Electrical properties of the red blood cell membrane and immunohematological investigation

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Hemagglutination is widely used in transfusion medicine and depends on several factors including antigens, antibodies, electrical properties of red blood cells and the environment of the reaction. Intermolecular forces are involved in agglutination with cell clumping occurring when the aggregation force is greater than the force of repulsion. Repulsive force is generated by negative charges on the red blood cell surface that occur due to the presence of the carboxyl group of sialic acids in the cell membrane; these charges create a repulsive electric zeta potential between cells. In transfusion services, specific solutions are used to improve hemagglutination, including enzymes that reduce the negative charge of red blood cells, LISS which improves the binding of antibodies to antigens and macromolecules that decrease the distance between erythrocytes. The specificity and sensitivity of immunohematological reactions depend directly on the appropriate use of these solutions. Knowledge of the electrical properties of red blood cells and of the action of enhancement solutions can contribute to the immunohematology practice in transfusion services.

Keywords: *Zeta potential; Erythrocytes; Optical tweezers; Agglutination*

Introduction

The red blood cell (RBC) membrane contains proteins and glycoproteins embedded in a fluid lipid bilayer that confers viscoelastic behavior. Sialylated glycoproteins of the RBC membrane are responsible for a negatively charged surface which creates a repulsive electric zeta potential (ζ)[1,2] between cells. These charges help prevent the interaction between RBCs and the other cells and especially between each other.[3] Hemagglutination is a physicochemical phenomenon involving several complex factors. Immune-mediated RBC agglutination has two basic stages, the non-visible sensitization stage, consisting of the binding of antibodies to antigen determinants on the RBC membrane and the visible agglutination stage, resulting from random collisions between antibody-coated and sensitized cells, thus promoting agglutination (Figure 1).[4]

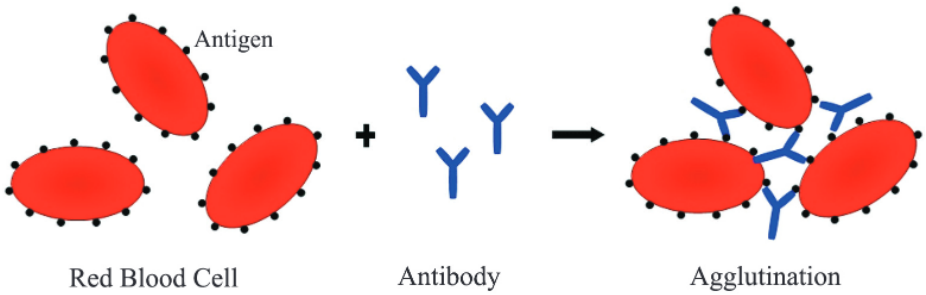

Figure 1 – Representation of the hemagglutination reaction. Blood group antigens and antibodies form a clumping of erythrocytes (modified from Parslow et al., 2004)[5]

These two stages depend on attracting intermolecular forces such as hydrophobic bonds, Van der Walls forces, electrostatic forces and hydrogen bonds, in addition to repulsive forces such as the zeta potential.[4] Repulsive force is generated by the negative charges on cell surfaces that occur due to the presence of carboxyl group in sialic acids in the erythrocyte membrane.[1,2] Clumping occurs when the aggregation force is greater than the force of repulsion.[1]

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First phase of agglutination

The bonding of an antibody to an antigen occurs due to the formation of multiple reversible intermolecular forces of attraction. These non-covalent forces are weak when compared to covalent bonds however the formation of multiple bonds produces a total binding energy strong enough to resist rupture. The strength of the bond between antigen and antibody combining site is termed antibody affinity and is dependent on the combined forces (attractive and repulsive forces). The attraction forces are dependent on the distance between the antibody combining site and antigen.[3] The non-covalent bonds involved in antigen-antibody reactions are hydrogen bonds, electrostatic forces, Van der Waals forces and hydrophobic bonds (Figure 2).

