Université de Liège

Faculté de Médecine

Sciences Biomédicales et pharmaceutiques

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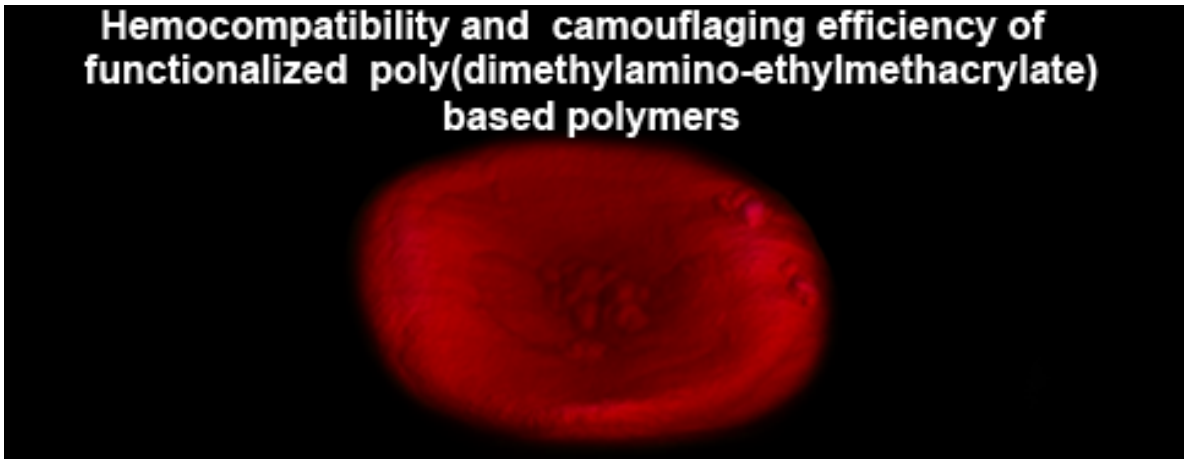
Universidad Autónoma de San Luis Potosí

Doctorado Institucional en Ingeniería y Ciencia de Materiales

Hemocompatibility and camouflaging efficiency of functionalized poly(dimethylamino-ethylmethacrylate) based polymers



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Thesis directed by

Professor Christian Grandfils.

Interfacultary Center of Biomaterials (CEIB), ULG, BE.

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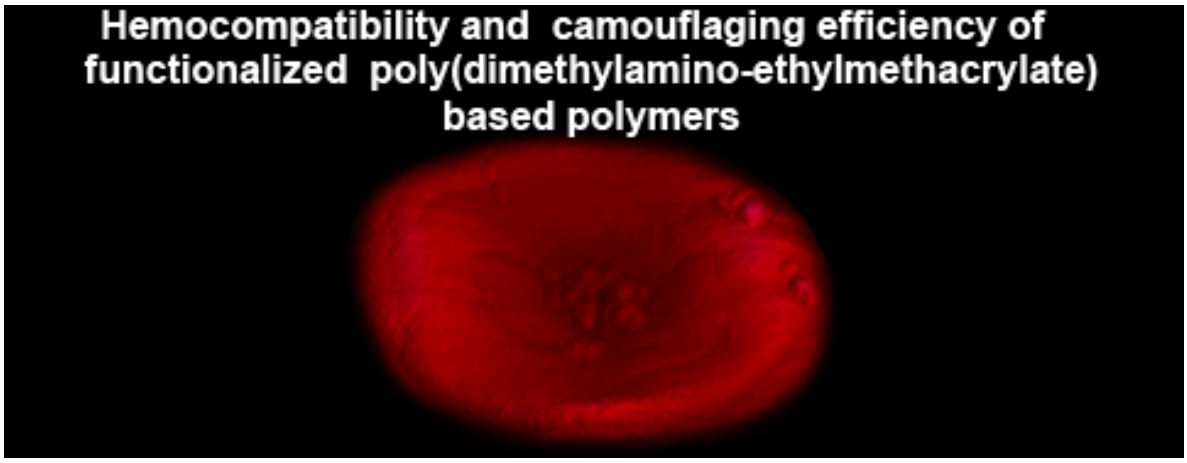

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Abstract

This doctoral thesis has been mainly focused on the interaction between poly (2-dimethylamino-ethylmethacrylate) (PDMAEMA) based polymers and human blood. Although PDMAEMA is a polycation widely reported for tailoring polyelectrolytes complexes for gene or drug delivery, little is known on its blood compatibility. With the final perspective to produce a universal blood, we have also investigated the immunomasking ability of this polymer family to cover red blood cells (RBCs) antigens. Hemocompatibility of the free form of our synthetic polycations was assessed in vitro following international standard tests (ISO 10993-4) and considering the following main parameters: hemagglutination, hemolysis, platelet size and number, blood coagulation, and the complement system. Our toxicological screening has highlighted that hemotoxicity can be avoided when these PDMAEMA-based polymers fit to specific chemical properties, in particular those regarding their Mw and composition. Some of our observations support the assumption that PDMAEMA homopolymers interact strongly with the surface of the RBCs but without disturbing the inner structure of the membrane.

Adopting a self-assembly process of our polycations to the cell plasma membrane surface, an event which should occur readily through the predominance of anionic sites present at the glycocalyx surface of the RBC's, we have assessed the immunomasking ability of PDMAEMA homopolymers differing in Mw. Their properties have been compared with PDMAEMA-PEG copolymers to measure the benefit expected from the polyether sequences through their steric hindrance ability. Adopting anti-glycophorin A (aGA) as membrane marker and using FACS to quantify the cell immunoprotection we observed only a partial camouflaging of RBCs for both homopolymers and copolymers. Unexpectedly, they also caused a sensitization of the immunorecognition of the GPA antigen. Although the PEG did not improve drastically the camouflaging ability, the architecture of the copolymers has been highlighted through the comparison between palm-tree and random copolymers. In addition to generate significant toxicological information on the hemocompatibility of PDMAEMA, our research has evidenced new interactions between our synthetic macromolecules and RBCs which would be valuable to future explore in depth in the frame of fundamental and applied research projects

Résumé

Notre travail de thèse s'est principalement focalisé sur l'interaction entre des polymères à base de Poly[2-(diMéthylAmino)Éthyle MéthAcrylate] (PDMAEMA) et le sang humain. Bien que le PDMAEMA soit un polycation largement rapporté pour la formation de complexes polyélectrolytiques dans le domaine de la libération de gènes et de médicaments, sa compatibilité avec le sang est mal connue. Dans la perspective à long terme de produire un sang universel, nous avons étudié la capacité d'immuno-masquage de cette famille de polymères pour recouvrir les antigènes exprimés à la surface des globules rouges.

L'hémocompatibilité de nos polycations synthétiques sous forme libre a d'abord été évaluée *in vitro* selon les tests standards internationaux (ISO 10993-4) et en considérant les paramètres principaux suivants : hémagglutination, hémolyse, nombre et taille des plaquettes, coagulation sanguine et activation du complément. Notre étude toxicologique a démontré qu'une'hémotoxicité peut être évitée si ces polymères à base de PDMAEMA répondent à des propriétés chimiques spécifiques, en particulier en regard de leur masse moléculaire et de leur composition. Certaines de nos observations confirment l'hypothèse selon laquelle les homopolymères de PDMAEMA interagissent fortement avec la surface des globules rouges sans toutefois modifier la structure interne de leur membrane plasmique.

Adoptant un procédé d'auto-assemblage de nos polycations en surface de la membrane plasmatique cellulaire, un processus qui devrait se réaliser facilement grâce à la prédominance des sites anioniques présents à la surface du glycocalyx des globules rouges, nous avons évalué la capacité d'immuno-masquage d'homopolymères de PDMAEMA de différentes masses moléculaires. Leurs propriétés ont été comparées à celles de copolymères PDMAEMA-PEG en vue de mesurer le bénéfice apporté par les séquences polyéther normalement susceptibles de fournir un effet répulsif par effet stérique. Adoptant la glycophorine A (GA) comme marqueur membranaire et le FACS pour quantifier l'immuno-protection de la cellule, nous avons observé un camouflage partiel des globules rouges, tant par les homopolymères que par les copolymères. Etonnamment, ces macromolécules de synthèse ont aussi induit une sensibilisation de l'immuno-reconnaissance de la GA. Bien que les séquences de PEO n'améliorent pas drastiquement la

capacité de camouflage, l'influence de l'architecture des copolymères a été mise en évidence par la comparaison entre copolymères de type « palmier » et copolymères statistiques.

En plus des informations toxicologiques importantes relatives à l'hémocompatibilité du PDMAEMA, notre recherche a aussi mis en lumière de nouvelles interactions intéressantes entre nos macromolécules synthétiques et les érythrocytes. Ces observations mériteraient d'être détaillées tant dans la perspective d'une recherche à caractère fondamentale que d'une valorisation dans le secteur biomédical.

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To people in Liège…

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Professor Grandfils, opened the door of their home to me in bad times (and in good times too of course). For all those big details, thanks a lot dear Chantal.

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beginning of this PhD story. During my PhD he gave me a lot of suggestions to improve my work and supported me when hard times came. Thanks a lot Elías.

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To Helena

Thanks, my lovely girl 'cause you´re always there by my side. Pues si, pues si, pues si…

To God

Oh thank God!

List of Abbreviations

aAab: anti-A antibody
aCD45: anti-CD45 antibody
aGPA: anti-glycophorin A antibody
AL: sodium alginate
APPT: activated partial thromboplastin time
ASTM: American Society for Testing and Materials
ATRP: atom-transfer radical polymerization method
BCT: Behring Coagulation Timer analyzer
BmPEG: mPEG-benzotriazole carbonate (bifunctional)
BRBCs: bovine red blood cells
BrPEI: branched poly(ethyleneimine)
BSA: bovine serum albumin
CH-PDCH: chitosan-phosphorylcholine
CHO-K: chinese hamster ovary cells
CL: cardiolipin
CmPEG: cyanuric chloride methoxy poly(ethylene glycol)
CyanMHb-m: cyanmethemaglobin method
DADMAC: diallyl-dimethyl-ammonium chloride
DMEM: Dulbecco's modified Eagle's medium
EATC: Ehrlich ascites tumor cells
ECO-RBCs: enzyme-converted O red blood cells
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
EM: electrophoretic mobility
EP: extrinsic pathway
FACS: flow cytometry
GPA: glycophorin
HA: sodium hyaluronate
Hb: hemoglobin

hBMECs: human brain microvascular endothelial cells

hESC: human embryonic stem cells

hKBEPC human KB epidermal carcinoma cells

HMW: high molecular weight

HUVECs: human umbilical vein endothelial cells

IP: intrinsic pathway

ISO: International Organization for Standardization

JTC: Jurkat T cells

LDH: lactate dehydrogenase

LbL: layer-by-layer method

LMW: low molecular weight

lPEI: linear poly(ethyleneimine)

MFI: mean fluorescence intensity

MRBCs: murine red blood cells

MTT: (3-(4, 5-dimethylthiazol-2-y1)2, 5-diphenyl tetrazolium bromide)

Mw: molecular weight

ncfRBCs: noncamouflaged red blood cells

PAMAM: poly(amidoamine)

PBS: phosphate buffer saline

PC: polycation

pcfRBCs: partially camouflaged red blood cells

PCs: polycations

PDCH: phospatidylcholine

PDDAC: poly(dially-dimethyl-ammonium) chloride

PDMAEMA: poly(2-dimethylamino-ethylmethacrylate)

PECs: polyelectrolyte complexes

PEG: poly(ethylene-glycol)

PEG-RBCs: pegylated red blood cells

PEI: poly(ethyleneimine)

PLL: poly-l-lysine

PS: phospathidylserine

PT: prothrombin time

PVP: poly(4-vinylpyridine)

Q-P(TDAE): partially quaternized poly[thio-1-(N,N-diethyl-aminomethyl) ethylene])

RBC: red blood cell

RBCs: red blood cells

rHb: released hemoglobin

rLDH: released lactate dehydrogenase

R rms: roughness value

SPA-mPEG: succinimidyl ester of methoxypolyethylene glycol propionic acid

sRBCs: sensitized red blood cells

SRBCs: sheep red blood cells

Content Index

1. GENERAL INTRODUCTION

1. Introduction

Polycations (PCs) are positively charged polymers when dissolved in a 7.4 pH medium. In that state, PCs interact spontaneously with negatively charged molecules such as proteins and nucleic acids. This ionic interaction is driven mainly by entropy resulting from the release of the counterions initially associated with the involved macromolecules (1) . As a consequence of this physicochemical event, polyelectrolyte complexes (PECs) are formed within solutions containing PCs and anionic molecules. PCs have been explored extensively when they form PECs, either as drug delivery systems (DDSs) carrying DNA (polyplexes) or DDSs for peptide/protein drugs (2-4). PCs are natural or synthetic polymers; both types are explored in preclinical research and some are already used in clinics. For example, both natural PCs protamine sulfate and chitosan are used to neutralize heparin in blood and to carry insulin, respectively (2-5). Chitosan, also a natural PC, has been used to form hydrogels and membranes for the treatment of skin regeneration (6). Synthetic poly (dimethylamino-ethylmethacrylate) (PDMAEMA) PC, sold under the trademark Eudragit, is another example of a PC that is applied daily for pharmacological use (7).

PCs are popular for drug delivery because they not only complex anionic drugs, but they also interact with the cell membrane of mammalian cells. That interaction facilitates adsorption of PECs onto the cell surface and cellular uptake of the complexes (2-4). In addition to the uptake of PECs, the PC–cell membrane interaction is a very useful means of modifying the cell membrane surface by adsorption of PCs (8). Membrane cell modification by PCs is a strategy that is commonly employed to cover or mask cell antigens (8-10). Hence, it is an attractive means of camouflaging antigens on the erythrocytes (red blood cells [RBCs]) because camouflaging RBC antigens can avoid health problems associated, for instance, with transfusion of incompatible blood groups, alloimmunization of chronically transfused patients, immune hemolytic transfusion reactions, and extremely acute transfusion-related lung injuries (11, 12).

Camouflage of RBC antigens by PCs is a promising strategy, but it is also a big challenge due to the diversity and high density of blood group antigens that must be masked. Moreover, it is also a challenge to maintain the biophysical properties of RBCs and to avoid triggering PC-induced hemotoxic responses. Hence, the study of PC-blood interaction is important not only for camouflaging antigens, but also for evaluating PCs employed as cationic carriers for IV administration or carriers for crossing the blood-brain barrier. Accordingly, our study is significant for both camouflaging of antigens and assessing PCs that are explored as cationic carriers.

1.1.1 Surface characteristics of RBCs: zeta potential and antigens

The negative electrical nature of RBCs is suitable to induce PC adsorption. Such electrical nature originates from extracellular antigens composed of glycosylated proteins (See Table 1.1) (12-14). Those glycosylated structures form a 10–15-nm deep, negatively charged barrier—the glycocalyx. This negative layer creates the zeta (ζ) potential, an electrokinetic potential that assures the stability of RBCs suspended in the blood and prevents their spontaneous aggregation (15). The glycosylated membrane proteins act also as blood group antigens. For instance, the A and B antigens, 2 of the most clinically significant blood groups, are glycosylated structures. However, not all blood group antigens are formed by carbohydrate structures; some have other chemical conformations (Table 1.1).

Blood group antigens are varied in function and numerous. To date, about 800 antigens have been identified, and most have been classified into 29 blood groups. According to their functions, blood antigens can be classified into 5 categories: transporters and canals, receptor and ligands, adhesion molecules, enzymes, and protein structures (15). Table 1.1 (Part 1 and 2) shows an overview of the blood groups including the name of the group, name of the antigen, component nature, function, membrane localization, and density.

