter 5 nm, sementara imej topografi yang divisualkan oleh FESEM menunjukkan bentuk polihedron. Kedua-dua imej EFTEM dan FESEM bagi PEG-AuNPs adalah beraglomerasi. Selain itu, PEG-AuNPs juga mempunyai purata diameter terbesar 156.2 nm serta saiz zarah dan keupayaan zeta paling sedikit iaitu -6.83 mv berbanding PEG4000 dan AuNPs. Terdapat perbezaan min yang ketara bagi tindakbalas aglutinasi RBC antara larutan PEG-AuNPs dan LISS pada fasa globulin anti-manusia (AHG) (nilai p=0.010). Walau bagaimanapun, hanya anti-D dan gabungan antibodi anti-Le^a dan anti-Le^b sahaja yang menunjukkan perbezaan min yang ketara dengan nilai p=0.026 dan p=0.036. Kesimpulannya, larutan PEG-AuNPs yang baru dibangunkan ini boleh digunakan sebagai larutan alternatif untuk mengantikan LISS dalam meningkatkan tindakbalas antara antigen-antibodi RBC.

SYNTHESIS AND CHARACTERISATION OF PEGYLATED GOLD NANOPARTICLES FOR ENHANCEMENT OF AGGLUTINATION REACTION IN ANTIBODY SCREENING TEST

ABSTRACT

Red blood cell (RBC) antigen-antibody agglutination is a crucial reaction in antibody screening tests (AST) to identify the presence of unexpected clinically significant antibodies in the plasma recipient. However, with the current method using low-ionic strength solution (LISS) as an enhancement medium for detecting antibody in AST, several drawbacks such as the missing detection of some clinically significant antibodies, and thus, can cause an adverse transfusion reaction. Hence, a new solution using polyethylene glycol with molecular weight of 4000 (PEG4000) conjugated with gold nanoparticles (AuNPs) was synthesised to produce a better RBC antigen-antibody agglutination enhancement capacity in AST. The synthesised PEG-AuNPs solution was characterised for optical properties by Ultraviolet-Visible Spectroscopy (UV-Vis), morphological characterisation by Energy-Filtered Transmission Electron Microscopy (EFTEM) and Field Emission Scanning Electron Microscope (FESEM), particle size and zeta potential analysis by Dynamic Light Scattered (DLS), and structural and composition of bonding material by Fourier-transform infrared (FT-IR) spectroscopy. Then, the RBC agglutination scoring using tube method was compared between the synthesised solution with standard LISS in AST using 120 samples consisted of 60 negative and 60 positive samples of antibody. The final colour of the solution changed to ruby red indicating the successful formation of PEG-coated with AuNPs. The synthesised solution exhibited a single maximum absorption peak at a wavelength of 529 nm, and it corresponded with the Surface Plasmon Resonance (SPR) absorbance

of AuNPs. The EFTEM images of PEG-AuNPs represent a spherical colloid with average size of 5 nm diameter, meanwhile the topographical image visualised by FESEM showed polyhedral shapes. Both EFTEM and FESEM images of PEG-AuNPs showed agglomeration. It was found that PEG-AuNPs had the largest average diameter of 156.2 nm and zeta potential of -6.83 mV. There was a significant difference of mean for RBC agglutination reaction scoring between PEG-AuNPs solution and LISS at anti-human globulin phase (AHG) (*p*-value=0.010). However, only samples with anti-D and combination of anti-Le^a and anti-Le^b antibodies specificities showed a significant different between both solutions (*p*-value=0.007 and *p*-value=0.036 respectively). In conclusion, the synthesised PEG-AuNPs solution can be used as an alternative solution for LISS in enhancing the RBC antigen-antibody reaction.

CHAPTER 1

INTRODUCTION

1.1 Research background

1.1.1 Pre-transfusion testing

Red blood cells (RBCs) transfusion is indicated in treatment of haemorrhage and to improve tissue oxygenation. However, before RBCs transfusion can be commenced, pre-transfusion testing is usually being performed to ensure the safety and compatibility of the blood components transfused. The pre-transfusion testing involves identification and blood sample labelling of the intended recipient's, followed by necessary testing before the blood components are issued for transfusion (Hillyer *et al*., 2018). Tests that involve in pre-transfusion testing include ABO and Rh (D) blood grouping for both donor and recipient, antibody screening test (AST) for unexpected antibodies in the recipient and cross-matching between donor and recipient samples. These tests usually take about one to two hours to be completed.

1.1.2 Problem with current method and consequence

However, if unexpected antibodies are present in the recipient's sample, it will be subjected to further test which is antibody identification. Most of the time, low-ionic strength solution (LISS) and anti-human globulin (AHG) are used for AST and antibody identification (Armstrong, 2020; Catherine *et al*., 2022). LISS commonly is ready to use and supplied at the optimal dilution and without further dilution. Example of LISS (tube technique) used in Malaysia is Gamma N-HANCE from Immucor Inc. Antibody identification may take hours to days to be completed, therefore, time is a concern especially if the recipient needs an urgent transfusion. Furthermore, the

current AST has numerous limitations, including missing detection of clinically significant antibodies in the recipient's plasma samples.

Clinically significant antibodies are capable of causing haemolytic transfusion reactions (HTR) and/ or haemolytic disease of the foetus and newborn (HDFN) (Catherine *et al*., 2022). These antibodies can cause intravascular haemolysis of the transfused antigen positive RBCs, shortened RBC's survival, and could significantly cause mortality and morbidity to the recipient (Korsak *et al*., 2020; Massey *et al*., 2022). These antibodies can be RBC antibodies against low-incidence antigens, antibodies that show dosage if the RBCs are not homozygotes, weak antibodies that react with RBCs from fresh donor units but may not react with the older donor's RBCs unit, and antibodies against compound antigens such as f antigen in Rh blood group system (Massey *et al*., 2022). A dosage antibodies phenomenon is where the strength of the antibody reaction varies depending on the quantity of the target antigen present on the RBCs. Hence, it is important that AST is performed with utmost reliable methods and procedures prior any blood transfusion to avoid any adverse transfusion reactions (Knowles, 2007; Maitta, 2018).