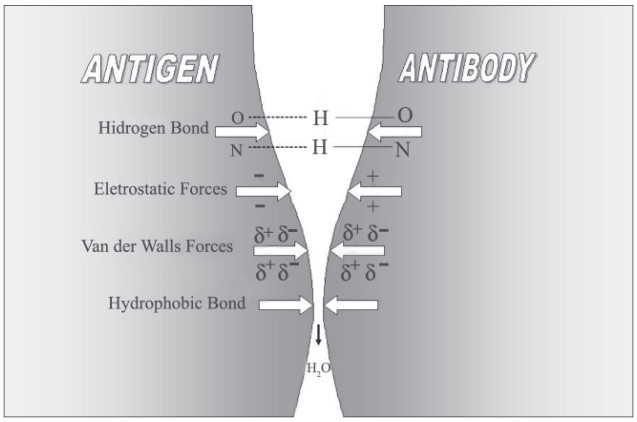

Figure 2 – Forces involved in antigen-antibody binding. Hydrophobic forces and van der Waals can be formed when antigens and antibodies are close to each other. Electrostatic interactions and hydrogen bonds do not require the antigen and antibody to be close (modified from Abbas & Lichtman, 2005)[3]

– Hydrogen bonding results from the formation of hydrogen bridges between two electronegative atoms. Common hydrogen bonds in antibody-antigen interactions are O-H-O, N-H-N, and O-H-N. All are characterized by a covalent bond between atoms. The reactions are exothermic and stronger at low temperatures, usually associated with a carbohydrate antigen (Figure 2).[3]

– Electrostatic force is a consequence of the attraction between positively charged amino acids ($-NH3^{+}$) located on the side chains and negatively charged groups for example the carboxyl group ($-COO^{-}$). The degree of ionization of molecules depends on the pH of the reaction.[6]

– Van der Waals Forces are nonspecific attractive forces and are generated by the interaction between electron clouds and hydrophobic bonds. These bonds occur as a result of minor asymmetry of atom charges resulting from electron position. Van der Walls forces rely on the association of nonpolar, hydrophobic groups so that contact with water molecules is minimized. Although these forces are very weak, they may become collectively important in an antigen-antibody reaction (Figure 2).[3]

– Hydrophobic Links are the main bonds formed between antigens and antibodies. When two hydrophobic groups (nonpolar) come together, these side chains interact and exclude water molecules from the area of interaction. These reactions are endothermic, therefore occur more strongly at higher temperatures and are associated with protein antigens (Figure 2).[3] The strength of the bond between antigen and antibody is measured as the free energy change. The reaction between antibody (Ab) and antigen (Ag) is reversible in accordance with the law of mass actions and may be written as follows (Figure 3):

$$[Ag] + [Ac] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [AgAc]$$

Figure 3 – Where k_1 and k_2 are the rate constant for the forward, and reverse reactions respectively. $[Ag]$, $[Ab]$ and $[AgAb]$ are the concentration of Ab, Ag and combined product $AgAb$, and K is the equilibrium or association contant.[7]

$$\frac{[AgAc]}{[Ag] \times [Ac]} = \frac{k_1}{k_2} = K$$

K reflects the degree to which the antibody and antigen bind to each other and the speed of the reaction. The degree of antigen-antibody uptake is predominantly influenced by the ionic strength and antigen-antibody proportions of the bonds.[7]

Second stage of agglutination

Once antibody molecules bind to antigens on the RBC surface, bonds between the antibodies and sensitized cells form with visually apparent agglutination. The formation of these chemical bonds depend on the size and physical characteristics of the antibody and the distance between cells.[8] Antibodies differ in their ability to agglutinate. IgM antibodies are considerably more efficient than IgG, this capability of IgM class of immunoglobulins is evidently more than sufficient to allow RBC agglutination in saline medium, whereas shorter IgG molecules are unable to bring about aggregation of erythrocytes in saline in spite of the fact that the first stage of RBC binding has occurred.[2] Two erythrocytes, suspended in isotonic saline water, cannot approach each other closer than 50-100 Å.[4]