Name	Antigen	Component nature	Function	Membrane localization	Density and density variation
ABO	A, B, AB,	Oligosaccharides (negatively charged)	Antigen	Surface	250,000 > 1,000,000
MNS	M,N,S,s,U,He,Mp,Vw2	GPA highly glycosylated (negatively charged) GPB	Could contribute to glycocalyx Interacts with B3 to increase anion transport	N-terminal extracellular domains, single membrane spanning domains and cytoplasmic C-terminal domains.	GPA: 1,000,000 GPB: 200,000
P	P1	Carbohydrate (negatively charged)	Glycocalix	Extracellular	500,000
Rh	D, C, E, c, e, f, Cw, V, G, 36 more	RhCE: RHD protein	Transport, structure	Transmembrane	100,000-200,000
Lutheran	Lua ,Lub, Lu3, Lu4, Aua ,Aub 12 more	Lutheran glycoprotein B-cam (negatively charged)	Receptors and adhesion molecules,	Integral membrane with 5 extra cellular domain	1500-4000
Kell	K, k, Kpa ,Kpb, Ku, Jsa ,Jsb 16 more	Kell glycoprotein (negatively charged)	Endopeptidase	Transmembrane domain and long extracellular domain	3,000-17,000
Lewis	Lea,Leb,Leab,LebH, ALeb,BLeb	Carbohydrate (negatively charged)	Receptor, Antigen	Extracellular	13,000-14,000
Duffy	Fya, Fyb, Fy3, Fy4, Fy5, Fy6	Fy glycoprotein (negatively charged)	Binds chemokines for remove them from bloo	Trasnmembrane with external domain	
Kidd	Jka,Jkb, Jk3	Kidd glycoprotein (negatively charged)	Urea transport	Transmembrane with external domain	14;000
Diego	Dia, Dib,Wra,Wrb,Wda ,Rba, 14 more	Band 3	HCO_3/Cl	Transmembrane long citosolic domain	1,000,000
Yt	Yta,Ytb	Acetylcholinesterase	Enzyme	Surface	10,000
Colton	Coa,Cob, Co3	Aquaporin	Water channel	Transmembrane	120,000-160,000

Table 1.1 (Part 1) Red blood cell antigens (Ref. 12-14).

Name	Antigen	Component nature	Function	Membrane localization	Density
Landsteiner - Weiner	LWa ,LWab,LWb	LW glycoprotein (negatively charged)	Adhesion/receptor	Surface	1000
Chido/rogers	CH1, CH2, Rg1 +6 more	C4A/C4B	Complement not strictly Ag	Surface	-
H	H	Carbohydrate (negatively charged)	-	-	-
Kx	Kx	Kx glycoprotein (negatively charged)	Homology with neurotransmission	Transmembrane	1000
Gerbich	Ge2, Ge3, Ge4, Wb, Lsa ,Ana ,Dha	GPC; GPD	Could contribute to glycocalyx Links membrane to skeleton	Transmembrane with extra and intra cell domain	C:135,000 D: 50,000
Cromer	Cra,Tca,Tcb,Tcc,Dra,Esa, IFC, WESa, WESb,UMC	DAF Glycoprotein (negatively charged)	Complement control protein	Transmembrane With extracellular domain	20,000
Knops	Kna,Knb, McCa,Sla,Yka	CRI:CD35 Glycoprotein (negatively charged)	Complement control protein	Single pass membrane	20-1500
Indian	Ina, Inb	CD44	Glycosaminogycan hyaluronan recpetor	Single pass Membrane protein	2000-5000
Ok	Oka	CD147 Glycoprotein (negatively charged)	Receptor and adhesion molecule	Single pass	-
Raph	Mer2	Tetraspanin	Integrity of basement membrane	Cross 4 times the cell membrane	-
Scianna	Sc1, Sc2, Sc3	Sc glycoprotein (negatively charged)	IgV	Trans,membrane and citosolic domain	-
Dombrock	Doa ,Dob,Gya ,Hy,Joa	Do glycoprotein (negatively charged)	ADP–ribosyltransferases,	surface	-
Xg	Xga,	Xga Glycoprotein (negatively charged)	Adhesion/receptor	Transmembrane with citosolic domain	9,000

Table 1.1 (Part 2) Red blood cell antigens (Ref. 12-14**).**

1.1.2 Universal RBCs

The ABO and Rh blood groups are the most clinically significant blood groups, although it cannot be ignored that other minor groups are also clinically important in cases of sensitization from of chronic transfusion. Usually, the ABO (A and B antigens) and Rh (D antigen) blood groups govern the selection of donor-receiver in blood transfusion. Evidently, an acceptor does not have antibodies reacting with the RBC donor. It means that donor and receiver must have the same blood group. In addition, a receiver can receive RBCs from a universal donor, meaning a group O donor. But when donor and receiver are mismatched, the RBCs interact with their corresponding antibodies and a complement system reaction is activated. This response induces an immunological mechanism leading to the formation of the membrane attack complex that subsequently promotes cell lysis (16).

Mismatching of donor-receiver must always be avoided. For this reason, blood transfusion clinics have compatibility testing that includes a set of extremely effective protocols for identifying and preventing use of incompatible blood; however, mismatched transfusions still occur (17). Hence, using universal RBCs is advisable to prevent risk of AB and Rh hemolytic transfusion reactions as well as having a large supply of universal RBCs units in a blood bank. This is not always possible because of the lack of blood donors (17). To solve the need of universal RBCs for transfusion, biomedical scientists have explored strategies to develop type O RBCs. For that goal, three main approaches have been investigated: 1) production of RBCs from different cell lines, 2) enzyme-modified RBCs (ECO RBCs), and 3) polymer-shielded RBCs. Although the 3 strategies seek the same goal, they are performed with very distinct methods, and each one has its own challenge.

1.2 Development of universal RBCs

1.2.1 Production of universal RBCs from different cell lines

In vitro growing of RBCs is a strategy for developing universal RBCs (Fig.1.1). Differentiation of primitive hematopoietic stem-and-progenitor cells (HSPCs) can be programmed to grow RBC precursors. For instance, both RBC precursor erythroid burst-forming units and erythroid colony-forming units have been grown in vitro. Early manipulation of these RBC precursors has allowed studying sequential expression of various blood group antigens including ABH, MN, P, and Lewis systems. Hence, the approach can be applied to develop type O RBCs (18). Another method investigated for production of RBCs is based on the differentiation of human embryonic stem cells (hESC) (19, 20). Using hESC, Lu et al (20) have grown mature RBCs. These authors have produced RBCs with phenotypes A Rh+, B Rh−, and O type/Rh. Moreover, the cells showed oxygen-carrying properties comparable with those of normal adult RBCs. These strategies are not limited to using HSPCs or hESCs because RBCs can be also produced from some mature cells. Szabo et al have reported (21) the conversion of human dermal fibroblasts to multilineage blood progenitors, including erythrocyte progenitors. Although RBC production from other cell lines is a promising strategy, the method has limitations because its large scale production requires large amounts of expensive cytokines and growth factors. Thus, the strategy is still far from reality for large-scale production of universal RBCs (19, 20).

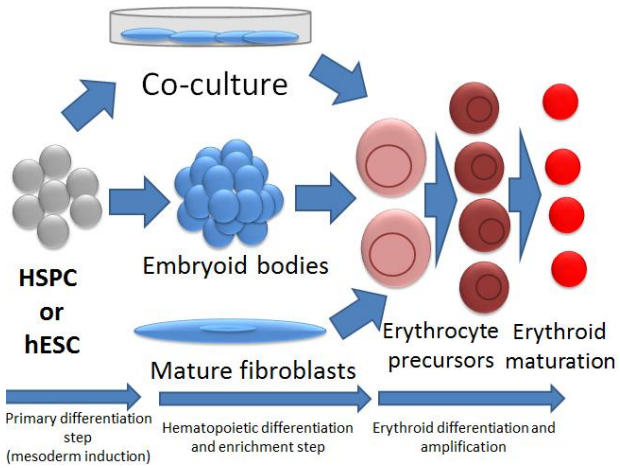

Fig. 1.1 Cell differentiation methods for growing of RBC's (Based on 19, 20).

1.2.2 Production of ECO-RBCs

Blood group antigens are distributed on the external surface of the erythrocyte so that if the antigenic sites of any blood groups are effaced from the cell surface, the immunogenicity of the RBCs will disappear. This principle has been applied to convert blood group A− or B− RBCs into blood group O by enzymatic cleavage of the antigenic sites. The resulting cells have been called "enzyme-converted O red blood cells (ECO-RBCs)." Enzymatic cleavage of blood groups A or B is based on the fact that AB blood group specificity is determined by the nature of monosaccharides at the extremity of the extracellular domain of the antigens. For the A antigen, the immunodominant monosaccharide is a terminal α1-3– linked N-acetylgalactosamine. For type B RBCs, the monosaccharide is an α1-3 linked galactose. For the H, a variation of the antigen in the ABO system, the terminal monosaccharide is an α1-2 linked fucose (22, 23) (Fig.1.2). Hence, when an enzyme removes the ending monosaccharide, the RBCs are transformed into an O blood group (22, 23).ECO RBCs have been produced from A and B RBCs; however, they have shown controversial results in clinical assays with variability in the patient-to-patient immune response. Moreover, a major disadvantage of the approach is that large-scale production of the enzymes is expensive and difficult (24).

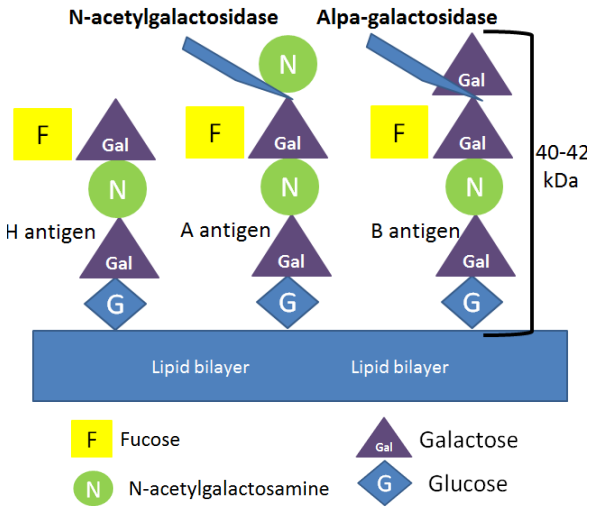

Fig. 1.2 Schematic representation of the structure of the A, B and H antigens. The enzymes N-acetylgalactosamine and the α-galactosidase "cut" the immunodominant monosaccharides for A and B antigens, respectively (Based on 22, 23).

1.2.3 Production of Polymer-shielded RBCs

1.2.3.1 Pegylation of RBCs

Tailoring a polymeric shield to cover RBCs is another strategy for developing universal RBCs, which relies on the fact that polymers can be bonded to the surface of the cell membrane. Consequently, polymer chains should cover and mask the blood group antigens. Moreover, the polymeric layer should favor the repulsion of antibodies. This approach promotes an "immunocamouflage" for the RBCs. Because RBCs covered by polymeric protection should be undetected or invisible to the immune system, they have been called "stealth RBCs" (24). Covalent PEGylation has been the most common method of producing stealth RBCs. It consists of covalent binding of polyethylene glycol (PEG) chains (which explains the generic name of the method) onto the erythrocyte surface. PEG is a nonionic molecule with a neutral charge, soluble in water by virtue of the hydrogen bonding of ~3 water molecules per ethylene oxide unit as same time as the terminal hydroxyl groups are available for coupling reactions(25). The particular physicochemical properties of PEG give to such molecule its ability to repulse antibodies. In an aqueous solution, the PEG chains attract water molecules to form a large hydration sphere that prevents antibody binding. Moreover, the rapid mobility and molecular flexibility of the PEG segments also contribute to limit any protein binding. As a consequence, the PEG chains form a shield around the RBC (Fig.1.3). The surrounding steric exclusion volume is of sufficient size to prevent the approach of large molecules such as antibodies (25, 26).

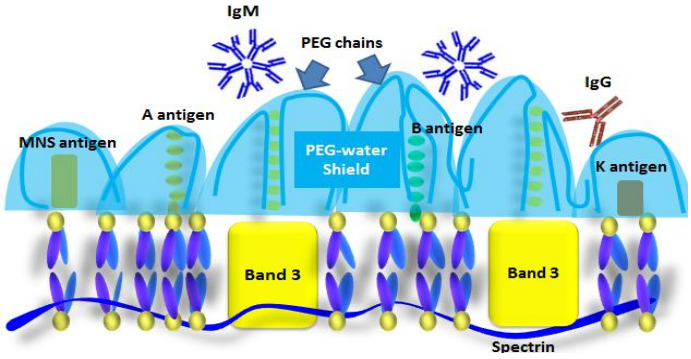

Fig. 1.3 Steric repulsion zone (blue sky) formed by PEG (blue sky lines)-water on the cell surface. The masked antigens cannot be contacted by the antibodies (IgM or IgG) (25, 26).

PEGylation has already been shown to be efficient in masking molecules for clinical application, such as PEGylated interferon-α2, which has encouraged the use of PEGylation for RBCs (22). PEGylated RBCs have been investigated for more than 10 years. During that time, several studies have evaluated the ability of PEGylation to mask antigens, the effect of PEG on the biophysical properties of RBCs, and the in vivo behavior of PEGylated RBCs. With respect to camouflage, results have been controversial because promising in vitro results have not been supported by in vivo tests (24). In vivo, masking is not totally efficient, and PEGylated RBCs have shown rapid clearance (24). On the other hand, a disadvantage of PEG grafting is that the method requires several steps, including the use of highly toxic reagents such as cyanuric chloride, N-hydroxysuccinimidyl ester of methoxypoly (ethylene glycol) propionic acid, and benzotriazole carbonate. Hence this strategy is still limited in practice because of the extensive and expensive purification procedures necessary to eliminate side effects.

1.2.3.2 Self-assembly of PCs polymers to mask RBC antigens

Blocking biological recognition by self-assembled polymers is another strategy for camouflaging blood group antigens. The approach consists of anchoring a copolymer consisting of a PC backbone and PEG moieties on the RBC surface. Thus, the PCs chain should self-assemble onto the negatively charged surface of the RBC; at the same time, the PCs should include PEG moieties to facilitate steric antibody repulsion. Early findings on self-assembly of PCs on RBCs were published by Elbert and Hubell (8). These authors observed that RBCs treated with 0.1% poly-l-lysine-graft-polyethylene glycol (PLL-PEG) with a molecular weight (Mw) of either 20,000 or 375,000) blocked wheat germ agglutinin-induced aggregation, concluding that inhibition of agglutination was due to physical protection originating from copolymers anchored to the RBCs, thus avoiding cell agglutination (8). Although these authors have discontinued exploring PLL-g-PEG abilities for camouflaging RBCs antigens, they laid the foundation for using electrostatically bounded polymers for developing immunologically silent cells (27).

1.3 Our strategy: Self-assembly of PDMAEMA based polymers for camouflaging

In this work we explored an alternative strategy to develop stealth RBCs. It relies on the adsorption of poly (dimethylamino-ethylmethacrylate) (PDMAEMA)-PEG copolymers onto the RBC surface (Fig. 1.4). This strategy consists of tailoring the copolymers capable of self-assembling at the erythrocyte surface, linking the cationic polymeric sequence (PDMAEMA) to the glycocalyx by ionic interaction. The other sequence, based on poly (ethylene glycol), should prevent both nonspecific interactions and specific recognition of the biological surface by steric hindrance. The use of self-assembly is analogous to the stabilization of aqueous colloidal dispersions by the adsorption of hydrophilic/hydrophobic block copolymers.