1.1.3 The prevalence of adverse transfusion reactions in Malaysia

In Malaysia, the reported adverse transfusion reactions (ATRs) cases per 10,000 blood components issued decreased from 60 cases in 2020 to 52 cases in 2021. Notably, mild allergic reaction was the most common (total of 3,457 cases for both years) followed by febrile non-haemolytic transfusion reaction (FNHTR) with total of 2,385 cases. There were only a total of 5 delayed haemolytic transfusion reaction cases reported (National Haemovigilance Coordinating Centre, 2023).

A study at a tertiary care center in Malaysia found the most common ATRs were allergic reactions (42.51%) and FNHTRs (37.17%), followed by acute hemolytic transfusion reactions (5.69%), transfusion-associated circulatory overload (2.23%), and transfusion-associated dyspnea (1.59%) (Mardania & Balalib, 2020). A retrospective study at Hospital Sultanah Bahiyah in Kedah, Malaysia from 2015 to 2017 found an overall ATRs incidence of 0.54% (1 in 187 transfusions), with 0.25% being allergic reactions and 0.22% FNHTRs (Ros *et al*., 2021). A retrospective study at Hospital Universiti Sains Malaysia from 2018-2021 reported a vasovagal reaction prevalence of 0.45% (159 out of 35,134 donors), with dizziness or mild vasovagal reactions being the most frequent (54.7%) (Ibrahim *et al*., 2023).

1.1.4 Gold nanoparticles

Among many types of nanoparticles (NPs), gold nanoparticles (AuNPs) have attracted tremendous interest used in clinical laboratories. AuNPs are known for its unique properties such as stable, high biocompatibility, optical electronic, good conductivity, non-toxic, and having large surface functionalisation area (Hammami *et al*., 2021). As AuNPs have relatively inert element and possess beneficial properties for tunable physical, chemical and optical properties, they are capable to react easily with a wide range of different biological macromolecules and organic molecules, and thus, have made them useful in many research fields. Most of AuNPs synthesis method are rapid, highly sensitive, and only needed a straightforward method used to synthesise with nanometer sizes (Fusco *et al*., 2017; Hammami *et al*., 2021). Besides, high electron density in AuNPs also have enabled them to enhance protein agglutination due to their capability in increasing the cellular uptake upon interaction with the cell membranes (Jazayeri *et al*., 2016; Draz & Shafiee, 2018).

Nevertheless, little attention has been devoted to AuNPs applications in transfusion medicine. Previous study had shown that AuNPs solution was capable to enhance agglutination reaction in the detection of weak B subgroup using slide test technique (Wiwanitkit *et al.*, 2007). Besides, the solution had also been used as an alternative enhancement potentiator for weak antigen potency on RBCs as it could increase 7.0 – 37.7% agglutination reaction for ABO grouping system and 12.1 – 50.9% agglutination reaction in ABO subgroups (Ammaranond *et al.*, 2011). Furthermore, the AuNPs solution could improve 37.2% agglutination reaction in AST compared to the standard enhancement reagent using LISS and it can also shorten the incubation time at 37 °C and during AHG phase, indicates the efficiency of the AuNPs solution in accelerating the agglutination process without compromising accuracy (Choktaweesak *et al.*, 2016).

1.1.5 Polyethylene glycol (PEG)

Besides the standard LISS, polyethylene glycol (PEG) is also used as enhancement media in Transfusion Medicine as it can remove water molecules in the in vitro environment to allow a greater probability of collision between antigen and antibody molecules. Thus, they can enhance the detection rate of clinically significant alloantibodies in patients' serum and make it a sensitive technique for the detection and identification of RBC antibodies (Wenz, 2019; Abdelmonem *et al.*, 2022). In addition, PEG can also enhance the affinity to AuNPs surface, prevent AuNPs aggregation with non-specific protein adsorption, thus can improve the performance of AuNPs in biological application (Reznickova *et al.*, 2017). Therefore, development of new alternative solution by combining the PEG and AuNPs may provide an alternative solution in enhancing the agglutination process during AST.

1.2 Problem statement

Several methods to facilitate, improve and expedite the AST include using of enhancing reagent such as albumin, LISS or PEG have been employed in transfusion medicine services. However, the present AST method may miss to detect some clinically significant antibodies such as antibodies with show dosage effect, such as anti-Fy^a, anti-Fy^b, anti-Jk^a, anti-Jk^b, anti-S and anti-s (Walker, 2018; Armstrong, 2020). Furthermore, these methods take about 30 to 45 minutes to be completed and the cost of these reagent is quite expensive (Basavarajegowda & Shastry, 2023).

1.3 Research justification

The aim of this study is to synthesis and characterise a newly solution using PEGylated gold nanoparticles (PEG-AuNPs) that may enhance the detection of the presence unexpected antibody and produce at least 10% better result of RBC agglutination reaction scoring when compared to standard LISS during AST. By using PEG-AuNPs as an enhancement reagent in AST, it would potentially decrease the overall AST process by at least five minutes and reduce the overall cost of AST by at least 30%.

1.4 Research objectives

1.4.1 General objective

To synthesis and characterise PEGylated gold nanoparticles solution for enhancement of agglutination reaction in antibody screening test (AST).

1.4.2 Specific objectives

1. To synthesise PEG-AuNPs solution that can enhance the RBC agglutination reaction in AST using one-step synthesis method.
2. To characterise the synthesised PEG-AuNPs solution for optical properties by using Ultraviolet-Visible Spectroscopy (UV-Vis), morphological characterisation by Energy-Filtered Transmission Electron Microscopy (EFTEM) and Field Emission Scanning Electron Microscope (FESEM), particle size and zeta potential by Dynamic Light Scattered (DLS), and structural and composition of bonding material by Fourier-transform infrared (FT-IR) spectroscopy.
3. To compare the RBC agglutination scoring between PEG-AuNPs solution with standard LISS.
4. To determine the sensitivity, specificity, positive predictive value and negative predictive value of agglutination score for the PEG-AuNPs in AST using descriptive statistics.