The distance between the RBCs is an important factor in RBC agglutination and depends of electronegative surface charges and the ionic cloud that normally surrounds them.[1]

Zeta potential

The zeta potential is a physical property which is exhibited by all particles in suspension. The development of

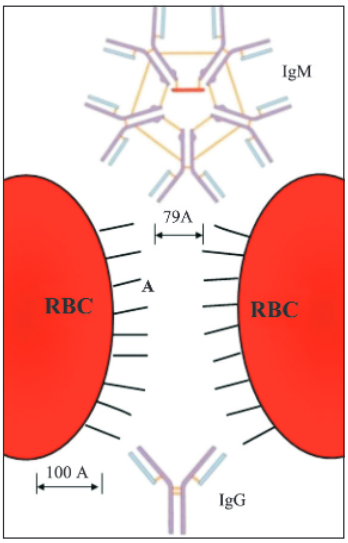

Figure 4 – The minimum distance for two red blood cells with IgG and IgM molecules to bind (scale 100Å) (Modified from Van Oss & Absolon, 1983).[4]

a net charge on any particle affects the distribution of ions in the surrounding interfacial region resulting in an increased concentration of counter ions, ions of opposite charge to that of the particle, close to the surface. Thus a double electrical layer exists around each particle.[9-12] The double layer is formed in order to neutralize the charged particle. There is a thin layer of counter ions strongly attracted to the charged particle, called the compact layer. The counter ions in the compact layer are immobile due to this strong electrostatic force. Counter ions outside the compact layer are mobile and called the diffuse layer (Figure 5).

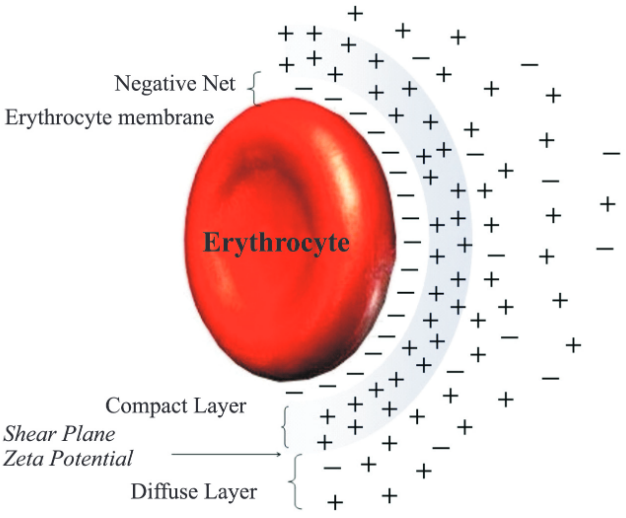

Figure 5 – Schematic representation of zeta potential. Erythrocytes (negative charges) in suspension causing a rearrangement of charges through the formation of two ionic layers that generate a electric potential difference between them, called the Zeta potential (Modified from Pollack & Reckel, 1977 and Rouger & Salmon, 1981).[1,13]

The zeta (ζ) potential is the electrostatic potential at the boundary dividing the compact layer and the diffuse layer. The zeta potential serves as an important parameter in characterizing the electrostatic interaction between particles in dispersed systems and the properties of dispersion as affected by this electrical phenomenon.[9-12]

The zeta potential (ζ) can be shown by an equation (Figure 6) and depends on electronegativity (charge) of the RBC (σ), the dielectric constant of the medium (D) and ionic strength (μ) [1,13]

$$Z = f\left[\sigma, \frac{1}{D}, \frac{1}{\sqrt{D\mu}}\right]$$

Figure 6 – Equation for zeta potential

Hemagglutination enhancement solutions

Several methods are used to enhance the second stage of agglutination and allow visualization of the reaction such as reducing the negative charge of surface molecules, reducing the hydration layer around the cell and introducing positively charged macromolecules that aggregate the cells.[14,15]