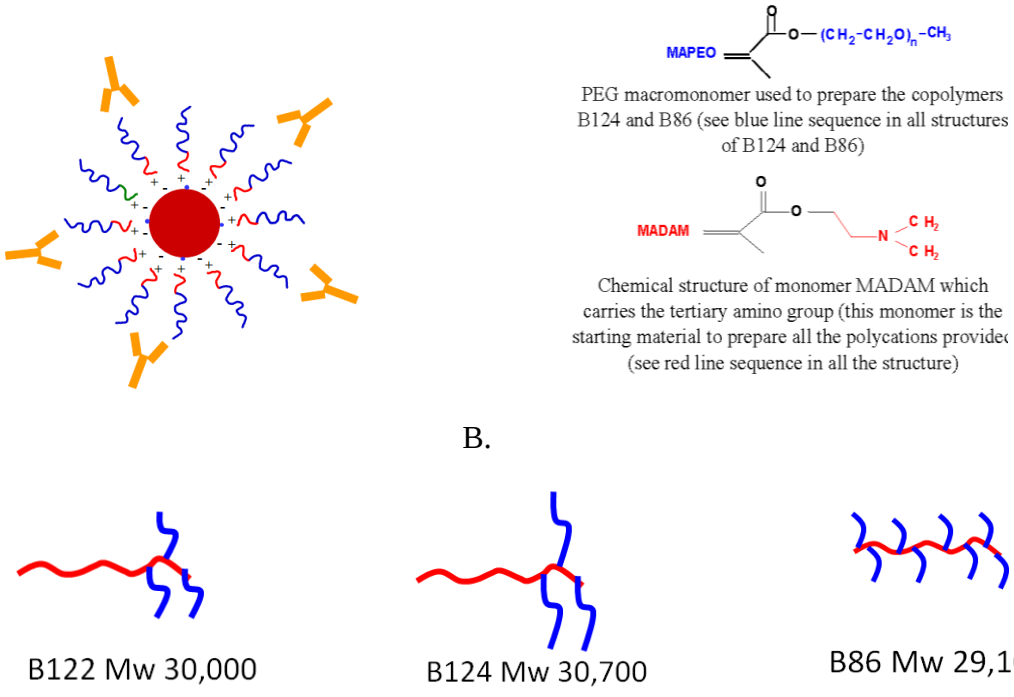

Fig. 1.4 A) Stealth RBC's scheme. PDMAEMA (Red) interacts with the RBC's surface while PEG (Blue) avoid binding of antigens. B) Chemical structure of the building blocks of the PDMAEMA based copolymers. C) Architecture of PDMAEMA-co-MAPEG copolymers.

Our approach has recently been investigated by Ch. Grandfils at the Interfacultary Center for Biomaterials (CEIB) at the University of Liège in collaboration with the Group of Applied Optics to Biology from the Universidad de Rosario (28). Compared with other strategies, our approach offers as a main advantage reliance on a single and simple step consisting of the physical addition of a polymer solution to a washed RBC suspension. If the copolymer is well designed with a view to promoting its quantitative adsorption on the RBC membrane, a reasonable amount of polymer (in terms of cost and toxicity) should b used to cover all the RBC surfaces (~7.5 mg/100 mL of RBC suspension [40 %]) (29).

1.4 Aim of the work

This PhD dissertation has as its primary main aim to evaluate the feasibility of the strategy. Accordingly, the project was divided into 3 secondary tasks: (1) to assess the in vitro hemocompatibility of PDMAEMA- based polymers, (2) to analyze the morphological and electrical change in the RBC membrane after adsorption of PDMAEMA, and (3) to control the functionality of PDMAEMA-based polymers to mask the blood group antigens on the RBC.

The study was performed within the framework of the European project Nanobiopharmaceutics (EU FP6 IP). Thus, hemocompatibility tests have been devised to verify the suitability of PDMAEMA in developing nanoparticles for drug delivery purposes (30). Moreover, hemocompatibility of several other polymeric nanoparticles tailored for coworkers was evaluated (31). This manuscript includes the most significant results and conclusions of the experiments performed for the PhD project. In addition, results obtained from experiments performed in collaboration with other research teams, and other items of interest about the project are mentioned. **All results and conclusions are strictly confidential.**

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2. HEMOCOMPATIBILIY OF PDMAEMA BASED POLYMERS

2.1 Introduction

In this research, we investigated the masking of antigens in red blood cells (RBCs) under in vitro conditions. On the basis of final clinical application, we first verified the in vitro hemocompatibility of the PDMAEMA-based polymers. Several negatively charged blood components such as RBCs, platelets, and distinct plasma proteins may interact with PCs, provoking such hemotoxic responses as RBC aggregation (1), hemolysis (2), complement activation (3), platelet aggregation (4), and changes in blood-brain–barrier permeability (5). Thus, evaluation of PDMAEMA-induced blood toxicity is a major goal in our research. The investigation of hemocompatibility is essential for our masking strategy but it is not limited to such propose; knowledge of PDMAEMA hemocompatibility is also significant in the field of drug and gene delivery because PDMAEMA is a popular PC for tailoring nanovectors (6-13). Hence, we devote this chapter to the hemocompatibility assessment of PDMAEMA-based polymers. First, we present a background about biocompatibility and hemocompatibility concept. Second, we justify the scientific and experimental bases of our study. And third, we present the experimental section with results and discussion following the format of Cerda-Cristerna et al (14, 15).

2.2 Biocompatibility and Hemocompatibility

Biocompatibility is a key concept in the development of materials contacting the human body. A material contacting living tissues must be well accepted by the tissues, thus implant material should have such physicochemical properties that avoid induction of adverse biological responses, in other words, be biocompatible. However, determining a material's biocompatibility is difficult. In a strict sense, a totally biocompatible material does not exist because inert biomaterials do not exist. If the biocompatibility of biomaterials is an ambiguous idea, then the study of biocompatibility is also ambiguous. To avoid ambiguity, biocompatibility should be a well-defined and well-understood concept.

In 1986, during the Consensus Conference on Definitions in Biomaterials (European Society of Biomaterials), "biocompatibility" was defined as "the ability of a material to

perform with an appropriate host response in a specific application" (16). Nowadays that definition seems out-of-date because it was based on ideas established between the 1940s and '80s. Recently, Williams proposed a new definition: "Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy" (17). The Williams definition better describes biocompatibility because it is a dynamic and multifactorial process involving a biomaterial, its function, and the host response (Fig. 2.1) (18).

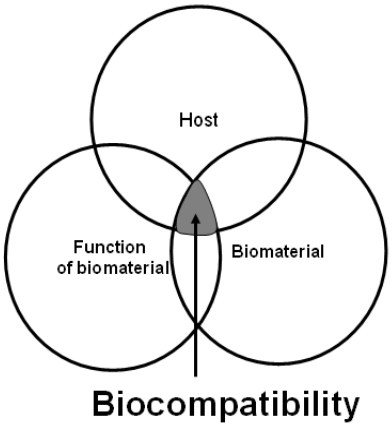

Fig. 2.1 Biocompatibility, a multi-factorial process on biomaterials (Based on 18)

Taking into account that biocompatibility is influenced by several factors, biomaterial-tissue interaction is challenging to evaluate. Thus, biocompatibility of biomaterials is studied according to the type of biological reaction needing evaluation. Accordingly, when one wishes to evaluate the interaction between a biomaterial and blood, one studies hemocompatibility, the science of testing for biomaterials performing a function in blood. At the same time, hemocompatibility assessment focuses on blood-biomaterial interactions and their consequences. Additionally, hemocompatibility is a characteristic of a biomaterial defined as "the property of the material to not provoke changes in blood functions, transformation of its components, or formation of thrombus" (19).

Although it is well known that biomaterials can alter blood, understanding the biology behind that is still far from complete (20). This is not surprising when one considers that blood is a tissue formed from millions of cells and large amounts of plasma proteins that may interact with biomaterials. When a biomaterial is exposed to blood, 2 important events occur on the foreign surface: first, protein adsorption and then, cell adhesion. In protein adsorption, the first to adsorb on the surface are the proteins in the highest concentration and smallest in size. Later, larger proteins displace the adsorbed proteins (21). This effect was first observed by Vroman et al ("Vroman effect") when fibrinogen was removed from a glass surface by high molecular weight (HMW) kininogen and coagulation factor XII (22). The Vroman effect governs protein adsorption of biomaterials; it also governs hemocompatibility. In addition to the Vroman effect, the physicochemical properties of biomaterials influence protein adsorption, for instance, hydrophobic or hydrophilic surface, surface energy, electrical charges, and surface topography (23). Protein adsorption always occurs on a material contacting tissue, and it can be undesirable because it may trigger biological cascades such as blood coagulation, complement activation, or fibrinolysis (23).

2.3 PC-cell membrane interaction and its effect on the cell

PCs are attracted to cells because of electrostatic interaction between the cationic groups at the polymer and the anionic domains of the cell membrane (24-26). Consequently, PCs adsorb onto the cell surface, which may induce physicochemical modifications of the plasma membrane. Change of the net electrical charge (27), rearrangement of the phospholipid bilayer (28), increase in permeability (29), and porous formation (30) occur at the cell membrane as a result of PC adsorption. In addition to membrane modification, PCs may also induce internal cellular changes. Perturbations of cytosolic organelles and cellular metabolism have been reported after PC uptake by the cell (31-33). The cell membrane can undergo several strongly correlated PC-induced alterations that presumably occur at the same time or within milliseconds (31, 33). Although it is difficult to determine when one or the other change occurs, clearly the cellular alterations occur in one direction: from the cell surface to the cytoplasm. Therefore, PC-induced effects can be ordered as follows: first, change of net electrical charge; second, rearrangement of the phospholipid bilayer;

third, increment of permeability and porosity. Evidently, internal cellular changes can be placed in the fourth position.

2.3.1 Effect of PCs on zeta potential of cells

Neutralization of negative electric charges on the cell membrane occurs because of PC adsorption. Accordingly, when adsorbed, the PCs induce changes in electrophoretic mobility (EM) or ζ potential value of cells. For instance, change in EM has been observed in Ehrlich ascites tumor cells (EATC) that were incubated with PLL (Mw 120,000). Compared with the EM control (100%), the EM of the PLL-incubated cells was reduced ~80% and ~50% with 10 µg/mL and 20 µg/mL PLL, respectively (29). Evidently, that dose/response reduction can be explained by the increment of cationic groups available to neutralize the anionic sites of the cell membrane (29). The effect of PCs on EM has also been reported by Grandfils et al (27). They observed that the EM of washed RBCs was decreased by spermine (Mw 348) and polyethyleneimine (PEI [Mw 10,000]). At 10 µg/mL, spermine and PEI changed the EM from −2.77 µm.cm/V.s to −1.06 and +1.80, respectively. Dissimilarity between the 2 results, as explained by the authors, was due to the difference between the value of the positive charge and the Mw of the PCs employed (27).

In addition to Mw and polymer concentration, flexibility of the polymer is another factor affecting the cell EM. For example, Singh et al noticed that rigid cationic cytochrome C (Mw 12,400) and lysozyme (Mw 14,300) failed to completely neutralize the glomerular epithelial cells (34). However, the flexible protamine (Mw 7,000) or PLL (Mw 3,500) completely neutralized those cells. Differences of PC behavior occurred because the cytochrome and lysozyme have rigid structures caused by crosslinking of cystine residues. Hence, after chemically breaking the half-cysteine residues, both cytochrome and lysozyme completely neutralized the cells as did the PLL and protamine (34).The role of zeta potential in cells is correlated with morphological stability, transport of molecules, and cell-to-cell electrostatic repulsion forces, among others. Thus, reduction of negative charges could affect cellular conditions. Reversal or neutralization of charges has been correlated

with increased membrane permeability (29, 34) and cell aggregation (1, 35), for instance. Because cell aggregation plays a significant role in hemotoxicity, the effect of reducing the ζ-potential of RBCs is also explained in the paragraphs below (2.4.1.1 Hemagglutination).

2.3.2 Effect of PCs on arrangement of phospholipid molecules

PCs can interact with negatively charged phospholipids in a lipid membrane, which may cause the membrane to undergo rearrangement of its structure. For example, Yaraslavov et al and Yaraslavov and Kabanov have observed PC poly(4-vinylpyridine) (PVP)–induced rearrangement of the phospatidylcholine (PDCH)-cardiolipin (CL) vesicle membrane (36-38). First, the PVP was adsorbed onto the surface of the vesicles, then the cationic groups induced transbilayer migration of the charged lipid molecules from the inner to outer leaflet of the bilayer (39). The PVP also induced migration of lipid molecules from the outer to the inner leaflet of the vesicle (PC-induced flip-flop) (36, 38). Interestingly, the lipids returned to their normal arrangement after desorption of the PVP (36). In addition to evidence obtained from studies of PVP and lipid vesicles, it has been noted that PCs induce externalization of lipid molecules in the cell membrane. In particular, it was observed that PCs cause translocation of phospathydilserine (PS), an anionic phospholipid component of the cell membrane inner leaflet. Moghimi et al (28) reported that PEI (20 µg/mL; Mw 25,000) induced redistribution of PS from the inner plasma membrane to the outer cell surface. In addition, PS translocation was correlated with cell death occurring after 1 h of PEI-cell contact. These authors concluded that binding of PEI molecules to the plasma membrane proteoglycans caused PS translocation with a subsequent membrane destabilization, inducing cell death. This series of events was considered a single phase of toxicity: PEI-mediated phase 1 cytotoxicity (28). Symonds et al have also observed that PLL induced PS translocation with subsequent cell death (40). PS exposition was identified on Jurkat-T cells (JTC), human umbilical endothelial cells (HUVEC), and hepatocyte-like cells incubated with at least 20 µg/mL PLL (Mw 27,400) for 1 h. In addition, all cell lines presented loss of viability correlated with perturbation of the membrane (40). PDMAEMA is another PC that has been seen to induce PS translocation. Jones et al observed that 2 µg/mL PDMAEMA (Mw 22,000) perturbed the cell membrane of human myelomonocytic

cells (hMMCs) (41). After 1 hr incubation, the cells presented PS exposition and cell death via necrosis (41).Thus, in view of the evidence about interaction between PCs and cell membrane phospholipids, it is clear that distinct PCs can induce PS translocation after a brief contact (1 h). Moreover, the exposition of the PS is strongly correlated with loss of membrane integrity and cell death.

2.3.3 Effect of PCs on membrane permeability

PCs can induce cytotoxicity mainly because of their cationic nature that induces cell membrane permeability (31, 32). This fact was clearly demonstrated by Choksakulnimitr et al when they noted that, compared with anionic and neutral polymers, cationic polymers induced a stronger cytotoxic effect (42). The researchers observed that 100 µg/mL protamine (no Mw reported) and PLL (Mw 39,800) induced ~80% cytotoxicity in brain endothelial cells (bECs), whereas neutral 100 µg/mL Dextran (no Mw reported), anionic BSA (Mw 67,000), and anionic Dextran-sulfate (Mw 8,000) induced <10% cytotoxicity in bECs. The cytotoxicity of PCs was correlated with the ability of their charges to membrane integrity (42). Change in cell plasma membrane permeability was identified early as a cytotoxic effect of PCs. In 1973, Mayhew et al demonstrated the correlation between increased membrane permeability and increased percentage of dead cells (EATC cell line) (29). These authors observed that 10 µg/mL PLL (Mw 170,000) promoted significant release of K^+ from the cells (from 0.20 to 0.01 mEq) and caused ~85% dead cells. But 10 µg/mL PLL (Mw 2,800) induced discrete release of K^+ (from 0.20 to 0.12 mEq) and ~10 % dead cells. Evidently, the Mw influenced the noxious behavior of the PLL: the lower the Mw the higher the membrane integrity (29). Nowadays the capability of PCs to induce cell membrane leakage is well known, and it is commonly evidenced by the release of lactate dehydrogenase (rLDH), a cytoplasmic enzyme marker. Accordingly, protamine (no Mw reported) and PLL (Mw 39,800) have induced about 80% rLDH in microvessel endothelial cells (bEC) incubated with 100 µg/mL of 1 PC or another (42).PEI (Mw 800,000) has induced about 70% rLDH in L929 fibroblasts incubated for 1 h with 1,000 µg/mL of the polymer (42),whereas PEI (Mw 25,000) has stimulated about 60 % rLDH from Jurkat T cells, hepatocytes, and HUVECs incubated with 30 µg/mL PEI after 8 h (42). PDMAEMA

is another PC inducing LDH release. PDMAEMA of different Mws (43,000; 112,000; 215,000; 527,000; 915,000) and concentrations (2–50 μg/mL) has shown ability to induct LDH release in human brain microvascular endothelial cells (hBMECs) (12). At 20 μg/mL and above, all these PDMAEMAs cause more than 70% rLDH (12).