1.5 Research questions

1. Has PEG-AuNPS solution successfully been achieved using one-step straightforward synthesis method?
2. What are the characteristics of the PEG-AuNPs solution synthesized by combining PEG4000 with AuNPs?
3. Can the PEG-AuNPs solution enhance the detection of unexpected RBC antibodies during AST and how does it compare to standard LISS in terms of RBC agglutination reaction scoring?
4. What are the differences in terms of sensitivity, specificity, positive predictive value, and negative predictive value of agglutination score between PEG-AuNPs and LISS?

1.6 Hypothesis of study

Alternative hypothesis : The synthesis of PEG-AuNPs solution is successful and has higher ability to enhance RBC antigen-antibody agglutination reaction when compared to the current standard LISS.

Null hypothesis : The synthesis of PEG-AuNPs solution is unsuccessful and there is no difference in the ability to enhance RBC antigen-antibody agglutination reaction between the PEG-AuNPs solution with LISS.

1.7 Scope of research

The scope of research defining the specific objectives, target population, variables, methods, and limitations is illustrated as in Figure 1.1.

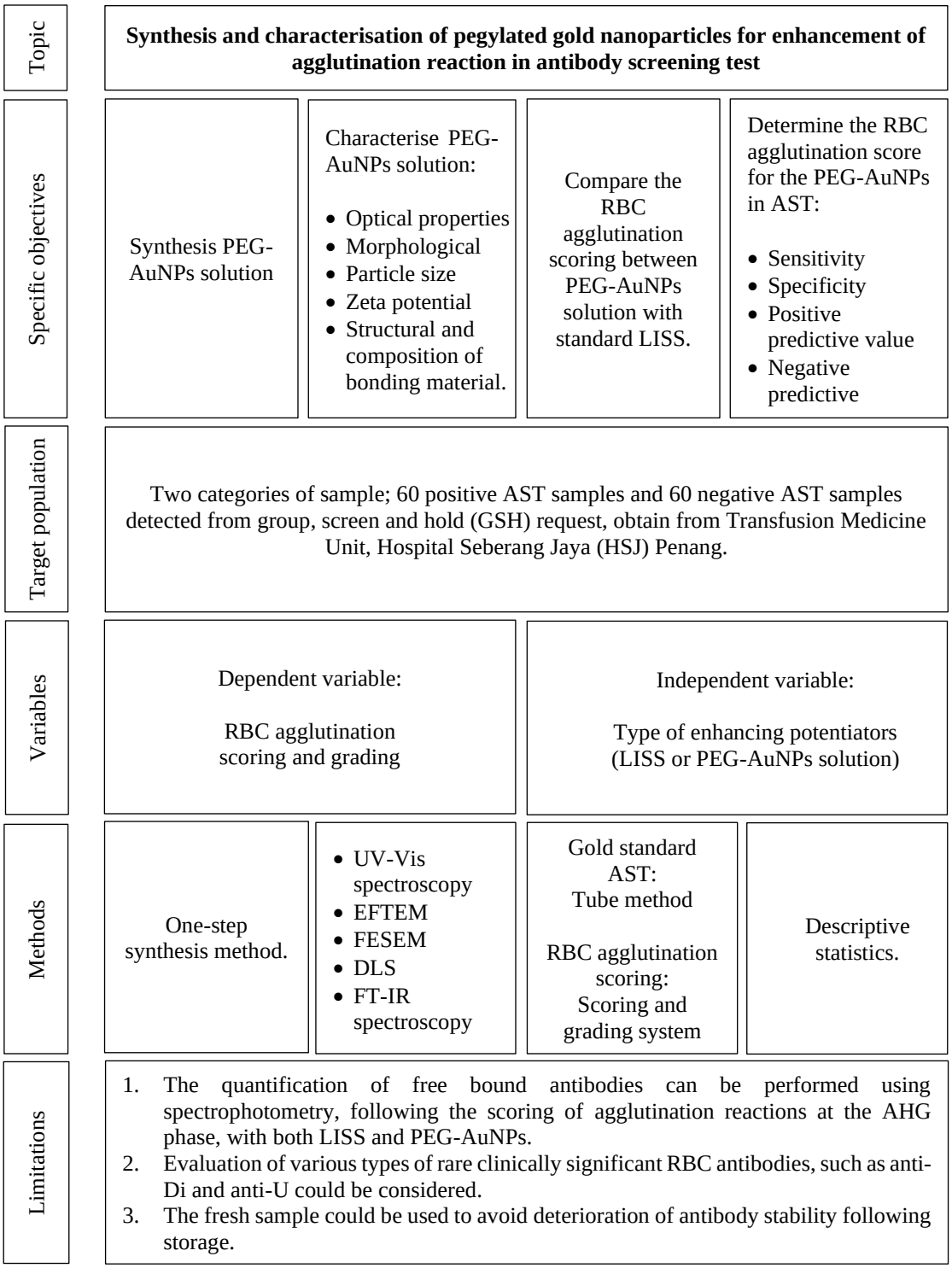

Figure 1.1 Research scope of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, literature review on brief definitions, structures, unique properties, applications in transfusion medicine of PEG and mechanism of PEGylation are discussed. The brief definitions, structures, unique properties, synthesis methods, characterisation techniques and applications in transfusion medicine of AuNPs also are explained. This is followed by description on RBCs agglutination reaction and overview of pre-transfusion testing.

2.2 Polyethylene glycol (PEG)

2.2.1 Definition and structure of PEG

PEG is a chemical compound derived from petroleum that composed of repeating ethylene glycol units, and condensation polymer of ethylene glycol and water (Shi *et al.*, 2021). Generally, the term of "polyethers" refers to polymers containing ether linkages in their main chain, while "glycol" refers to polyether polyols with one or more functional end-groups such as a hydroxyl (-OH) group. PEG has both a polar oxygen atom and a nonpolar $(CH_2)_2$ group that often generated by anionic chain transfer polymerisation (Zia *et al.*, 2017). The molecular formula of PEG is $(C_2H_4O)_{n+1}H_2O$ and its structure is shown in Figure 2.1.

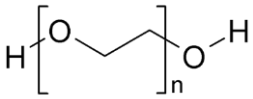

Figure 2.1 Molecular structure of single PEG chain (Ringsdorf, 1975)

Generally, common PEG is linear or branched in structure, varies in shape and it is identified by its chain length, which is directly determined through average molecular weight (Mw) in g/mol (Hong *et al.*, 2020). PEG is available in a variety of Mw from 200 g/mol to 20,000,000 g/mol (Khanteesa *et al.*, 2020). The number in the names of PEGs denotes their average Mw. For instance, PEG4000 is generally refers to a formulation containing a mixture of oligomers with an average Mw of 4000. Table 2.1 shows the physical properties of PEG at room temperature according to their Mw (Mastropietro *et al.*, 2017).