Another way to improve the agglutination is to decrease the zeta potential. This is possible by increasing the dielectric constant, changing the composition of the medium by adding macromolecular substances such as albumin,[1] Dextran,[15,16] or polyethylene glycol among others.[8]

Low ionic strength saline solution (LISS)

Low ionic strength saline solution consists of a salt solution with a lower sodium chloride concentration. The solution described as LISS[17] has 20% less sodium chloride compared to normal isotonic solution. The formula described is made up of 0.17M saline (180 mL), 0.15M phosphate buffer (20 mL) and 0.3M sodium glassine (800 mL).[17] By lowering the number of sodium (Na^+) and chloride (Cl^-) free ions in the solution, the ionic layer around the erythrocyte is reduced which promotes non-covalent bonds that are dependent on the distance between antibody and antigen sites on the RBC membrane. The rate of association between antigens and antibodies will be required greater and so less incubation time is needed for the formation of antigen-antibody complexes.[18]

The magnitude of the zeta potential depends on the net charge density of surrounding cations, that is, on the ionic strength. Thus, a decrease in ionic strength will result in a reduction in the thickness of the double layer due to increased cations density resulting in the need to maintain electrical neutrality. This further reduces the zeta potential.[1]

Proteolytic enzymes

Several proteolytic enzymes are used in agglutination reactions. These include bromelin, chymotrypsin, dispase, ficin, neuraminidase, pepsin and trypsin. However, papain and bromelain are most frequently used in immunohematology. The papain enzyme (EC 3.4.22.2) is related to the cysteine protease (C1) family. It consists of a single polypeptide chain of 212 single non-glycosylated amino acids, 3 disulfide bridges and one sulfhydryl group which is required for its activity. This enzyme is extracted from the latex of Carica papaya.[19,20] The bromelain enzyme (EC 3.4.22.32) is a endopetidase extracted from the latex of the pineapple plant stem (*Bromeliaceae*). It is related to the family of cysteine protease C1 and has a single chain, glycosylated amino acid cysteine with seven bridges and probably three disulfides.[19,20] Proteolytic enzymes or proteases catalyze the cleavage of peptide bonds between amino acids of proteins. Proteases are a large family and can be categorized as exopeptidases and endopeptidases according to the point at which they break the peptide chain. Exopeptidases catalyze cleavage of the end of polypeptide chains both at the aminoterminal or carboxyl end of the peptide, releasing peptides and amino acid residue. Endopeptidases catalyze hydrolysis of peptide bonds in the inner regions of the polypeptide chain between the amino and carboxy terminal regions. They can be subdivided according to the reactive group at the active site involved in catalysis into serine-, cysteine-, aspartic- and metallo-endoproteinases.[19,20] Serine peptidases have a serine residue involved in the active center, the aspartic peptidases have two units of aspartic acid in its catalytic center. Cysteine-proteases have an amino acid cysteine and metallo-proteases use a metal ion in the catalytic mechanism.[19,20] The mechanism used to cleave a peptide bond involves making an amino acid residue (peptidases of serine, threonine and cysteine) or a water molecule (aspartic acid and peptidases glutaminic acid and metalloproteases) nucleophilic so that it can attack the peptide carboxyl group.[19,20] Enzyme-treated cells are frequently used in antibody screening and antibody identification procedures in immunohematology, to remove glycoprotein fragments from the RBC membrane, thus enabling greater proximity between the RBCs and a better access of antibodies to blood group antigens (Table 1).[18] The removal of sialic acid directly reduces the electrical charge of the erythrocyte surface which causes a reduction in zeta potential (Table 2).