Increased membrane permeability is explained by cationic charges on the PCs modifying the characteristics of the cell membrane, consequently affecting its integrity. As mentioned above, a more specific explanation would be on the basis of the PCs' ability to induce PS translocation (28,41). Pore formation is another event that can induce membrane permeability. Hong et al have observed that 2.8–280 μg/mL poly(amidoamine) (PAMAM) (Mw 28,260) induced permeability in human KB epidermal carcinoma cells (hKBEPC) and Rat2 cells (RT2C) (43). Interestingly, leakage of LDH from both hKBEPC and RT2C was correlated with the presence of holes induced by the PAMAM. The pore size was estimated to be from 15 to 40 nm, allowing the release of LDH (135–140 kDa; ~4.3 nm radius). Although LDH was released, it was insignificant (<30%), thus, the membrane presented nanoscale alterations, and it was not cytotoxic (43). Nanopore formation has also been observed in cells contacting PAMAM and PEI. Interestingly, pore formation by both polymers at noncytotoxic concentrations (<6 μg/mL) was a reversible event (30). Chen et al observed that membrane porosity formed on a timescale of 1–100 ms, but the membrane recovered its original state after 5 ± 2 s and 12 ± 4 s for PEI-formed pores and PAMAM-formed pores, respectively (30). The authors explained that the membrane's retarded recovery after contacting PAMAM was due to the PC's stronger interaction with the membrane, increasing membrane stability as the polymer coated and stabilized the pore (30). Evidence of pore formation helps to explain a possible mechanism for PC-induced permeability. But, as noted by Hong et al (43) and Chen et al (44), nanopore formation was not correlated with significant cytotoxicty.

2.3.4 Effect of PCs on cell metabolism

Pore formation and disruption of the cellular membrane are linked to PC uptake by the cell. The latter process can be mediated by at least 2 internalized routes, vesicular internalization (eg, endosomes) and plasma membrane perturbation with internalization (33). After the PCs adsorb onto the cell surface, the polymer may penetrate the cell membrane to reach the cytoplasm. In the case of cationic nanovectors, they release their content into the cytoplasm; hence, the free PC lies in the cell (24, 25). On the other hand, free PCs (ie, not complexed with anionic molecules) can also penetrate the cell membrane to lie in the cell; however, cytoplasmic pathways of free PCs have been poorly investigated, although it is well known that PCs can affect cellular metabolism. In this respect, cell viability is frequently evaluated by measuring metabolic activity with the MTT assay which measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase; hence, the more the MTT is reduced, the lower the cell viability. Accordingly, several PCs have shown the ability of decreasing cell metabolism. Star-shaped PDMAEMAs (Mw 51,000) and branched PEIs (Mw 25,000) have inhibited cell metabolism of Chinese hamster ovary (CHO-K1) cells. Both polymers at a concentration range 250–500 µg/mL reduced cell viability from 100% to 10%. The same concentration range of linear PEI (Mw 25,000) and linear PDMAEMA (Mw 17,000) has reduced cell viability to 90% and 40%, respectively (13). PLL is another PC capable of inhibiting cell viability. In colon carcinoma cells, PLL (Mw 1,509) at 10 and 100 µg/mL reduced cell viability to 80% and 0%, respectively (45). On the other hand, when PLL was added to leukocyte carcinoma cells, the cytotoxic effect was even stronger, causing 0% viability at 10 µg/mL (45).

PC toxicity on cell metabolism cannot be unrelated to its toxic effect on the cell membrane; the former follows the latter, and evidently a strong correlation exists between them, as demonstrated by Fischer et al (2). These authors observed that PEI (600,000), PLL (3,600), and poly (diallyl-dimethyl-ammonium chloride) (DADMAC; Mw 500,000) induced cytotoxicity correlated with membrane damage (>50% rLDH) and decreased metabolic activity (< 50%). Interestingly, release of LDH occurred after 1 h of incubation, but cell

activity decreased after 3 h incubation, showing that the mechanism of cytotoxicity had begun at membrane levels, continuing into the cell (2).

In addition to the ability of PCs to inhibit cell metabolism, they induce specific damage in mitochondria; consequently, PCs can induce cell death by apoptosis. The mitochondrion is the central regulatory element in stress-induced cell death. When a stimulus perturbs the mitochondrion membrane, the proapoptotic protein cytochrome c (Cyt-c) is released from the mitochondrial membrane, cleaving caspase-9. The activated caspase-9 later activates caspase-3, which initiates apoptosis via the mitochondrial pathway (31). This series of events can be activated by PCs. For example, PEI (Mw 25,000) contacting Jurkat T cells formed pores on the mitochondrial membrane, releasing Cyt-c. PEI at 10 and 20 µg/mL induced 20% and 45% caspase activation, respectively (28). That series of events was named "PEI-mediated phase 2 cytotoxicity," which is the phase following PEI-mediated phase 1 cytotoxicity (see paragraphs above) (28). Interestingly, PLL-induced apoptosis preceded events similar to those observed during PEI-mediated phase 1 cytotoxicity (ie, PS translocation and cell membrane permeability). With respect to apoptosis activation, low Mw (LMW) PLL (2,900) and HMW PLL (27,400) also induced apoptosis via the mitochondrial pathway. It was observed that either LMW or HMW PLL interacted directly with the outer mitochondrial membrane, resulting in redistribution of cardiolipin and release of Cyt-c (33). Chitosan is another PC that can trigger apoptosis via mitochondria (46). Chitosan (no Mw reported)-induced apoptosis was identified in human bladder tumor cells that released caspase-3 when contacting 100 µg/mL of the polymer. Interestingly, when a caspase-3 inhibitor was added to the cells contacting chitosan, apoptosis was not completely inhibited as expected. This finding suggested that, in addition to caspase-3 activation, there is another pathway participating in chitosan-induced apoptosis. But it still has not been explored (46).

Polymer	Mw	Concentration (µg/mL)	Cell line	Cytotoxicty	Proteins in culture medium
L- PLL (40)	27,400	10-30	JTC	rLDH % >40	Serum-free medium
L-PLL (2)	3,600	100	L929F	rLDH% ~70 MTT < 10 %	DMEM, 10% fetal calf serum
L- PLL (42)	39,800	100	BrEC	rLDH % ~80	Serum Free Media
L-PLL (45)	NR	10-100	cCC	MTT <80%	Earle's Minimum Essential Medium, 10% v/v fetal calf serum
Br-PEI (28)	25,000	30	JTC, THLE, HUVEC	rLDH > 50%	Serum-free medium
Br-PEI (47)	800,000	1000	L929F	rLDH% ~70	Serum-free medium
Br-PEI (13)	25,000	250-500	CHO-K1	MTT < 10%	Serum-reduced medium
Br-PEI (2)	600,000	100	L929F	rLDH% < 50 and MTT < 5 %	DMEM with 10% fetal calf serum

Table 2.1 Overview of distinct cytotoxicty studies comparing PLL and PEI. L- (Linear), Br- (Branched)Jurkat T cells (JTC), L929 fibroblats (L929F),bBrain entothelial cells (brEC), colon carcinoma (cCC), THLE-3 hepatocytes (THLE), human umbilical vein endothelial cells (HUVECs), Chinese Hamster Ovary cells (CHO-K1). NR: no reported. Dulbecco's modified Eagle's medium (DMEM).

In view of the above, it is quite likely that PCs perturb cells. However, despite PCs' well-known cytotoxic properties, it is difficult to determine precisely which PC is more toxic and which is less, because biocompatibility depends on several factors such as polymer nature, polymer architecture, Mw, polymer concentration, and even in vitro culture conditions. An example of that situation in presented by the Table 2.1; overview shows that PLL and PEI have been studied under different variables and their cytotoxicty response varies from 5 to 80%. Hence, biocompatibility of PCs should be considered a property that depends on particular conditions. This situation should not limit PC use, but should offer opportunities and freedom to generate new polymeric architecture, composition, and sequences to regulate PC-cell reactivity. Moreover, the biocompatibility of PCs has not been widely explored in vivo and it should be realized that in vivo conditions could

decrease the toxic response because of the protective action of anionic molecules (proteins) that might interact with the PCs. Additionally, if in vitro tests require local administration of PCs, their in vivo use should require local administration with a wide distribution of the polymer in the organs or body; thus, the dissemination of PCs should decrease local toxic effects.

2.3.5 PDMAEMA and its effect on cells

PDMAEMA-based PCs have been widely investigated for application as cationic carriers and have emerged as one of the most effective PCs to transfect cells (6-9, 11, 12, 24, 25, 48, 49). However, as does any PC, PDMAEMA may induce cell toxicity. Jones et al (41) have observed that PDMAEMA (Mw 22,000) provoked PS exposition and decreased cell viability (<10%) in hMMCs incubated with 1 µg/mL and 2 µg/mL of the PC, the former concentration and the latter depolarized mitochondria in 20% and 25% of cells, respectively. Because no caspase enzyme or Cyt-c activities were detected, cell death was correlated with alterations in the membrane and mitochondria; also, necrosis was identified as the toxic process occurring (41). Layman et al have also observed cell damage due to PDMAEMA (12). PDMAEMA in distinct Mws (43,000–915,000) and concentrations (2–50 µg/mL) induced over 70% rLDH in hBMECs (12). In addition to PDMAEMA-induced membrane damage, metabolic damage has also been induced by PDMAEMA, as reported by Schalon et al (13).

Although PDMAEMA has induced cytotoxicity, that polymer is considered a gold standard for gene delivery because of not only its efficacy in cell transfection but also because it has shown more biocompatibility than PEI, another gold standard and the most popular PC for cell transfection. Dubruel et al have observed that PDMAEMA (Mw 93,000 and 201,000) polyplexes did not affect cell viability (98%–108%) of COS-1 cells, whereas PEI (Mw 25,000) polyplexes reduced cell viability to 65%–79 % (50). Additionally, PDMAEMA and PEI polyplexes induced <10% and 16%–27% hemolysis, respectively (50). Moreover, Robbens et al have observed that PDMAEMA was also more biocompatible than PEI.

When human hepatocyte cells were incubated with PDMAEMA (Mw 300,000) and PEI (Mw 25,000), the half-maximal inhibitory concentration (IC50) for cell viability was 21 µg/mL for PDMAEMA and 7 µg/mL for PEI (51).

2.4 Hemocompatibility of polycations

As described in the previous section (2.3 PC-cell membrane interaction and its effect on the cell) PCs can produce several effects on certain mammalian cells. The effects of PCs on cells are governed by the interaction between the cationic charges and negatively charged cellular elements, in particular those related to membrane domains. Because PCs can potentially interact with any negatively charged blood element, a description of all possible interactions is beyond the scope of this section. Herein we describe the effect of PCs on the blood elements and biological cascades that were evaluated in our study: RBCs, platelets, blood coagulation, and complement activation.

2.4.1 Red blood cells and polycations

2.4.1.1 Hemagglutination

Thrombosis is defined as an "in vivo phenomenon resulting in the partial or complete occlusion of a vessel or device by a thrombus (52)." Formation of a thrombus is an undesirable event because it may provoke a cerebrovascular accident or stroke—and even death—because of blocking blood circulation (52). Although a thrombus is generally formed by a mechanism related to blood coagulation, it can be also formed by RBCs (52).An RBC thrombus originates from RBC aggregation or hemagglutination, an event that may be induced by PCs. Hemagglutination is a response to the adsorption of cationic polymers on the RBCs. Such adsorption neutralizes the negative charges on the RBCs (27, 29) and hemagglutination occurs (1, 35, 53).The effect of PCs on neutralizing and aggregating RBCs was early observed by Yu and Pearson in 1975 (54). The authors incubated sheep RBCs (SRBCs) with DEAE-dextran (Mw 2,000), polybrene (no Mw

reported), spermine (Mw 348), and PLL (Mw 70,000). After 60 minutes of incubating the RBCs with the PCs at 37°C, all the PCs had induced hemagglutination in a dose-dependent manner. The inhibition of anionic charges was also evaluated in neuraminidase-treated RBCs. Because neuraminidase is an enzyme that removes charges from salicylic acid groups, the neuraminidase-treated RBCs also exhibited hemagglutination. Hence, either with PCs or with neuraminidase, a lowering of the negative charges led to diminished repulsion and facilitated cell adhesion (54). Today it is well known that most PCs induce RBC aggregation and that the aggregativeness of PCs depends not only on the cationic charges but also on factors such as polymer concentration and Mw. Moreau et al (1) and observed that PLL (Mw 24,000 and 124,000) poly(diallydimethylammonium) chloride (PDDAC) (Mw not reported), PDMAEMA (Mw 193,000), and partially quaternized poly[thio-1-(N,N-diethyl-aminomethyl) ethylene] (Q-P(TDAE) (Mw 130,000) provoked hemagglutination in washed RBCs (1). The aggregation strength depended strongly on molecular weight, but when PCs of the same Mw were used in distinct concentrations, the aggregation showed an augmentation of strength correlated with the increment of polymer concentration. Interestingly, the in vitro results obtained by Moreau et al were extrapolated to in vivo observations; all the tested PCs showed induction of toxicity in mice. After injection of the PCs, the mice showed acute toxicity with shock, distress, and convulsive movements—some mice died (1). The maximal nontoxic dose was 10 mg/mL for PLL (Mw 24,000); 5 mg/mL for PLL (Mw 124,000), 4 mg/mL for PDDAC, 2 mg/mL for PDMAEMA, and 1.5 mg/mL for Q-P (TDAE). The authors presumed that the aggregation of RBCs induced blocking of vessels, which was reflected in acute and lethal toxicity (1).

2.4.1.2 Hemolysis

Hemolysis is the “release of Hb from erythrocytes, either by destruction or through a partially damaged but intact cell membrane” (52). This cell damage is one of the most common PC-induced hemotoxic responses. A variety of PCs have shown hemolytic properties. The list includes PLL (1, 2), PAMAM (2), PDMAEMA (50, 55), and PEI (56, 57). Compared with the number of studies exploring the mechanism of cell membrane

damage in mammalian cell lines, the number of studies investigating the mechanism of RBC membrane damage is small. However, the mechanism of PC-induced hemolysis is analogous to the loss of membrane integrity in mammalian cells (2.3 section PC-cell membrane interaction and its effect on the cell). Presumably, hemolysis can be promoted by PS translocation, increased permeability, and formation of nanopores. The PS translocation can be an expected alteration expected because cationic molecules may induce such membrane alteration in RBCs. It has been observed that lead (Pb+) provoked PS exposition in washed at 1–3 μM concentration (58). Hence, considering the cationic nature of PCs and that PCs have induced PS translocation in distinct mammalian cells, it can be expected that PCs stimulate PS exposition on the RBCs; thus, PCs increasing permeable properties of the membrane can induce hemolysis. That correlation has been clearly identified for PEI (Mw 600,000). In the L929 fibroblast, concentrations of 100 μg/mL PEI and 1,000 μg/mL PEI induced ~55% rLDH and ~80% rLDH, respectively. In the other hand, when washed RBCs were incubated with the same PEI at the same concentrations, the lower concentration induced 9.02% ± 0.10% released hemoglobin (rHb) while the higher concentration induced 92.67% ± 1.34% rHb (2). In relation to PC-induced nanopore formation and cell membrane damage, that correlation has been presumed in RBCs' contacting PCs. Sovadinova et al have observed that amphiphilic methacrylate copolymers containing primary ammonium groups (Mw of copolymers: 2,300–2,800) as cationic functionality induced nanopores in washed RBCs (59) leading to colloid-osmotic lysis. Evidence showed that the pores in the RBC membrane were not large enough to allow release of Hb molecules (~6 nm) but the pores allowed the release of the fluorescent dye calcein (~1 nm) to the external phase. Hence, hemolysis occurred because the polymers produced nanosize pores, leading to an influx of small solutes into the cells and causing irreversible rupture or global destabilization of the cell membrane. The other major finding related to the identification of pore formation was heterogeneousness in terms of cell distribution. The researchers interpreted that this finding could be due not only to differing ratios of polymers bound to individual cell membranes, but also to cell-to-cell variations in their sensitivity to pore formation (59). This interesting mechanism could be used to explain PC-induced hemolysis, particularly for linear and low molecular weight PCs such as those employed by Sovadinova et al. Based on the evidence about PC-induced cell

membrane damage and considering that the PC-cytotoxicity mechanism is similar in different cell lines (although they can be different in strength), it can be presumed that PC-induced hemolysis is a consequence of the following events: PS translocation, increased membrane permeability and nanopore formation.