Table 2.1 Physical properties of PEG and their Mw (Mastropietro *et al.*, 2017).

Molecular weight (Mw)	**Physical properties**
Between 200 - 600 Mw	Viscous and colourless liquid
Between 700 - 900 Mw	Semi-solid
1000 Mw and above	White free-flowing powder or flakes

The low molecular weight PEGs have more hydroxyl groups compared to their structure, and as the molecular weight of PEGs increases, the number of hydroxyl groups remains significant (Khanh *et al*., 2022). PEG is chemically synonym with polyethylene oxide (PEO) or polyoxyethylene (POE) but they are different by their relative Mw and their monomer or parent molecule (ethylene glycol, ethylene oxide or oxyethylene) (Zia *et al.*, 2017). PEG Mw below 20,000 is often being used in biomedical fields while PEO above 20,000 Mw is commonly used in polymer chemistry fields due to their effect of chain length and chemical properties (Thomas & Ajitha, 2019; Fam *et al.*, 2020; Sanchez-Cano & Carril, 2020). The optimal PEG for

RBC antigen and antibody reaction was found to be at 4000 Mw in a 20% suspension of phosphate buffer saline (PBS) (Nance & Garratty, 1987; Emam *et al.*, 2021).

2.2.2 Unique properties of PEG

PEG is an ideal choice for various applications due to its many unique properties. PEG is inert, synthetic, hydrophilic, thermally stable, and has a high biocompatibility (D'souza & Shegokar, 2016). PEG has high solubility in water and organic solvents especially in ethanol, acetonitrile, benzene, and dichloromethane. However, it is insoluble in diethyl ether and hexane (Zarrintaj *et al.*, 2020). PEG in water acts as a very mobile molecule allowing the exclusion of water and other polymers.

PEG is considered as a greener option, due to its non-toxic, non-volatile, non-immunogenic, and non-antigenic properties. Furthermore, PEG also does not harm active proteins or cells although it interacts with cell membranes and it resists recognition by the immune system (Turecek & Siekmann, 2020). Due to its safety profile, PEG has been recognised as safe material from the Food and Drug Administration (FDA) of United State and European Medical Agency (EMA) (Padín-González1 *et al.*, 2022). Thus, this makes PEG the ideal material to be added in media and conjugated with other molecules without interfering cellular functions or targeted immunogenicity (Turecek & Siekmann, 2020).

2.2.3 Mechanism of PEGylation

The PEGylation is the conjugation process of linear PEG via functional group with other interest biomolecules or nanoparticles (NPs) (Harris *et al.*, 2001; Shi *et al.*, 2021). PEGylation has the benefit of effectively prevent the aggregation induced by change in thermal and pH stability of protein in aqueous solutions (Lee *et al.*, 2015; Lundahl *et al.*, 2021).

There are three common strategies for PEGylation with NPs surface; physical adsorption, chemical conjugation, and molecular self-assembly as shown in Figure 2.2 (Shi *et al.*, 2021). The physical adsorption is the most popular conventional strategy, which promotes simple and easy-to-control condition by functionalising the surface of NPs with PEG. This strategy is based on hydrophobic interactions or electrostatic adsorption, in which PEG absorbs the hydrophobic or charged groups. In contrast, chemical conjugation is based on conjugation of the terminal group of PEG onto the exposed group of the NPs surface by chemical bonding (covalent coupling). This strategy uses moderate chemical reaction conditions and has high yield of production. In comparison, molecular self-assembly mainly involves solvent diffusion (nanoprecipitation) or evaporation (emulsification). In this strategy, the hydrophilic PEG is conjugated to hydrophobic polymers or lipids to produce spherical NPs by self-assembly with or without other compounds in water. The hydrophobic part of the polymer comprises the core of the NPs, while the outer layer of particles is PEG coating (Shi *et al.*, 2021).

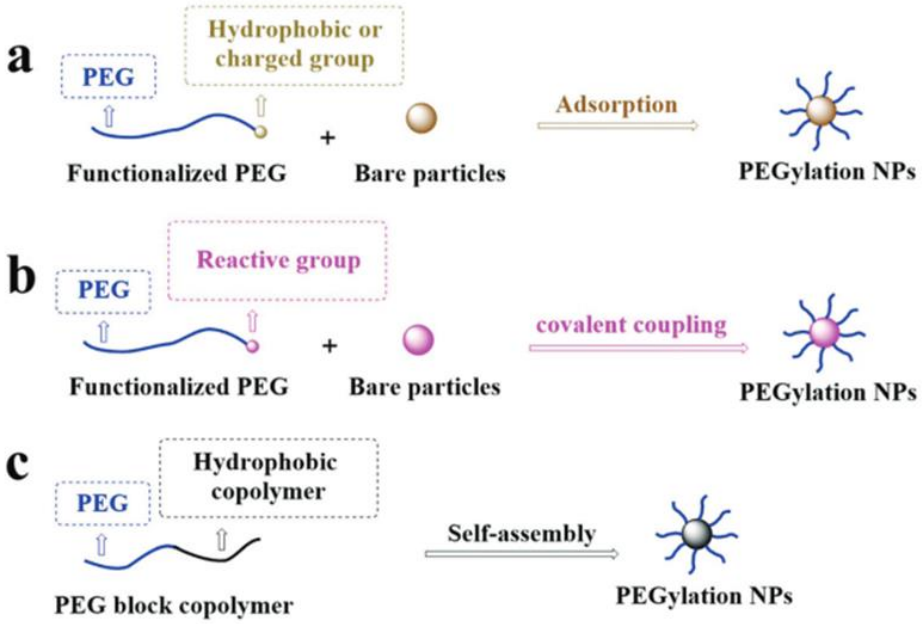

Figure 2.2 Three common strategies for PEG coating with NPs surface; (a) by physical adsorption (b) by chemical conjugation (c) by molecular self-assembly (Shi *et al.*, 2021)