Table 1 - Description of the effects of enzyme treatment by proteases in the RBC membrane (Modified from Daniels & Bromilow, 2007[18])

Enzyme treatment of red cells by cleavage of glycoprotein from the red cell membrane		
Action	Effect	Result
Sialic acid is the major contributor to RBC negative charge	Reduces net negative charge	Allows cells to be closer
Glycoproteins are hydrophilic and attract water molecules	Water molecules need to be shared by neighboring RBC	RBC closer to each other
Glycoprotein structures protrude from RBC membrane surface	Reduced steric hindrance	Antigens more accessible to antibodies
RBC antigen carriers	Lose specific antigens (MNS and Duffy system)	Undetectable antibodies of these specificities

Table 2 - Effects of enzyme treatment of red blood cells using bromelain, chymotrypsin, dispase, ficin, neuraminidase, pepsin and trypsin

Results were obtained from the zeta potential measurements (ζ) (Modified from Omi et al., 1994[21])		
Enzyme	Zeta potential ζ (mV)	% Zeta potential Reduction
Bromelain	-6.05	55.8
Chymotrypsin	-7.91	42.3
Dispase	-9.04	34.0
Ficin	-4.38	68.0
Neuramidase	-1.31	90.4
Papain	-8.52	37.8
Trypsin	-7.28	46.8
Normal	-13.70	0

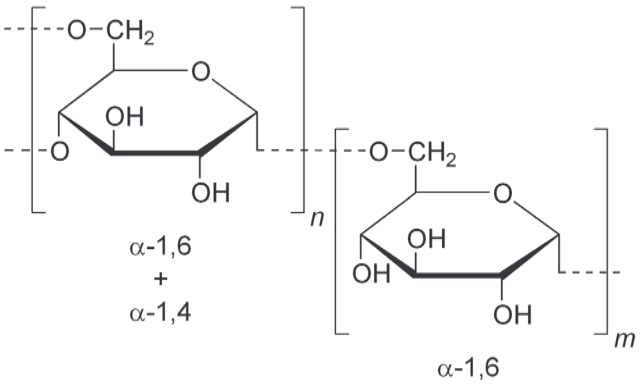

Figure 7 – Segment structure of the molecule of dextran

Dextran

Dextran is a polysaccharide carbohydrate produced by fermentation of sucrose by *Leuconostoc mesenteroides* that converts sucrose to dextran (Figure 7). It can be used as a substitute for plasma in hypovolemic patients, but can induce RBC aggregation.

The mechanisms involved in dextran-induced erythrocyte aggregation are not yet fully understood and two processes have been proposed as inducers of aggregation: The formation of bridges between cells by polymer molecules (the "bridging" mechanism)[22-24] and condensation of cells induced by osmotic gradients due to depletion of macromolecules in the intercellular space. [22,25,26]

Polyethylene glycol

Polyethylene glycol (PEG) is a water soluble linear polymer, prepared by the polymerization of ethylene oxide which is commercially available over a wide range of molecular weights. PEG has the following structure:

$$HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2OH$$

PEG is soluble in water, methanol, benzene, dichloromethane and is insoluble in diethyl ether and hexane; this solubility characteristic is often described as amphiphilic.[27] The hydroxyl group of the PEG molecule provides a site for covalent bonds with other molecules and/or surfaces. PEG does not denature proteins or prevent the approach of other small molecules. It is substantially non-antigenic and non-immunogenic and has been widely used in biotechnology and biomedicine, especially in the exclusion of other aqueous polymers in the solution, avoiding complete solubilization. It does not cause a decrease in cell activity and proteins are biodegradable and nontoxic.[27] PEG in water acts as a very mobile molecule allowing exclusion of water and other polymers,[27] thus the action is to remove water from the surface of RBCs, increasing antibody concentration and promoting the binding of antibodies with antigenic sites.[28]

Conclusion

Immunohematological research is based on specific reactions between antibodies and antigens. IgG antibodies are unable to approximate RBCs without the addition of enhancer substances that promote agglutination. The specificity and sensitivity of immunohematological reactions depend directly upon the appropriate use of these substances. A better knowledge of the electrical properties of RBCs and the mechanism of action of different solutions may contribute to the standardization of hemagglutination tests.

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