2.4.2 Platelets and polycations

Platelets have as a major function the formation of a platelet plug when the integrity of a blood vessel has been altered. The platelets are anuclear, disc-shaped cells with a diameter of 2–4 μm, numbering from 150,000 to 400,000/mL, and they have a lifespan of 8 to 14 days (60). Platelets present a period of inactivity in normal conditions, but when endothelial damage occurs, they are activated to form a platelet clot collaborating in the stopping of bleeding. Platelets are extremely sensitive to activation not only by endothelial damage but also by biomaterials (23). In addition to activation of platelets, biomaterials can affect the number of those cells by promotion of platelet aggregation; this situation can be stimulated by PCs. Because platelets are negatively charged cells, the PCs can adsorb onto their membrane surface. Hence, PCs can induce platelet aggregation and possibly platelet aggregation which has been reported in the literature. Coller showed that the PC polybrene (no Mw reported) induced aggregation of washed platelets (4). When the platelets were incubated with 2 μg/mL, 4 μg/mL, and 6 μg/mL, the EM (100%) was reduced to ~85%, ~70%, and ~55%, respectively. The effect of EM reduction was correlated with the platelet aggregation because the polymer concentration also increased the aggregation (4). In addition to that biophysical action, PCs activate platelets by other mechanisms. PLL, polybrene, and protamine at 1.5–10 μg/mL (no Mw reported) have provoked platelet aggregation by activating the endogenous mechanism of platelet aggregation (61).Evidence has proven that there was a latent period of aggregation, which depended on the dose of the polymer; moreover, the aggregation process was sensitive to cyanide, an inhibitor of cell respiration that requires calcium ions (61). Furthermore, the aggregation was sensitive to EDTA. No aggregation was observed when the chelating agent EDTA was added to the platelets before the PCs; the aggregation was inhibited because calcium ions mediate the endogenous mechanism of platelet aggregation (61).

2.4.3 Blood coagulation and polycations

PCs can alter hemostasis because of electrostatic interaction between the PCs and clotting factors. This interaction can be expected because some clotting factors have an acidic pI at physiological pH 7.4, for instance, factor VIIa, prothrombin, factor IX, antithrombin III, and factor XII (62). Hence, PCs can perturb steps in the coagulation cascade, causing a delay or inhibition in coagulation. This phenomenon has been already observed in vitro. Chu et al found that protamine (Mw 4,500) delayed prothrombin time because it blocked factor VII (FVII)(63). The control time (11.1 ± 0.3 s) was significantly (<0.05) delayed at 13.2 ± 0.2 s, 15.1 ± 0.2 s, and 16.3 ± 0.04 s with 9, 18, and 36 mg/mL, respectively. That response occurred because protamine interacted with FII and blocked the contact of FVII with the initiator of the extrinsic pathway—the tissue factor (TF) (63). These authors also observed that PEI (Mw 750,000) inhibited extrinsic blood coagulation (64). Prothrombin control time was 12.4 ± 0.3 s, while PEI samples with 6.7 µg/mL and 9 µg/mL showed a prothrombin time of 14.1 ± 0.5 s and 14.4 ± 0.2 s, respectively; these two samples later showed statistically significant differences when compared with control. Chu et al presumed that PEI blocked the interaction between the enzyme thrombin and the substrate fibrinogen; consequently, the prothrombin time was delayed (64). Their report provides evidence that PCs can have detrimental effects on blood coagulation because of the PC–blood factor interaction.

2.4.4 Complement system and polycations

The complement system is a humoral mechanism focusing on identifying and eliminating foreign molecules or bacteria from the body. This defensive system destroys and removes substances either by lysis or by mediated leukocyte function in inflammation and innate immunity. The complement system performs its function by regulating approximately 30 plasma- and membrane-bound proteins (receptors or regulators) (65). The proteins split into 3 pathways: (1) the classical, (2) the lectin, and (3) the alternative. Each pathway is initiated in an independent way; however, they are not distinct, but they overlap. For instance, the alternative pathway amplifies both the classical and lectin pathways. Moreover, they all

converge at the formation of the C3a convertase complex, forming the membrane attack complexes (65). Although the complement system is a major defense line against pathogenic organisms, this defense mechanism can also be activated by biomaterials (66).

Biomaterial-induced complement activation is an undesirable effect that should be avoided because it may provoke rapid clearance of the biomaterial and inflammatory reactions (66). Hence, PCs, as any biomaterial, may activate the complement system (3, 67). Ward et al have demonstrated that PLL (Mw 20,000)-bound C3 that is one of the complement activator proteins. The activated C3 was also detected on the PLL, because C3 binding was inhibited by heat, indicating that the binding was due to activation of complement and not solely to nonspecific binding. Detection of C3 suggested that the complement was activated by the alternative pathway (67). PLL-induced complement activation was also observed by Plank et al (3). The authors evaluated the complement activation with a hemolytic assay without any discrimination between classical and alternative pathways. In that way, the effect of each PLL test on the ability of serum to lyse antibody-sensitized RBCs was evaluated and a CH50 value for that PLL on the serum was obtained by linear regression. Hence, the percentage of $CH50_{max}$ was plotted as a function of the concentration of the positive charge in the assay mixture. This test allowed evaluating the effect of the Mw and concentration of the polymer charges on complement activation. Thus, Plank et al evaluated the ability of distinct PLLs (Mw 1,000, 4,000, 6,000, 25,000, and 50,000) to activate the complement and observed that the strongest complement activation was achieved by the samples incubated with the largest PLL, so that strength of activation was correlated not only with Mw but also with the number of amine groups in each polymer chain (3).

2.5 PDMAEMA based polymers and hemocompatibility

PDMAEMA-based PCs have been widely investigated for applications as cationic carriers and have emerged as one of the most effective PCs to transfect cells (9, 13, 51, 68). Although different cytotoxicity effects of PDMAEMA have been reported in the literature, surprisingly the toxic effect of PDMAEMA on blood has been studied in only a limited number of tests, focusing mostly on hemagglutination and hemolysis. Moreover, those

studies have been limited to evaluation of PDMAEMA as polyplexes but not as free polymers (Table 2.2). To better highlight the lack of information on PDMAEMA hemocompatibility, we have summarized the main conditions that have been considered in toxicological studies (see Table 2.2).

Authors	PDMAEMA evaluated on the study	Sample	Test
Lin et al 2008 (55).	PDMAEMA (Mw 24,000) polyplexes PEG-b-PDMAEMA (Mw 19,000) polyplexes PEG-b-PDMAEMA (Mw 21,000) polyplexes	Washed Human RBC	RBC aggregation Hemolysis test
Yancheva et al 2007 (62).	PDMAEMA (Mw 24,000) polyplexes Quaternized (Q) PDMAEMA Mw 35,000 Quaternized (Q) PDMAEMA Mw 45,000 QPDMAEMA-*N*-carboxyethylchitosan (CECh)	Whole blood	Coagulation tests
Pirotton et al 2004 (68).	PDMAEMA (Mw 37,500) polyplexes P(DMAEMA-b-MAPEG) (Mw 24,100) polyplexes	Rat blood Human blood	RBC and platelet counting Hemolysis and RBC aggregation
Verbaan et al 2004 (48).	PDMAEMA (Mw 7700) polyplexes PDMAEMA (Mw 7700)-b-PEG polyplexes PDMAEMA (Mw 1927) PDMAEMA (Mw 1927)-b-PEG polyplexes	Washed Mice RBC	RBC aggregation
Dubruel et al 2003 (50).	PDMAEMA (Mw 93,000; 110,000; 166,000; 201,000) polyplexes	Washed Bovine RBC	Hemolysis test
Verbaan et al 2003 (69).	PDMAEMA (Mw 160,000) polyplexes	Washed Mice RBC	RBC aggregation
Moreau et al 2002 (1).	PDMAEMA (Mw 130,000)	Washed Human RBC	RBC aggregation Hemolysis
Verbaan et al 2001 (70).	PDMAEMA based polyplexes. No information about the Mw	Washed Mice RBC and plasma	RBC aggregation

Table 2.2 Overview of the literature related to the hemocompatibility of PDMAEMA

Most studies have evaluated the hemocompatibility of PDMAEMA-based polyplexes but not the hemocompatibility of free-form PDMAEMA (noncomplexed). However,

bioreactivity of these 2 entities should differ. Variations in the toxicology of complex and noncomplex PCs have already been reported (8, 31, 32). Hence, the use of free-form PDMAEMA allows us to evaluate and understand the interaction between the above-named polymer and blood elements.

When evaluated, free-form PDMAEMA hemocompatibility tests have been limited to only one polymer. To date, only 2 studies from the same research team have reported on the hemocompatibility of free-form PDMAEMA (Mw 130,000) (1, 53). These studies only evaluated hemolysis and RBC aggregation. Hence, a comparison of hemocompatibility for distinct polymers is important in understanding the influence of the physicochemical properties of PDMAEMA on blood.

Most hemocompatibility tests have been done on washed RBCs but not on whole blood (see table 2.2). Washed RBC suspensions represent an ideal medium in which to evaluate the specific interaction of RBCs with PDMAEMA. However, in whole blood there is a large presence of cells and plasma proteins that can interact with PCs. This interaction will evidently affect the behavior of PDMAEMA on blood. Thus, evaluation of hemocompatibility on whole blood can better mimic in vivo responses after IV administration.

Red blood cell aggregation and hemolysis are the most common tests carried out in hemocompatibility studies of PDMAEMA (see Table 2.2). To date, no study has evaluated the ability of PDMAEMA to induce or inhibit the complement system. Additionally, information about the effects on blood coagulation is poorly described in the literature. Furthermore, there is scarce information on the action of this PC on human platelets. Hence, blood coagulation, platelet behavior, and complement activation as major components of blood reactivity should be also considered for the screening of hemocompatibility. A review of the literature shows that PDMAEMA hemocompatibility is still a point that should be investigated in depth. It also highlights the major point of our investigation and future in vivo applications of PDMAEMA-treated RBCs.

2.6 Scientific method for the hemocompatibility study of PDMAEMA based polymers

2.6.1 Aim of the study

In an effort to reinforce our knowledge of the hemocompatibility of the free form of PDMAEMA, we undertook the present study to evaluate the hemoreactivity of 3 distinct PDMAEMA homopolymers varying in Mw between 10,000 and 40,000. A copolymer with a P (DMAEMA-co-methacrylate) end functionalized polyethylene glycol macromonomer (MAPEG) palm tree–like structure was also evaluated. PEI was used as a positive reference to compare its hemoreactivity. Hemagglutination, hemolysis, platelet number, blood coagulation, and the complement systems were assessed in normal human whole blood according to ISO 10993-4.

2.6.2 Hypotheses

H_1: Hemagglutination increases in relation to the increment of polymer concentration, molecular weight, and incubation time.
$H_{2:}$ PDMAEMA-induced hemolysis increases in relation to the increment of polymer concentration, molecular weight, and incubation time.
H_3: The percentage of blood clot formation and platelet number decreases in relation to the increment of PDMAEMA concentration.
H_4: The percentage of complement activation increases in relation to the increment of PDAMEMA concentration.

2.6.3 Rationale behind the selection of the techniques used to assess the hemocompatibility of the PDMAEMA based polymers. The ISO 10993- 4: Biological evaluation of medical devices-Part 4, selection of tests for interactions with blood.

ISO 10993-4. Biological evaluation of medical devices. Part 4: Selection of tests for interactions with blood is the recommended guide for evaluating hemocompatibility (52).This booklet describes 3 different approaches for hemocompatibility testing: (1) in vivo, (2) ex vivo, and (3) in vitro *(52).* In vivo tests focus on biological responses involving

several factors and complex responses that are not easily reproduced in vitro. Ex vivo tests are performed when the intended use of the device is ex vivo, for example, external communicating devices. In vitro experiments allow a detailed examination of specific factors involving hemocompatibility. In order to perform any of these 3 approaches, the ISO 10993-4 (52) classifies into 5 categories the methods for testing hemocompatibility: (1) thrombosis, (2) coagulation, (3) platelets, (4) hematology, and (5) immunology. Ideally, these 5 categories should be evaluated when a biomaterial intended to contact blood (71).

The hemocompatibility tests were selected on the basis of the 5 categories recommended for testing biomaterial hemocompatibility. Below we briefly describe and justify the specific techniques that have been optimized and used for the framework of this study. All the tests were based on typical methods for evaluating hemocompatibility; however; it is essential to remark that all methods were adapted and standardized in order to adapt the test to our requirements and get reliable results.

2.6.3.1 Thrombosis: evaluation of hemagglutination

Hemagglutination was evaluated by adopting a method based on observation by optical microscopy. We adapted an evaluation score based on a classical cross-score with a guide describing the aggregation signs as reported by Straton and Renton (72) (Table 2.3). We do not use the smear method in preparing samples because we noticed that the tangential stress applied during smear preparation promotes dissociation of cell aggregates, giving false negative results. On the other hand, we observed that, using a cover slide on a sample dropped on a glass slide, the normal local pressure caused by the cover slide was enough to significantly alter the cell aggregates, giving rise to a false positive. Hence, (a) we adapted the conditions of sample preparation, and (b) we adapted evaluation of the hemagglutination by combining image data analysis and the cross-score. We observed our samples by dropping aliquots of them into phosphate-buffered sodium. This technique offers the advantage of observing the samples in suspension without inducing any mechanical solicitation. Additionally, it allows observing either single cells or aggregate bodies as individual entities. It facilitates the scoring of the samples. The main drawback of

our method is cell sedimentation, which occurs within 1 minute after dropping the sample, thus, it is necessary to image the samples in a short time. Details of the methodological steps are described in the hemocompatibility assessment section (2.7 sections).

Score	Description
-	Single cells, any aggregation
+ R	Few single cells, presence of cell rolls and small aggregation bodies and presence of roleaux (R).
+ +	Few single cells and medium aggregation bodies
+ + +	No cells and medium aggregation bodies
+ + + +	Large aggregation bodies
+ + + + +	Large aggregation body filling visual field

Table 2.3 Score to evaluate hemagglutination

2.6.3.2 Hemolysis: cyanmethemaglobin method (CyanMHb-m)

We used (CyanMHb-m) to evaluate the hemolytic properties of PDMAEMA. The method is based on the oxidation of Hb and formation of hemoglobin-cyanide (HbCN), which has a broad absorption maximum at 540 nm (73). The CyanMhb-m measures the material-induced hemolysis that is reported as a percentage of Hb released into the supernatant normalized by the total Hb available at the beginning of the test ([free Hb concentration/total Hb concentration] x 100) (73). To reach the total Hb concentration, a lysing reagent is used to release Hb from the RBCs; the reagent also decreases the turbidity (source of interference and false absorbance) from protein precipitation while a HiCN standard solution is used to perform a calibration curve to get the right measure (73).

The CyanMHb-m selected was the *Standard Practice for Assessment of Hemolytic Properties of Materials* (ASTM designation F 756-00) (73). This standard recommends using rabbit blood, but we modified it by using human blood, which was more in accord

with our use of polymers. The advantages of CyanMHb-m are easy preparation, the availability of a primary reference standard (HiCN), and the use of a lysing agent (73). In addition, the released Hb is easily measured by absorption; the broad absorption band of HiCN allows using simple filter-type photometers as well as narrow band spectrophotometers. A disadvantage is that the cyanide solutions are toxic if inhaled or ingested. In our case, the procedure was performed under strict controls of work safety, and all experiments were performed under air extraction.

2.6.3.3 Platelet: Platelet counting by a Coulter Multisizer II

Platelet counting was performed by a Coulter Multisizer II. Counting by Coulter is performed when cells pass through an aperture in a glass tip and the system detects a sudden and momentary increase in resistance (a partial interruption of the current flow). The formed current is monitored for irregularities by an oscilloscope-type device and the data are digitized in a computer as particles. One drawback of the Coulter lies in the fact that this system counts particles but not cells. It means that the equipment does not differentiate a cell from cell debris or cell aggregates; in all cases, the system counts particles within a specific voltage window preselected by the user. Blocking of the aperture in the tip can also occur if large cell aggregates are present (74). Another disadvantage of this methodology is the need to separate the platelets from the RBCs by centrifugation. The procedure allows isolation of the rich platelet fraction, but it can eliminate platelet aggregates that were possibly generated by the biomaterial.