2.2.4 Applications of PEG in medicine and transfusion medicine

The versatility of PEG has made it as the most used biopolymers with vast number of applications in medical fields (Gheorghita *et al.*, 2021). Initially, PEG was introduced in 1977 as a polymer for covalent attachment to bovine serum albumin and liver catalase proteins, with the aim of improving drug delivery applications. This innovation demonstrated PEG's potential to enhance the solubility and stability of pharmaceutical compounds (Abuchowski *et al.*, 1977). In 1986, the first PEGylated protein, a modified version of the enzyme asparaginase, received approval from the FDA for clinical use. This milestone marked a significant advancement by reducing immunogenicity and prolonging circulation time in the bloodstream (Weissig *et al.*, 2014). The introduction of PEGylated interferon-alpha in 1994 for the treatment of hepatitis C further exemplified the benefits of PEGylation, as it improved therapeutic

efficacy and minimized side effects compared to non-PEGylated formulations (Fried *et al*., 2002).

In 2000, the FDA approved PEGylated liposomes (Doxil), a chemotherapy agent primarily used in the treatment of various cancers, including breast cancer and Kaposi's sarcoma (Ahmed *et al*., 2005; Gabizon *et al*., 2003; Laginha *et al*., 2005). By 2005, significant progress had been achieve in the development of PEGylated monoclonal antibodies (mAbs), which are antibodies modified by the attachment of PEG chains. This modification aims to enhance therapeutic efficacy and pharmacokinetics by improving solubility, stability, and circulation half-life (Fisusi *et al*., 2020).

However, the emergence of anti-PEG antibodies in 2012 raised significant concerns within the drug delivery and therapeutic fields. These antibodies, which target PEG commonly used to enhance the efficacy of therapeutic agents, can lead to accelerated blood clearance and reduced therapeutic effectiveness (Qi & Samuel, 2015). In 2015, the FDA approved multiple PEGylated products, including enzymes and monoclonal antibodies, thus reinforcing PEG's role in contemporary medicine (Ivens *et al*., 2015). Research on the application of PEG in nanoparticle formulations for drug and gene delivery intensified in 2020, resulting in improved therapeutic outcomes (Ryals *et al*., 2020; Zhang *et* al., 2022). Since 2023, ongoing studies have been investigating the implications of pre-existing anti-PEG antibodies on patient treatment and exploring the development of non-PEG alternatives (Mareike *et al*., 2023; Sherif *et al*., 2024). An overview of the application of PEG in medical development, particularly through PEGylation, along with key milestones, is illustrated in Figure 2.3.

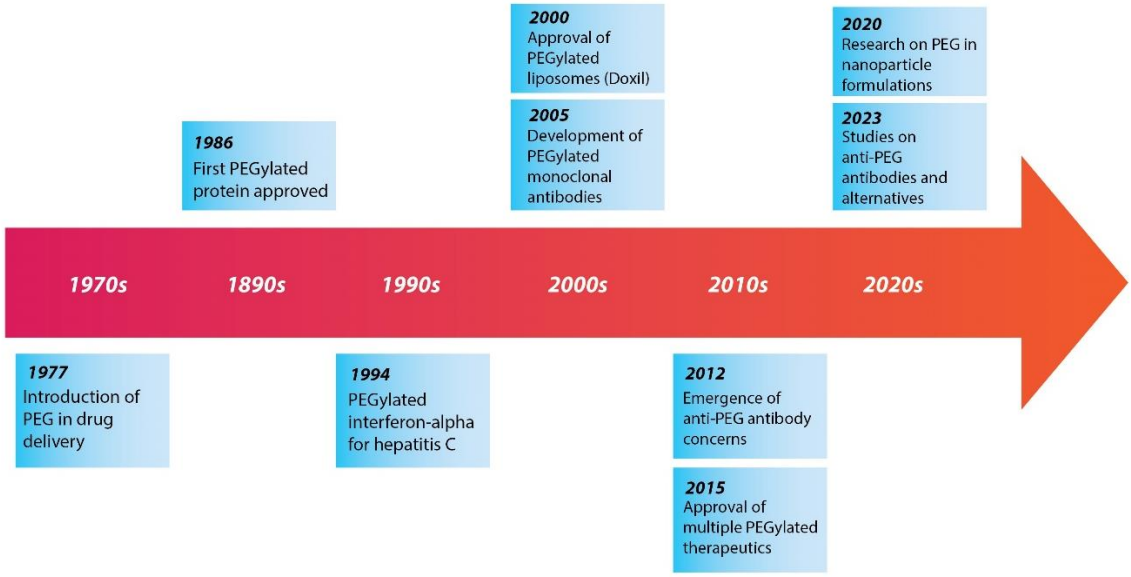

Figure 2.3 Timeline of PEG in medicine development (Adapted from Abuchowski *et al*., 1977; Ahmed *et al*., 2005; Fisusi *et al*., 2005; Fried *et al*., 2002; Gabizon *et al*., 2003; Ivens *et al*., 2015; Laginha *et al*., 2005; Mareike *et al*., 2023; Qi & Samuel, 2015; Ryals *et al*., 2020; Sherif *et al*., 2024; Weissig *et al*., 2014; Zhang *et al*., 2022)

In transfusion medicine, PEG is used as enhancement media as it can remove water molecules from *in vitro* environment to allow a greater probability of collision between RBC antigen and antibody molecules. Thus, PEG can enhance the detection rate and identification of clinically significant RBC antibodies in the patients' serum (Wenz, 2019; Abdelmonem *et al.*, 2022). The evaluation of PEG solution was first described by Nance and Garratty (1987) in transfusion medicine using PEG4000 Mw to detect and enhanced the weak antigen and antibody reactions. In their study, PEG was shown to have greater effect in enhancing weak types of antibodies (antibodies that showed 2+ reactive in LISS technique at the AHG phase of testing) (Nance & Garratty, 1987; Emam *et al.*, 2021). Thereafter, many other studies had looked into the potential application of PEG in transfusion medicine.

In 1990, de Man and Overbeeke evaluated the PEG for detecting RBC antibodies in multitransfused patients, including those with sickle cell disease (SCD).