For platelet counting, the blood is typically anticoagulated with EDTA. Although this anticoagulant is well suited to count blood cells, it is well known that EDTA impairs platelet aggregation and activation. Hence, we used citrate as the anticoagulant; in this way, the functionality and possible aggregation of the platelets was kept. However, different observations performed at CEIB have revealed that the use of citrate could be a major source of difficulties when performing platelet counting (including when using more automated equipment, such as Cell-Dyn Emerald (Abbott Diagnostics, Louvain-la-Neuve, Belgium) (75). Although details of these observations are beyond the scope of this PhD

work, this information should be kept in mind in any assessment of a hemocompatibility study.

2.6.3.4 Blood coagulation: Automated Blood Coagulation Timer (BCT) to evaluate Prothrombin time and Activated Partial Thromboplastin Tisular

Blood or plasma coagulation tests are in vitro reproductions of the events resulting in fibrin polymer formation, with or without the presence of platelets. Hence, the tests measuring fibrin formation are suitable indicators to have a valuable general overview of blood coagulation (52). In our laboratory we have adapted the Dade Behring BCT to test the effect of polymeric-based biomaterials on blood coagulation. Two major tests have been standardized to evaluate the extrinsic and intrinsic pathways, prothrombin time (PT) and activated partial thromboplastin time (APPT), respectively

PT evaluated the extrinsic pathway (tissue factor activated). The test starts when 1 part citrated plasma is added to 2 parts calcium chloride/thromboplastin suspension, and a consequent fibrin polymerization marks the endpoint of the test. For the PT test performed in the BCT, the formation of fibrin is detected by a change in the optical density of the sample. In the test, the tested plasma is compared with laboratory-prepared or commercial-control plasma; thus the ratio of the clotting time of test plasma to the control plasma gives an estimate of the efficiency of the clotting factor function in the extrinsic pathway. The rationale of using the PT for biomaterials is based on the fact that materials may decrease the activity of a component of the extrinsic or common pathway affecting the PT (76). The APPT evaluates the intrinsic pathway**.** Citrated platelet poor plasma is exposed to recalcified platelet membrane substituted. Two parts test plasma is mixed with 2 parts activator/phospholipid emulsion and incubated at for 3 minutes at 37°C. A plasma control is compared with the test plasma. Later, 2 parts $CaCl_2$ are added to the previous mixture and the timing starts. It is stopped when fibrin polymerization is detected and measured by the BCT. The rationale of using the APPT for biomaterials is based on the fact that materials may decrease the activity of a component of the intrinsic pathway affecting the APTT (77).

2.6.3.5 Immunology: complement activation evaluation by Human C3a ELISA

C3a detection is one of the most common tests used; it can be performed in the manner of an enzyme-linked immunosorbent assay (ELISA). Either in plasma or serum, the C3a anaphylatoxin is cleaved to the C3a-desArg form by the serum carboxypeptidase-N enzyme. Thus, the C3a-desArg gives a reliable measurement of the level of complement activation (78). In our experiment, we used a commercial kit to measure C3a-desArg (BD OptEIA). The principle of the test, as described by the manufacturer, is a solid phase sandwich ELISA. It is performed with a monoclonal antibody specific for human C3a-desArg coated on a 96-well plate. The wells are washed and then a mixture of biotinylated polyclonal antihuman C3a antibody and streptavidin-horseradish peroxidase is added. This mix produces an antibody-antigen-antibody "sandwich." Later, the wells are again washed and a substrate solution is added to produce a blue color in direct proportion to the amount of C3a-desArg present in the initial sample. Finally, a stop solution changes the blue color to yellow and it is spectrophotometrically read at 450 nm (78). This test has been used in our laboratory, showing reliable results; the disadvantage is that it is very sensitive to contamination or natural light.

2.7 Hemocompatibility assessment of poly(2-dimethylamino ethylmethacrylate) (PDMAEMA)-based polymers

This section describes the experimental hemocompatibility assessment of 4 PDMAEMA based polymers and a PEI. The materials and method, results, and discussion points are presented using the structure as the article published by Journal of Controlled Release. Introduction has been omitted taking into account that an extensive introduction has been already described.

2.7.1 Materials and methods

2.7.1.1 Materials

Two-(dimethylamino)ethyl methacrylate (DMAEMA) and poly(ethylene glycol) α-methoxy, ω-methacrylate (MAPEG), Drabkin's reagent (cyanmethemoglobin), Brij 35, and bovine hemoglobin were purchased from Sigma-Aldrich (Bornem, Belgium). Branched PEI, Mw 10,000, was purchased from Polysciences Europe (Eppelheim, Germany). Human C3a ELISA Kit for quantification of Human C3a-des-Arg was purchased from Becton Dickinson (Erembodegem, Belgium). Thromborel® S (Human thromboplastin, containing calcium for prothrombin time) was acquired from Dade Behring (Marburg, Germany). STA®-C.K. Prest® 2 (kaolin, for determination of the activated partial thromboplastin time APTT) was purchased from Diagnostica Stago (Asnières sur Seine, France). All other chemicals and reagents used were of analytical grade. Phosphate buffered saline (PBS), pH 7.4, the final composition, was prepared with KH_2PO_4, 1.4 mM; Na_2HPO_4 ,10 mM; NaCl, 137 mM; and KCl, 2.7 mM.

2.7.1.2 Synthesis and characterization of PDMAEMA

PDMAEMA and P(DMAEMA-b-MAPEG) were synthesized by solvent-free, atom-transfer radical polymerization (ATRP) (68) After polymerization, the polymers were purified in 3 successive steps consisting of chromatography realized on alumina support, precipitation in heptane, and dialysis against MilliQ (18 MΩ.cm) water, employing a cellulose membrane (cut off at 10,000). The purified polymers were dried by lyophilization. Relative average Mw (Mn and Mw) was determined by size exclusion chromatography in THF/triethylamine (TEA) (2.5 %) against polystyrene standards. The molar fraction in MAPEG was determined by ^{1}H NMR spectroscopy in CDCl3. Absolute molecular weight and polydispersity of the PEI has been analyzed by SEC-MALS according to Jiang et al. considering a dn/dc of 0.210 dL/g (79).

2.7.1.3 Blood sample collection

Human blood was obtained from the Red Cross Transfusion, Central Hospital, The University of Liège. Blood was collected from healthy donors in 4.5-mL tubes containing 3.2% sodium citrate. Experiments were done within 2 hours after collection. This study received the approval of the Ethics Committee of the Medical Faculty of Liège.

2.7.1.4 RBCs aggregation

Briefly, in micro Eppendorf tubes (200 µL), 1 volume of polymer solution was diluted in 9 volumes of whole blood. In view to assure a rapid and reproducible homogenization of the polycation solution in whole blood we adopted the following detailed procedure. One volume of the polycation solution was transferred within an Eppendorf tube. Nine volumes of whole blood were injected rapidly (less than 1 sec) within the polycation solution with a micropipette. Immediately after, the mixture was homogenized by 3 up-and-down aspirations. Samples were incubated for 15, 60, 120, and 240 minutes at 37°C under horizontal roller mixing (35 rpm). After each incubation time, 10 µL of each sample was diluted in 990 µL of PBS in 1.5 mL-Eppendorf tubes. From this suspension, 40 µL was dropped into a 96-well multiplate. Samples were immediately imaged with an inverted microscope (Inverso-TC, CETI, Kontich-Antwerpen, BE) at ×25 magnification. Three images of each sample were acquired with a digital camera (VisiCam 5.0, VWR, Leuven, BE) for scoring according to size of cell aggregates. A cross score was adapted from Straton and Renton (72). Two independent experiments were performed to support our results. Two distinct polymers batches were used to perform the tests.

2.7.1.5 Hemolysis test

The hemolysis test was adapted from Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM designation: F 756-00 (73). Polymer solutions and blood were prepared and incubated as described in section 2.7.1.4. After incubation, the samples

were centrifuged for 5 minutes at 600 g at room temperature (RT). Supernatants were collected and mixed with cyanmethemoglobin reagent. The released hemoglobin was measured at 540 nm in a microplate reader (Anthos HT III, type 12600, Anthos, Salzburg, AU). A calibration curve was established using bovine hemoglobin as the standard. Saponine (0.8 mg/mL) and PBS were used as positive and negative controls, respectively. Total hemoglobin released from whole blood diluted in the cyanmethemoglobin reagent was determined as 100% hemoglobin release. Hemolysis was expressed as the percentage of hemoglobin released (% rHb) to total content. Tests were done in triplicate. Three distinct experiments with distinct blood donor samples were performed to support our data. In view to assess the influence of plasma protein on the hemolytic action of the polycations, this test was also realized according to the same procedure but using washed red blood cells (a 45% RBC's suspension) instead whole blood. Two independent experiments were performed to support our results. Two distinct polymers batches were used to perform the tests.

2.7.1.6 Platelet counting

One volume of polymer solution was diluted in 9 volumes of whole blood in 1.5-mL Eppendorf tubes. Samples were incubated at 37°C for 15 minutes under lateral agitation at 250 rpm. After incubation, 125 µL of blood incubated with the polycation was diluted in 3,000 µL of Isoton II. This suspension was centrifuged at 850 rpm for 90 seconds at RT to obtain platelet-rich plasma (PRP). The supernatant was diluted in 40 mL Isoton II in a 50-mL Falcon tube. Platelet size distribution and counting were determined with a Coulter Multisizer II, assigning threshold limits between 1.0 and 3.7 µm. Samples incubated with PBS (pH 7.4) were used as negative control. All tests were done in duplicate. Two independent experiments were performed to support our results. Two distinct polymers batches were used to perform the tests. A One-Way ANOVA and Tukey-Kramer HSD were performed to identify possible significant statistical differences between concentrations.

2.7.1.7 Evaluation of blood coagulation: extrinsic and intrinsic pathways

Whole blood and polycation solutions were mixed and incubated as described in section 2.7.1.4. Samples were centrifuged for 5 minutes at 2,000 g at RT. After supernatant collection, prothrombin time (PT) to evaluate Extrinsic Pathway (EP) and activated partial thromboplastin time (APTT) to evaluate Intrinsic Pathway (IP) were measured directly with a Behring Coagulation Timer analyzer (BCT) (Dade Behring, Deerfield, IL, USA). In both tests plasma samples are first recalcified to reverse the effect of citrate anticoagulant and then supplied with the specific activators of coagulation. Then, time of clot formation is measured. Clotting ability of the control plasma is normalized to be 100%. The longer it takes plasma to clot, the lower is its clotting ability and the lower is the resulting test value expressed in % to the control plasma. Kaolin reagent was used as a positive control and PBS as negative control. Measurements were done in duplicate. Two independent experiments were performed to support our results. Two distinct polymers batches were used to perform the tests. A One-Way ANOVA and Tukey-Kramer HSD were performed to identify possible significant statistical differences between concentrations.

2.7.1.8 Complement activation

Whole blood and polycation solution were mixed and incubated as described in 2.6. After incubation, ethylenediaminetetraacetic acid (EDTA) (1 mM final) was added to stop any complement activation. Samples were centrifuged for 5 minutes at 2,000 g at RT. Supernatants were stored at –80°C for future analysis. Complement activation was estimated using the Human C3a ELISA kit for quantification of Human C3a-desArg (Becton Dickinson). Absorbance was measured at 450 nm with a microplate reader (Anthos HT III, type 12600). Concentration of C3a was expressed in ng/mL and as a percentage of activation of the blood control incubated and treated in the same manner. Zymosan was included as the positive control. Measurements were done in duplicate. Two independent experiments were performed to support our results. Two distinct polymers batches were used to perform the tests. A One-Way ANOVA and Tukey-Kramer HSD were performed to identify possible significant statistical differences between concentrations.

2.7.2 Results and discussion

2.7.2.1 Synthesis and characterization of PDMAEMA

Table 2.4 shows the characteristics adopted for the PDMAEMA-based polymers synthesized as for the commercial PEI. To simplify their use, the linear PDMAEMA homopolymers were named P1, P2, and P3, while the palm tree PDMAEMA-b-MAPEG copolymer and PEI were coded P4 and P5, respectively. It should be stressed that ATRP was particularly advantageous to assess the toxicological properties of well-defined PDMAEMA-based polymers with well-controlled molecular length and architecture. According to the procedure reported by Pirotton et al (68), we were able to synthesize the PDMAEMA of a LMW polydispersity and a block copolymer such as PDMAEMA-b-MAPEG. Control of the molecular features of these macromolecules is particularly important because their hemoreactivity can be significantly affected by their Mw, according to their polydispersity. To avoid any side reactivity of the blood with monomer, catalyst, or initiator residues, special attention was paid to extensively purify these polymers, having been submitted to 3 successive and complementary extraction steps. After purification, Cu residue, determined by ICP/MS was founded 158 +/- 17 ppm. Monomer residue, estimated from ^{1}H.NMR analysis, was below 0.1%.

Polymer	Name	Mw	PDI
PDMAEMA	P1	10,000	1.19
PDMAEMA	P2	26,400	1.17
PDMAEMA	P3	40,000	1.43
PDMAEMA-b-MAPEO	P4	30,700	1.18
PEI	P5	12,300	1.51

Table 2.4 Codes, Mw, and polydispersity index (PDI) of the polymers used in the experiment

2.7.2.2 RBC aggregation

RBC aggregation is a highly undesirable phenomenon that can induce serious circulatory disorders, even lethal toxicity (1). Thus, absence of hemagglutination should be considered a main criterion for blood compatibility. In our experiment, a particular effort was made to promote a rapid homogenization of the polycation solution with the RBC suspension to prevent their high local concentration. Accordingly, the procedure of mixing the polycation solution with the RBC suspension has been standardized, that is, the order of reagent addition, volume ratio of polycation solution to RBC suspension, concentration of the stock polycation solutions, rate of addition, mixing conditions during and after polycation solution, and mode and geometry of polycation addition. All these factors can affect the total surface of RBCs accessible to the polycations by controlling the initial and local concentration of free polycations.

The hypothesis proposed was supported by our results; the hemagglutination incremented in relation to the increment of polymer concentration, Mw and incubation time. Figure 2.8 includes representative images of RBC aggregates observed in the presence of some of the PDMAEMA. These observations have been quantified according to the score proposed by Straton and Renton (72) (Fig. 2.2, Table 2.5). These data clearly validate that the Mw, polymer concentration, incubation time, and structure of polycations strongly influenced RBC aggregation. The influence of Mw is clearly shown by the evolution of the P1, P2, and P3 scores. It is worthwhile to stress that whatever the time and concentration, no RBC aggregates were detected in the presence of the lowest molecular P1. By comparison, an aggregation of RBCs was observed at 200 and 500 µg/mL for polycations P3 and P2, respectively. Interestingly, if cell aggregation proceeds relatively quickly, that is, after the first 15 minutes of incubation with the polycation solution, an evolution in the extent of hemagglutination is observed after 4 hours of incubation.

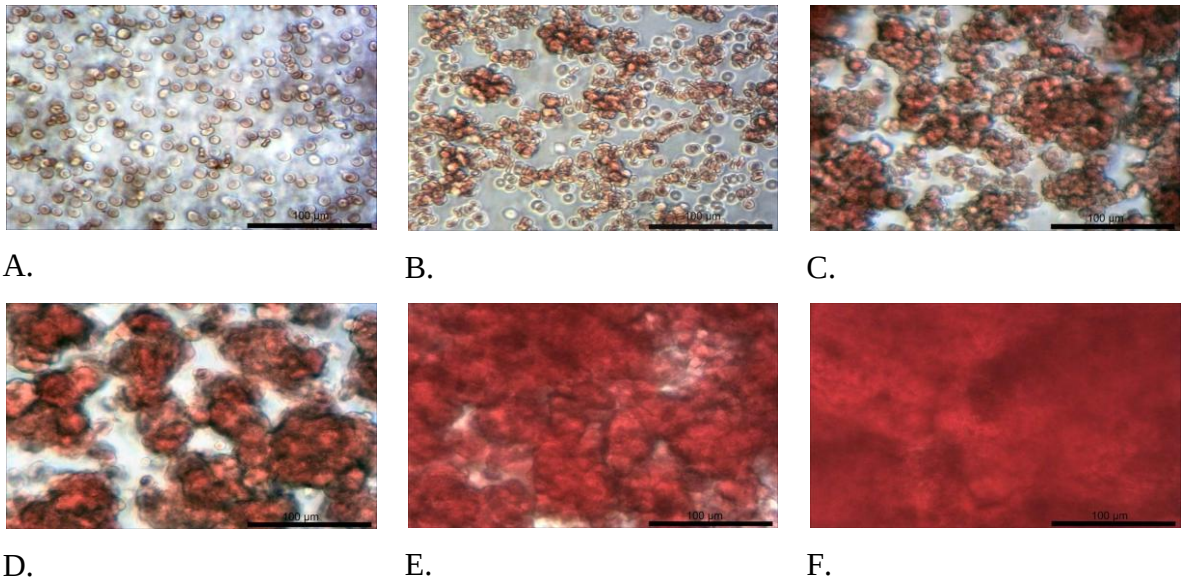

Fig. 2.2. Micrographies (25 x, scale bar 100 µm) representative of the hemagglutination scores. A. Score (-) Negative control (PBS) ; B. Score (+ R) Few single cells, presence of cell rolls (R) and small aggregation bodies (P3 200 µg/mL - 15 minutes); C. Score (++). Few single cells and medium aggregation bodies (P2 500 µg/mL - 15 minutes); D. (+++) No cells and medium aggregation bodies (P2 1000 µg/mL - 15 minutes) ; E. (++++) Large aggregation bodies (P3 1000 µg/mL - 15 minutes) ; and F. (++++++) Large aggregation body filling visual field (P3 1000 µg/mL - 240 minutes).