This study found that PEG was more sensitive than LISS, albumin, and saline methods in identifying antibody specificity (de Man & Overbeeke, 1990). A comparison retrospective study between PEG and bovine serum albumin (BSA) in AST had reported that PEG increased the detection rate of clinically significant antibodies (such as anti-D, anti-E, anti-C, anti-c, anti- Fyb, and anti-Jka) by 46% and decreased the detection an insignificant antibodies (such as anti-Leb and anti-P1) by 24% (Wenz *et al*., 1990). A similar finding regarding the type of antibodies was observed in a study with non- Caucasian (Japanese) population, with 18.56% difference antibodies were detected before transfusion (Okutsu *et al.*, 2011). In addition, other than anti-D, anti-C, and anti-E, anti-e also was detected by PEG as compared to the BSA in Rh(D) negative pregnant women's serum (Trkuljić *et al.*, 2000).

A previous study had found that PEG was capable to detect 35 clinically significant antibodies and 10 clinically insignificant antibodies, as compared to LISS which was only capable to detect 15 clinically significant antibodies and 33 clinically insignificant antibodies. By using PEG, it can increase 133% detection of clinically singnificant antibodies and reduce 70% insignificant antibodies detection (Barrett *et al.*, 1995). Moreover, PEG showed an ability to retain high sensitivity, lowering the number of false-positive reactions and decrease 2.7% detection of doubtful clinically significant antibodies compared to albumin and papain (Reisner *et al.*, 1996). Besides, PEG was also used as an alternative solution to LISS and whenever weak antigen-antibody reactions are encountered (Weldy, 2014).

Recent study between PEG and LISS using microcolumn gel technique showed PEG can enhance 41.4% of the RBC antigen antibody reaction especially in Rh system (69%), Duffy system (17.2%), Kidd system (10.3%), and Lewis system (34%) (Xiao-hua *et al*., 2022). A previous study in the US showed samples with low titers (≤1:8)

true anti-D alloantibodies, 76% had less than 2+ aglutination strength, with the remaining 24% had a stronger strength of 3+ when using PEG as an enhancement medium in AST in early pregnancy women who received Rh immunoglobulin (RhIG) (Walhof *et al.*, 2022). Besides, to preserve RBC for *in vitro* immunohematology testing in the next 70 days, PEG also was also added into the RBC storage solutions to reduce haemolysis and haemagglutination reactivity of the Rh, Duffy, Kidd, Lewis, MNS, and P1 antigens (Tang *et al.*, 2023).

The cost recovery of utilising PEG as an enhancement reagent in antibody screening tests can be influenced by three primary factors: the type and quantity of PEG employed, the intricacy of the testing procedure, and the expenditure of additional materials and equipment required for the testing. Generally, a 10 µl bottle of LISS reagent is priced at approximately MYR 46 and can be used for around 100 tests for AST. Therefore, the cost per test of using 100 µl of LISS for an antibody screening test is approximately MYR 0.46.

2.3 Gold nanoparticles (AuNPs)

2.3.1 Definition and structure of AuNPs

Advances in NPs technology particularly the dynamicity of NPs synthesis have given great advantages in many fields, including diagnostics, therapeutic and preventive medicine (Haleem *et al.*, 2023). The term "nano" is derived from the Greek word meaning "very small" and NPs refers to material between 1 to 100 nm in size (Bayda *et al.*, 2020). Among many types of NPs, AuNPs have attracted tremendous interest in clinical laboratory.

AuNPs can be divided into three main groups based on their size and surface functionality; gold (Au) colloids, monolayer protected clusters (MPCs), and small Au

clusters (Louis & Pluchery, 2012). Au colloids are large particles with a size range of 10 to 100 nm and are usually formed after the reduction synthesis process. MPCs are 1 to 10 nm particles surrounded by a monolayer of highly organic ligands such as thiols. In contrast, small Au cluster consisted of few atoms and usually in monodispersed state (Imaoka & Yamamoto, 2019).

The colour of AuNPs is wine-red compound with antioxidant properties compared to gold particles which are yellow inert solid. AuNPs have a different size range from 1 nm to 8 μm and are presented in several forms like spherical, nanorods, irregular shape, sub-octahedral, octahedral, icosahedral multiple twined, decahedral, tetrahedral, nanotriangles, hexagonal platelets, and nanoprisms as shown in Figure 2.3 (Giljohann *et al.*, 2014; Bharadwaj *et al.*, 2021). The AuNPs morphology depends on their preparation method, rising temperature, and salt concentration. However, the spherical AuNPs are widely used as they involve a straightforward method to synthesis (Hammami *et al.*, 2021).

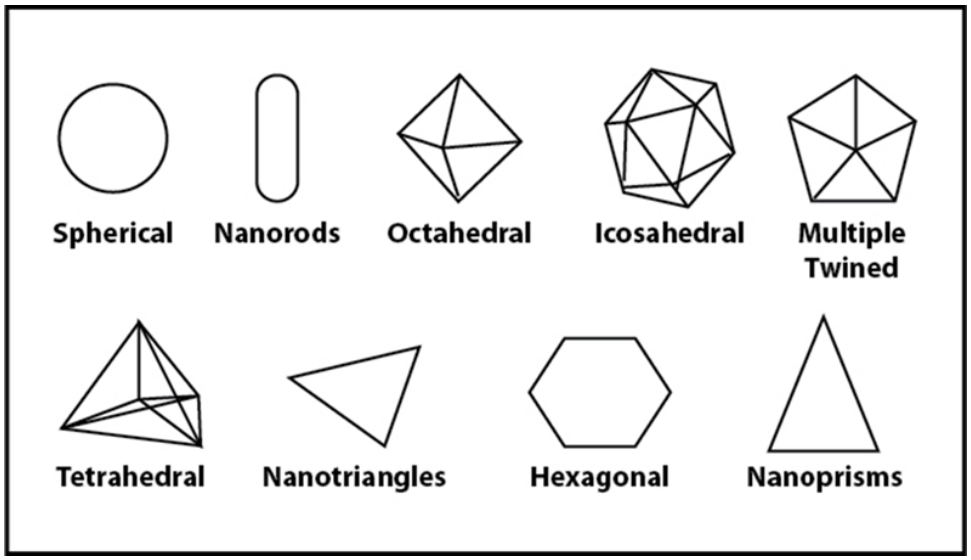

Figure 2.4 Various shapes of AuNPs (Adapted from Giljohann *et al.*, 2014; Bharadwaj *et al.*, 2021).