	15 minutes					60 minutes					120 minutes					240 minutes				
	µg/mL					µg/mL					µg/mL					µg/mL				
	10	100	200	500	1000	10	100	200	500	1000	10	100	200	500	1000	10	100	200	500	1000
P1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P2	-	-	-	++	+++	-	-	-	++	+++	-	-	-	++	++++	-	-	-	++++	++++
P3	-	-	+R	+++	++++	-	-	+R	+++	++++	-	-	+R	+++	++++	-	-	+++	++++	+++++
P4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+R

Table 2.5 Aggregation score for the polymers evaluated.

This evolution in aggregability of RBC's having the Mw and concentration of polycation clearly verifies the physico-mechanical mechanism(s) responsible for erythrocyte aggregation. Our observations support the assumption that aggregation of RBC's requires at least the combination of 2 physico-chemical mechanisms. The first relies on a decrease of the Zeta potential of the cells by neutralization of the negative charges present on the glycocalyx from the plasma membrane surface (1). The second, which is facilitated by the first event with a decrease in electrical repulsion forces, depends on a physical bridging of

RBCs through macromolecule chains physically attached to adjacent cells (80). Charge neutralization is well known to increase with an increment in polymer concentration (1, 29). Based on the Mw dependence highlighted in this study, it should be noted that the critical length needed to promote the physical cross-linking between erythrocytes should be within the nanometer size scale, taking into account the relatively small difference in Mw needed to be efficient (10,000 vs 26,400). Although longer molecular lengths are typically reported to promote bridging coagulation in latex coagulation (81), our results can be explained by the fact that these studies are carried out in a physiological saline medium which therefore decreases the Debye length involved in electrostatic repulsion. Additionally, the surface charge neutralization afforded by the polycations reduces the Zeta potential of the RBCs and therefore facilitates this hemagglutination process. The increase in hemagglutination with time of incubation also supports the mechanism of cell bridging since it can be anticipated that the cell networking process is the result of at least 2 events: polycation adsorption on the cell membrane and cell-to-cell cross-linking. It has been reported elsewhere that to proceed within a time scale of some minutes (27), cell bridging after adsorption on the cell membrane could proceed only after the polymer chain is rearranged to favor cell-to-cell interaction.

It is also of interest to point out the relatively high PDMAEMA concentration required to induce hemagglutination. Based on the assumption of a quantitative adsorption of the polycation molecules on the RBC membrane surface and roughly estimating that the surface covered by polycation molecules of Mw 20,000 should be ~75 nm^2, it follows that a theoretical polycation concentration of 75 µg/mL of whole blood should be required to cover all the RBC surfaces. This discrepancy can be explained by a partial neutralization of the polycations through their interaction with plasma proteins. Albumin and other plasma proteins with an acidic isoelectric point may indeed interact with polycations leading therefore to a competitive interplay between plasma proteins and RBCs (1, 35, 53). This protective effect afforded by plasma proteins against RBC aggregation and reported elsewhere for other polycations (1), does not overcome the action of higher Mw PDMAEMA homopolymers above a concentration threshold between 250 and 500 µg/mL.

The difference in the hemoreactivity of P4 is also of interest. Despite its higher Mw (ie, 30,700), no aggregation was observed, whatever the duration and polycation concentration assessed. This lack of hemoreactivity can be explained by the steric protection afforded by the PEG moieties. It can be anticipated that, if orientated correctly toward the surface, the PEG chains should reduce the cross-linking action mediated by the polycation segments. Our data correspond, therefore, to the results reported by other authors dealing either with other polycations or with polyelectrolyte complexes (55, 68, 82, 83)

If charge neutralization and cell bridging can be expected from the interaction of RBCs and polycations, whatever their chemical nature, other mechanisms could also be involved in the interrelationship of these polymers with cells. It was therefore relevant to compare the results of our study with other polycation studies. For this propose, we also included a PEI with a Mw of 10,000 and a branched structure. Except for a weak aggregation observed at the highest concentration assesed (1,000 µg/mL 240 minutes after incubation), no other macro- or microscopic changes were detected in the other experimental conditions. Although it has been reported that PEI induces hemagglutination (84, 85), all the studies were done using a higher Mw PEI, that is, 25,000 to 800,000. If PEI behaves in the same way as PDMAEMA, the lack of aggregability of the PEI assessed in our study can be explained by its insufficient length to support cell bridging.

2.7.2.3 Hemolytic properties

Having demonstrated the aggregability of RBC's by PDMAEMA homopolymers, we were interested in evaluating the extent of cell damage due to changes in membrane permeability or in membrane fusion. For this purpose, hemolytic properties were evaluated using the ISO Standard Practice for Assessment of Hemolytic Properties of Materials (52, 73). According to this test, the hemolytic properties of biomaterials were classified as a percentage of hemoglobin (% rHb) after incubating whole blood with a biomaterial. Materials were classed as nonhemolytic, slightly hemolytic, or hemolytic when rHb was 0%–2 %, 2%–5, or >5 %, respectively. Based on this classification, as highlighted in Fig.

2.3, all the PDMAEMA-based polymers were non-hemolytic whatever their concentration or Mw and irrespective of the duration of incubation time. Based on this result, the hypothesis for this experiment was rejected because PDMEMA-induced hemolysis was not influenced by the variables applied (polymer concentration, Mw and incubation time). These findings differ from the toxicological data reported earlier by Moreau et al (1) and Dubruel et al (50). These later authors demonstrated that PDMAEMA could induce a lysis of erythrocytes, depending on the Mw, concentration, and incubation time. However, the experimental conditions employed by these researchers differ from those of our study mainly by the fact that their hemolytic studies were realized in a saline buffer with washed RBCs and not in whole blood (50). The difference in hemoreactivity might well be explained by the protection afforded by the plasma proteins that bind polycations and form polyelectrolyte complexes with a reduced affinity for the RBC's (53). In order to support this explanation, we performed a hemolysis test incubating RBC's previously washed in PBS with the PDMAEMA solutions. The results from this test (Figure 2.4) demonstrated that in saline medium PDMAEMA induced up to 6 times more hemolysis in washed RBC's than in whole blood and this independently of the Mw and composition of this polycation. These data therefore clearly support the protective action afforded by the plasma proteins through their charge neutralization properties.

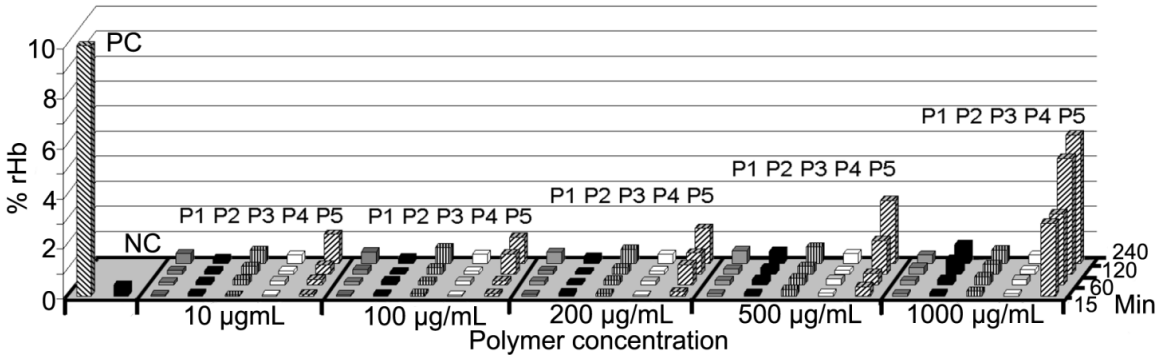

Fig 2.3 Evolution of the percentage of released hemoglobin (% rHb) after whole blood incubation vs polycation nature and concentration. Results express the average of 2 independent experiments.

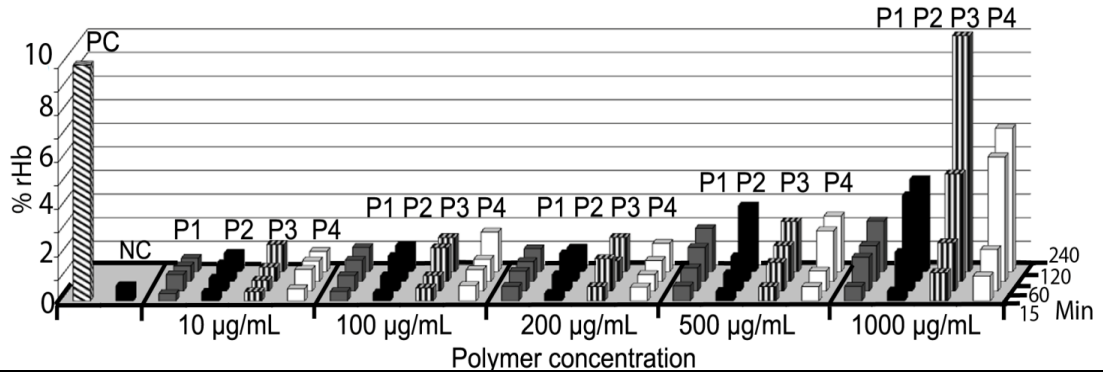

Fig 2.4 Evolution of the percentage of released hemoglobin (% rHb) after incubation with washed RBCs vs PDMAEMA nature and concentration. Results express the average of 2 independent experiments.

As we mentioned in the previous section, even in the presence of the strongest aggregation score (P3+++++), no hemolysis was observed. This variation in hemoreactivity could be explained by the fact that cell aggregation involved only the external layer of the RBC membrane, while the induction of hemolysis should disturb the inner part of the membrane structures sufficiently to affect cell permeability (1). In this respect, comparing the hemolytic properties of PDMAEMAs and PEIs is of major interest. As Figure 2 illustrates, PEI induced hemolysis in a concentration-and-time-dependent way. This hemolytic behavior of PEI has already been reported by Fischer et al (2). Based on their experimental findings, these authors suggested that the cytotoxic action of PEI could be related to its binding to proteoglycans present at the surface membrane. According to Moghimi et al this adsorption process could induce a membrane destabilization with a rapid redistribution of phosphatidylserine from the inner plasma membrane to the outer cell surface (28). Although this mechanism has not been demonstrated on RBC's per se, it does not by itself explain the difference of hemoreactivity observed between the PDMAEMA and the PEI. We can only guess why the ionic interaction of a polycation with the cell glycocalyx would affect these alterations in the membrane integrity while others do not. This difference in hemoreactivity between PEI and PDMAEMA allows us to suggest that PEI could better penetrate into the RBC membrane with its consequent cell lysis, while in opposite fashion, PDMAEMA could absorb more weakly on the cell surface, keeping a more open conformation with flexible polymer segments able to bridge adjacent RBCs. This difference in polymer adhesion on the RBCs would be of interest for future assessment by using

molecular probe microscopy such as atomic force microscopy. If at this stage we have no direct experimental proof of a mechanism of action between these two polymer families, there are nevertheless some theoretical and experimental observations which also support the actual interpretations of our data. As already discussed for protein opsonisation, the difference existing in charge density between PEI and PDMAEMA can also support their difference of ability to penetrate within RBC membrane. Surface charge is well-known to be an important factor regulating the mobility of a charged particle in the polycation-induced transmembrane migration of lipid molecules (28). Moreover, Fischer et al found also that besides the molar mass, the charge density represents a key parameter for the interaction with biological membranes with as consequence a significant effect on cell toxicity (2). The driving force for this interaction with cell membrane and perturbation results from the electrostatic attraction between the polyelectrolyte and the headgroups of the lipids. A charge density threshold to be membrane-disruptive has been evidenced by Kügler et al (84). Due to its difference in pKa, but also due to its higher hydrodynamic diameter, PDMAEMA should have therefore fewer propensities to penetrate within the erythrocyte bilayer and induce cell lysis.

2.7.2.4 Platelet counting

Compared with other blood cells, platelets are some of the most sensitive cells to react with foreign body surfaces, giving rise to their activation or aggregation. In particular, their adhesion is well-known to be mediated through conformational change of their glycoprotein integrin receptor GPIIb/IIIa responsible for platelet aggregation (85). In addition, changes in the amount of GPIIb and the translocation of P-selectin from α-granules to the platelet surface membrane underlie platelet-leukocyte aggregation, respectively (85). The results of our study, seen in Fig 2.5 clearly demonstrate that a least a 20% decrease in platelet number was already observed in te presence of such a low PDMAEMA concentration as 10 µg/mL. At 100 µg/mL, platelet counting achieved values up to 20% of control. Surprisingly, and in contrast to observations done with RBCs, the Mw of PDMAEMA has no major influence on platelet aggregation. The result supports the hypothesis proposed for this experiment, because there is a correlation between the

increment of polymer concentration and the decrease of number of platelets. In the other hand, comparison with data acquired with PEI is also worthwhile. Only a 20 % reduction in platelet number occurred with this polycation at the highest concentration assessed (100 µg/mL). Based on these different observations and by comparison with RBC behavior, it is difficult to anticipate the mechanism underlying this platelet aggregation. If a similar physical-chemical explanation could be suggested other than RBCs, that is, charged neutralization by the polycation combined with the bridging process, we can only guess why these events are so sensitive compared with the RBCs and why they were not affected by the Mw of the polycations and by the presence of a PEG segment (P4) as observed for erythrocyte aggregation. If, at this stage, we cannot rule out this mechanism (as suggested by Yancheva et al (62) when comparing the action of quaternized PDMAEMAs with the nonquaternized form of the same polymethacrylate or by Coller when studying polybrene effect on platelets (85), other mechanisms of action might be involved.

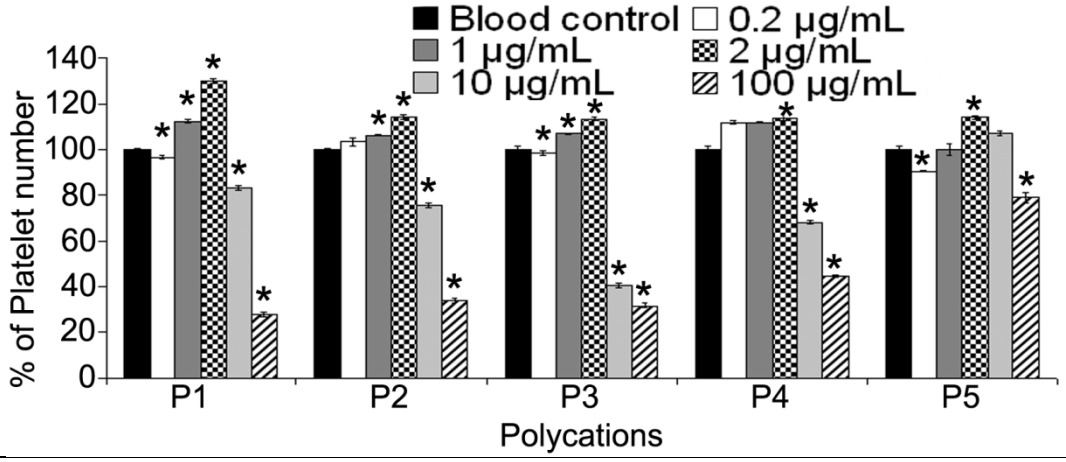

Fig. 2.5 Evolution of platelet after incubation of whole blood with the polycations vs polycation nature and concentration. Platelet counting in blood control was normalized as 100% platelet number. Bars with (*) show statistically significant differences in comparison to the blood control ($P < 0.05$).