2.3.2 Unique properties of AuNPs

AuNPs are known for their unique properties such as stable, high biocompatibility, non-toxic, and have large surface functionalisation area (Hammami *et al.*, 2021; Qiao & Qi, 2021). As they have relatively inert elements and possess beneficial properties for tunable physical, chemical, and optical properties, they are capable to react easily with a wide range of different biological macromolecules and organic molecules and thus, have made them useful in many research fields. Most of AuNPs' synthesis methods are rapid, highly sensitive, and straightforward method with nanometer sizes (Fusco *et al.*, 2017; Meena *et al.*, 2020). Besides, the high electron density in AuNPs also has enabled them to enhance protein agglutination due to their capability in increasing the cellular uptake upon interaction with the cell membranes (Draz & Shafiee, 2018).

The outstanding optical feature of AuNPs is unique Surface Plasmon Resonance (SPR), which is correlated with the cumulative absorption of conduction electrons (Hammami *et al.*, 2021). It is found in the visible region as well as in the near-infrared region (NIR), depending on AuNPs' size, shape and environment including surface chemistry, aggregation phase, and property of the surrounding medium (Elahi *et al.*, 2018; Bian *et al.*, 2019). The SPR can detect the changes in the AuNPs aggregation phase and demonstrate their specific colours. For instance, a solution containing spherical AuNPs presents as a red-purple colour of 1 to 100 nm range size with relative absorption peak from 500 to 550 nm (Hammami *et al.*, 2021). When AuNPs' shape or the surrounding medium are modified, the colour may change slightly (Amendola *et al.*, 2014; Daruich De Souza *et al.*, 2019).

2.3.3 AuNPs synthesis method

The synthesis method for AuNPs can be classified into top-down or bottom-up methods based on the raw material preparation as shown in Figure 2.4 (Elahi *et al.*, 2018; Jamkhande *et al.*, 2019).

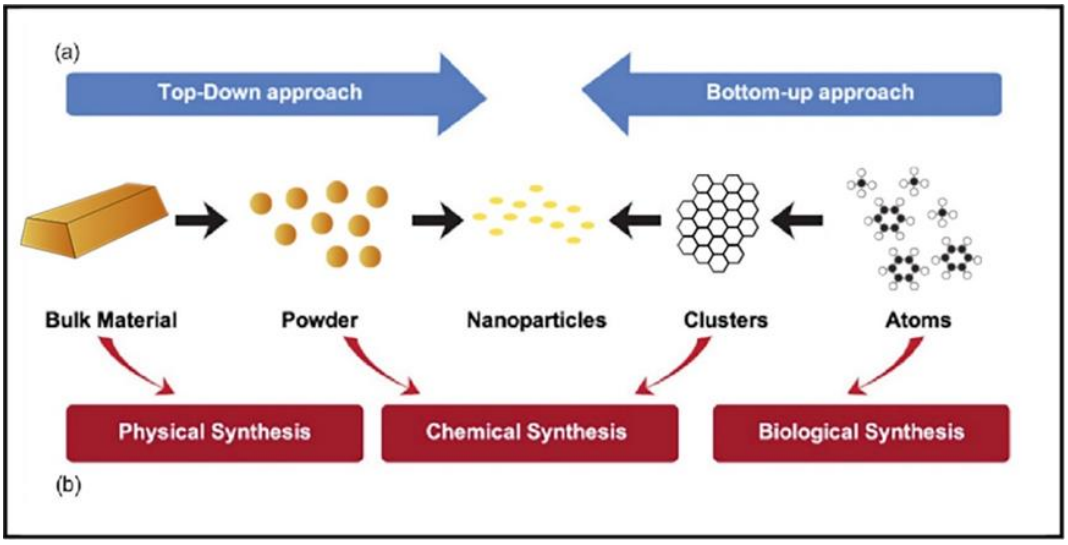

Figure 2.5 AuNPs synthesis mechanisms (Adapted from Elahi *et al*., 2018; Jamkhande *et al*., 2019).

The top-down approach begins from the bulk material of Au and is further broken down to produce NPs (Kumari *et al.*, 2019). For instance, mechanical milling, laser ablation and ion sputtering are examples of top-down approach methods (Jamkhande *et al*., 2019). The top-down method is straightforward to perform but it is not suitable for preparing informal shapes and very small size particles. Additionally, this method will also change the chemistry and physiochemical properties of AuNPs (Khan *et al.*, 2019).

In contrast, the bottom-up approach begins from atoms or molecules to form NPs (Khan *et al.*, 2019; Kumari *et al.*, 2019). Nanostructured building blocks are formed to produce the final AuNPs (Hammami *et al.*, 2021). For instance, physical vapour deposition method (solid state), chemical reduction method (liquid state), and

spray pyrolysis (gas phase) are categorised as bottom-up approach (Kumar *et al.*, 2018). This method requires straightforward synthesis preparation. Besides, size, morphology, and structure of AuNPs can also be controlled when using this method (Hasnidawani *et al.*, 2016; Hu *et al.*, 2020). Apart from bottom-up and top-down approach, AuNPs synthesis can also be categorised based on their synthesis methods; chemical, physical, and biological synthesis (Elahi *et al.*, 2018; Hammami *et al.*, 2021).

2.3.3(a) Chemical synthesis method

The chemical method is the most common approach for AuNPs synthesis. The reductant and stabiliser are two critical components in this method (Herizchi *et al.*, 2016; Amina & Guo, 2020). The reductant agents act as a chemical reducer (example of reductant agents include sodium citrate, sodium borohydride and diborane); the stabiliser acts to prevent the accumulation of the particles (Liu *et al.*, 2016; Daruich De Souza *et al.*, 2019).

The well-established chemical method synthesis of AuNPs was described by Turkevich (1951) which was known as nucleation-diffusional growth or single-phase synthesis. This method involved boiling at high temperatures, approximately 90°C, of tetrachloroauric acid solution with trisodium citrate ($Na_3C_6H_5O_7$) which acts as both stabilising and reducing agents (Ba Fakih *et al.*, 2020). Initially, the developed AuNPs were spherical with 10 to 20 nm in diameter in size but with subsequent improvement in the adjustment of ratio of Au to sodium, as well as the sequence of reagents addition, the produced AuNPs size were smaller with narrow size distribution (Teimouri *et al.*, 2018). However, this nucleation method is limited to only small size AuNPs preparation, specifically around 5 nm to 30 nm in diameter, and it is difficult to

interpret the surface chemistry of the developed AuNPs (Stiufiuc *et al.*, 2013; Teimouri *et al.*, 2018).

Subsequently, in 1994 Brust and Schiffrin had developed the two-phase synthesis method for the synthesis of alkanethiolate-protected AuNPs. In this method, $AuCl_4$ is transferred from an aqueous phase into toluene using tetraoctylammonium bromide (TOAB) as the phase-transfer catalyst. Then, an alkanethiol and sodium borohydride (NaBH4), as a reducing agent, were added to the toluene solution under vigorous stirring, resulting in the rapid formation of small AuNPs protected by alkanethiolate ligands. The reaction can be represented by the following equation: AuCl4- + 3 RSH + 3 e- → Au(SR) + 4 Cl- + H+, where R represents the alkyl group of the alkanethiol (Kulu *et al.*, 2020; Borsley *et al*., 2023). This method was able to produce a smaller spherical AuNPs size in the range of 1.5 to 5.2 nm and able to combine the AuNPs with other biomolecules such as organic solvents and thiolate molecules, thus increasing the stability of the product (Booth *et al*., 2017; Sengani *et al*., 2017; Teimouri *et al*., 2018).

Consequently, a one-step rapid synthesis method by combining the AuNPs with unmodified PEG in the presence of sodium hydroxide (NaOH) was created by Stiufiuc *et al.* (2013). In this research, PEG has dual functionality; as a reducing and stabilising agent. The spherical shape of 5 nm PEG-coated with AuNPs was rapidly produced within 2 to 10 minutes (Stiufiuc *et al.*, 2013; Niţica *et al.*, 2018).

Meanwhile, the production of various shapes (sphere, oval and rod) and expansion of AuNPs by the seed-mediated method was proposed by Wuithschick *et al*. (2015). This method used a strong reducing agent (sodium borohydride), weak reducing agent (ascorbic acid), and structure-directing agent to prevent further aggregation and nucleation of AuNPs (Wei *et al.*, 2021). This method can improve the

AuNPs' growth and control their structure properties, size, and form by adjusting the concentration of seed, reducing agent, and structural-directing agent (Kumari *et al.*, 2019). By introducing a controlled number of Au seeds into the growth medium, the particles are allowed to grow, resulting in AuNPs with sizes ranging from 20 to 110 nm. The process involves the controlled addition of Au seeds, which act as nucleation sites for the growth of AuNPs. The presence of citrate and Au in the reaction mixture facilitates the growth process, leading to the formation of monodisperse nanoparticles (Podlesnaia *et al*., 2021).

2.3.3(b) Physical synthesis method

Physical synthesis is another preferred method to synthesise AuNPs. It involved controlling the size distribution of the AuNPs (Ramalingam, 2019). The γ-irradiation, microwave irradiation, sonochemical, ultraviolet radiation, laser ablation, thermolytic, photochemical, ion implantation, electron beam, and optical lithography are amongst the techniques used in the physical synthesis method (Yang *et al.*, 2017; Ramalingam, 2019). However, laser ablation and ion implantation are the most common technique employed.

By choosing an appropriate parameter and type of aqueous solution in laser ablation technique, the surface area, aggregation, shape, size and properties of AuNPs can be controlled (Isa *et al.*, 2022). In this method, no surfactant is required to be added into the liquid media (Balachandran *et al.*, 2022). However, this method is not suitable for prolonged usage of laser as the sample will block the laser path and might lead to the reduction of ablation rate (Hu *et al.*, 2020). Ion implantation is another method that has been extensively used in the physical synthesis method. This method is used to prepare constant and fewer impurity nanocomposites, where ions are introduced into only a material's surface layer to a depth of several micrometers, while maintaining

the expected physical and chemical properties of the final product of AuNPs. It involved injecting the Au element into near-surface area in a vacuum chamber (Fnu, 2019).

2.3.3(c) Biological synthesis method

The biological synthesis method is also known as biosynthesis or biomimetic or green synthesis (Elahi *et al.*, 2018; Jamkhande *et al.*, 2019). This method is environmentally sustainable due to the use of natural and non-toxic products such as plants and microorganisms such as bacteria, fungi, algae, and yeast as tabulated in Table 2.2 (Elahi *et al.*, 2018; Kumari *et al.*, 2019; Kalimuthu *et al.*, 2020). The biomolecules from these living organisms function as reducer and stabiliser agents to reduce the Au chloride to form nanomaterials (Karthik *et al.*, 2016; Mustapha *et al.*, 2022).

Table 2.2 Different types of natural material for AuNPs synthesis in biological synthesis (Elahi *et al.*, 2018; Kumari *et al.*, 2019; Kalimuthu *et al.*, 2020).

Plant	Bacteria	Fungi	Algae	Yeast
Cinnamommum camphora (Camphor tree)	*Klebsiella pneumoniae*	*Penicillium chrysogenum*	*Spirulina platensis*	*Saccharomyces cerevisiae* (Baker 's yeast)
Cacumen platyclad (Dried herb)	*Bacillus subtilis*	*Aspergillus clavatus*	*Sargassum swartzii*	*Hansenula polymorpha*
Triticum aestivum (Bread wheat)	*Stenotrophomonas maltophilia*	*Rhizopus oryzae*	*Tetraselmis kochinensis*	Yeast Extract Mannitol

The biological synthesis is divided into bioreduction and biosorption mechanisms (Moodley *et al.*, 2020). In bioreduction, the microorganisms enzymes, such as *Staphylococcus aureus*, can reduce the Au ions into stable and inactive form while in biosorption, the cell wall of the organisms, such as *Weisellaoryzae species*, can fasten the Au cations in media to form stable AuNPs (Rauf *et al.*, 2017; Kumari *et*