Although higher platelet percentage have been observed for intermediate polycation concentrations, these variations, although statistically significant different, can be ascribed to the evolution in adhesion ability of platelets using citrate as anticoagulant. It should be indeed stressed that if platelet counting seems a relatively simple biological assay when adopting EDTA as anticoagulant, citrate selected here in order to preserve the

activation/aggregation of platelets give rise to more variations in counting especially within the first hour after blood sampling.

2.7.2.5 Coagulation

Several proteins are associated with both coagulation cascades, the intrinsic and extrinsic pathways. Hence it could be expected that hemostasis is affected if polycations form the polyelectrolyte complexes with key proteins governing these coagulation pathways. In our study, activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured in order to evaluate the effects of polycations on intrinsic and extrinsic pathways, respectively. From the data summarized in Tables 2.6 and 2.7, it can be observed that all the polycations within a concentration range of 10 to 100 µg/mL strongly increased the APTT and PT. These results support the hypothesis proposed for this experiment, the data shows that there is correlation between increment of polymer concentration and blood coagulation inhibition. For all 3 PDMAEMA homopolymers at a concentration of 100 µg/mL, the intrinsic pathway was no longer measurable under the standard conditions of analysis. By comparison, inhibition of the IP induced by the PEI is even stronger (a reduction already observed at 1 and 2 µg/mL). The extrinsic pathway was also significantly reduced in the presence of the PDMAEMAs, although not totally inhibited at 100 µg/mL in contrast to the action of PEI. Interestingly, the efficiency of EP inhibition decreases when the Mw of the homo PDMAEMAs is raised. Here and in contrast to platelet reactivity, the benefit in the steric shielding of PEG (see P4) is highlighted both for APPT and PT. In agreement with the results obtained by Yancheva et al (62) with either PDMAEMA (Mw 24,000) or their quaternized forms, the inhibition of intrinsic and extrinsic pathways of coagulation can be readily explained by the acidic pI of several of the key proteins involved in the coagulation cascades allowed to generate polyelectrolytes complexes. Among these proteins, fibrinogen, factor IX, prothrombin, factor X, and antithrombin III deserve particular mention as key factors in the activation of all humoral blood reactions (ie, Hageman factor XII).

APTT (%)	P1	P2	P3	P4	P5
Blood control	100	100	100	100	100
Positive Control	52 *	49 *	50 *	51 *	54 *
0.2 µg/mL	100	100	99	100	99
1 µg/mL	100	96	100	100	100
2 µg/mL	100	71	90	100	100
10 µg/mL	39 *	40 *	40 *	43 *	30 *
100 µg/mL	0 *	0 *	0 *	15 *	30 *

Table 2.6 Evaluation of intrinsic pathway after whole blood incubation with polycations. Activated partial thromboplastin time (APTT) has been assessed with a Behring Coagulation Timer analyzer (BCT) (Dade Behring). Blood control was automatically normalized as 100% by the BCT. A decrease in APTT % corresponds to an inhibition of the pathway compared with blood control.

PT (%)	P1	P2	P3	P4	P5
Blood Control	100	100	100	100	100
Positive Control	130	130	130	130	126
0.2 µg/mL	110	114	102	104	95
1 µg/mL	124	128	124	114	92
2 µg/mL	126	129	127	115	88
10 µg/mL	64*	71*	92*	118	43*
100 µg/mL	25*	25*	25 *	25 *	25*

Table 2.7 Evaluation of extrinsic pathway after whole blood incubation with polycations. Prothrombin time (PT) has been assessed with a Behring Coagulation Timer analyzer (BCT) (Dade Behring). Blood control was automatically normalized as 100% by the BCT. A decrease in PT % corresponds to inhibition of the pathway compared with blood control.

2.7.2.6 Complement activation

The complement system is a major mechanism in the immunogenic response to facilitate the elimination of bacteria, viruses, and other foreign bodies. About 30 proteins of the plasma or those linked to the surface of the immune cells may be involved in this complex immunological machinery (65). Activation of this system can proceed according to 3 possible pathways involving multistep protein cleavages, but which differ both by the nature of the triggering element and by the first steps involved in initiating this activation.

These 3 enzymatic cascades have a common enzyme: C3 convertase, which cleaves the C3 protein in the anaphylotoxin called C3a, the biological parameter considered in our in vitro study to assess complement activation. Complement system activation is a major parameter to evaluate since it has been shown than polycations can activate such cascade (3, 67). Figure 2.6 highlights the level of complement activation (expressed in percentage of the normal blood incubated) observed in human blood samples incubated with PDMAEMA-based polymers or PEI. Zymosan, a mannan-rich, insoluble cell-wall polysaccharide of *Saccharomyces cerevisiae,* was employed as positive control for all tests. Before analyzing and discussing the data, it should be stressed that the level of activation observed for the zymosan control differs from polymer to polymer because each polymer has been evaluated separately, thus using different blood samples. This variation in positive control can therefore be assigned to an interindividual variation in complement activation as already reported by Bergh et al (86). Analysis of the level of C3a detected in blood incubated with the 5 polycations leads to the conclusion that no significant activation was observed, whatever their Mw and composition. Referring to the hypothesis (point 2.3.2), it was rejected by the results because there was no relation between the activation of complement and polymer concentration. However, in the case of the homopolymer P2, statistically significant differences were noticed. Taking into account that no dose/response can be derived from these data and they don't fit with the responses observed for the other polymers, we can only anticipate that these variations are linked to subject to subject variations. Compared with coagulation alteration, these observations open the question of the lack of reactivity of C3 protein, which has a pI of 5.7. Considering the high concentration and diversity of proteins present in plasma, the easiest way to support our data relies, of course, on the competition between these proteins for the foreign polymers. Compared with our data, those reported by Ward et al (67) showed complement activation for PEI 25,000 at a low concentration of 0.01 to 1 µg/mL. Although their PEI differs from the polycations evaluated in our study, it opens the question of the purity of the polycations evaluated and of the possible difference in this biological assay in function of the method adopted.

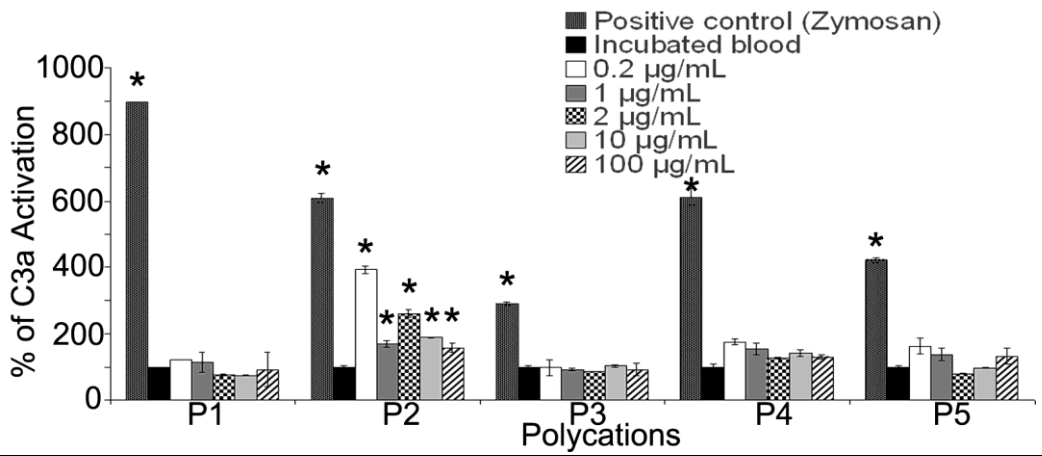

Fig 2.6 Evolution of the complement activation after incubation of whole blood vs polycation nature and concentration. Complement activation has been determined based on C3a assay (ELISA). C3a activation in in blood control was normalized as 100% platelet number. Bars with (*) show statistically significant differences in comparison to the blood control ($P < 0.05$).

2.7.3 Conclusions

The hemocompatibility of 4 PDMAEMA-based polymers and 1 PEI was evaluated according to the 5 categories of tests recommended in ISO-10993-4. To our knowledge, no other studies have evaluated the hemocompatibility of free PDMAEMA and PEI following such recommendations. In contrast to most of the hemocompatibility studies of PDMAEMA and PEI, our experiments were performed on whole blood to take into account the humoral response. Employing well-defined and extremely purified polymeric materials, our data have allowed us to draw clear relationships between the macromolecular properties of this polycation family and their hemoreactivity. Based on these systematic and extensive observations, we are in a position to propose, or confirm, the mechanisms underlying the different blood reactions. If most of them can be explained on the basis of nonspecific ionic interaction with the various polyanionic domains presented at the surface of various cellular and humoral blood elements, the sensitivity of some the reactions observed, in particular platelet aggregation, lead us to suggest that more complex mechanisms could be altered involving some specific biochemical signals. As outlined at the beginning of our study, it should be reemphasized that the hemoreactivity of PDMAEMA cannot be extrapolated to the same polycation once associated with the polyelectrolyte complexes. In addition, we

have shown that PEGylation of the polycation sequence is also a strategy that masks the reactivity of the cationic sequence.

Interestingly enough no major differences were observed in the humoral ractions of PEI and the various PDMAEMA evaluated, thus either regarding coagulation and complement activation. Difference in opsonisation behavior between PEI and PDMAEMA could be expected due to the existence of several macromolecular differences between these two polycation families. It is indeed well-known that polyelectrolyte complexe condensation capacity is not only related to the charge density of the interacting polymers but is also function of charge distribution, polymer backbone flexibility, and hydrophobicity/hydrophilicity balance (87). In the particular case of the comparison between branched PEI and PDMAEMA their architecture and accordingly flexibility largely differs. With an extensive branched structure as is typically the case for commercial PEI this polymer has an amino group ratio 1°:2°:3° equal to 1:1:1 (88). Compared to the linear structure of PMDMAEMA, the PEI architecture promotes therefore the condensation of the macromolecules which explains the exponential factor of the Mark–Houwink parameters of 0.26 as reported by von Harpe et al. (88). This value, similar to the data reported by Park and Choi (89) is noticeably low and mainly affected by the solvent quality and/or different degree of branching can be ascribed to the very compact structure of branched PEI. By comparison the value reported for linear PDMAEMA is significantly higher i.e. 0.5 and 0.6 (90). The charge density of the polycation is also expected to play a key role for their interaction with proteins. Polymer charge density is usually taken as the ratio between the amount of charged and total monomers. With a pK value ranging between 8.2 to 9.9 in function of the Mw and branching level (89), branched PEI has a high density of amine groups, is spite that it has been measured that two thirds of them could remain unprotonated in physiological environment (91). By comparison PDMAEMA has a mean pKa surrounding physiological pH, ie. 7.4 (92). This discussion should not been limited to thermodynamical parameters but should also take into consideration the kinetics aspects related to the dynamics of the macromolecule association-dissociation in aqueous mixtures. Although this topic remains mainly under-investigated in literature these exchange, addition reactions, and structural reorganization of either primary complexes or

supramolecular organization can largely contribute to modify the opsonisation behavior of polycation in blood. Function of the final outcome of this interplay between plasma proteins, the preferential adsorption of key opsonins in the activation of the classical biological cascades of the blood (such as Hageman Factor or Factors C3, B and D) should be definitely taken into consideration.

Definitively, if some of our observations have allowed us to support some mechanisms in hemoreactivity, they have also raised additional questions for which answers can be provided only by using additional biochemical and biophysical methodologies required to highlight the molecular and cellular events involved.

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3. CAMOUFLAGING OF BLOOD GROUP ANTIGENS

3.1 Introduction

In 1900, Karl Landsteiner, at 32, performed the first of a series of experiments exploring the matching of red blood cells (RBCs) and plasma, leading to the discovery of the ABO blood group system. The experiment consisted of tests mixing blood and serum samples collected from either human donors or animals. From these tests, Landsteiner made interesting observations and described that "the sera of healthy individuals not only have an agglutination effect on animal red cells but also on human red cells from different individuals. This phenomenon can be related to the dissolving capacity of serum for red cells as occurs in various diseases" (1, 2). The following year Landsteiner continued exploring his first finding; he used serum and RBCs from 29 people including his own and from 4 colleagues. Then he performed a simple and reproducible experiment to determine why RBCs agglutinated when coming in contact with certain sera. Landsteiner mixed each batch of collected serum with each RBC sample and noted the results in blocks of 5 or 6 different sera and RBCs, in all, 144 combinations. About those results, he wrote, "The sera in most cases could be separated into 3 groups. In many cases sera of group A react with red cells of another group, B, but not with group A; on the other hand, A red cells are affected in the same way by serum B. The sera of the third group (C) agglutinate red cells A and B; but C red cells are not affected by sera from A and B. Naturally the red cells must be considered insensitive to the agglutinins which are present in the same serum." He concluded, "I want [to] mention that the observation explains the varying consequences of therapeutic blood transfusions in humans" (2). Landsteiner's findings strongly influenced medical science and stimulated development of the field of blood transfusion. Because of the significance of discovering the ABO system, Landsteiner won the 1930 Nobel Prize in Physiology.

Since Landsteiner's time scientific investigation has demonstrated that many other blood groups exist on the RBC membrane. To date, 29 blood groups have been discovered; of all these groups, the ABO and Rh major systems are the most clinically important for blood transfusion, although the importance that such minor groups as Kell, Kidd, Lewis, and MSN have for chronically transfused patients cannot be ignored. Because of blood group

antigens, donor-receptor matching is required to avoid immune hemolytic transfusion reactions after blood transfusion; however, although transfusion matching is done with highly reliable tests, mismatched transfusion still occurs (3). To solve this problem (as explained in Chapter 1, strategies for developing universal RBCs have been explored. These include production of RBCs from different cell lines, enzyme-modified RBCs (ECO RBCs), and polymer-shielded RBCs. Due to its easy clinical applicability, this latter approach will be explored within the frame of our PhD dissertation.

3.2 Production of universal red blood cells by polymer-shielded RBCs

This strategy relies on the fact that protective polymers can be bonded to the surface of the RBC membrane. The polymer chains are consequently expected to cover and mask the blood groups' antigens; moreover, the polymeric layer's physicochemical properties favor repulsion of the antibodies. This approach promotes an "immunocamouflage" for the RBCs. Because erythrocytes covered by polymeric protection should be undetected or "invisible" to the immune system, they are called "stealth" or "silent" RBCs. Nowadays polymer shielding is performed by two major methods, polyethylene glycol ((PEG)ylation) of RBCs or self-assembly of cationic polymers.

3.2.1 PEGylated RBCs.

PEGylation of RBCs has been performed and explored by distinct research teams during the past 2 decades. The first works about the topic were published between 1996 and 1997 (3, 4). Since then, the method of RBC-PEGylation has maintained its original basis. It consists of inducing the binding of functionalized PEG chains with a functional group of proteins on the RBC membrane. Generally, cyanuric chloride methoxy PEG (CmPEG) is the most common polymer used for RBC PEGylation (also called PEG derivatization). The CmPEG is a monofunctional polymer that reacts with ε-amino groups of lysine residues and to a lesser extent with amino-terminal groups in proteins. Briefly, to perform derivatization, the RBCs (~1.5×10^9 cells/mL) are incubated with a CmPEG alkaline

solution (pH 8) for 30–60 minutes at 4°C (3, 4). As result of the CmPEG derivatization, covalent bonds are formed between the CmPEG and the RBC surface proteins, which occur mainly between the CmPEG and the band 3 anion transport channel proteins (5, 6), although CmPEG binds to many other unidentified membrane proteins (7).

PEGylated RBCs (PEG-RBCs) have been produced by different research teams. Among them, the Albany team headed by M.D. Scott (Albany Medical College, NY, USA) has been pioneering the technique. In 1997, Scott et al. reported their first findings on PEGylated RBCs (5). They modified Type A, B, and AB RBCs (human, sheep, and mouse) with CchmPEG (Mw 5,000).

The morphology, structure, and oxygen affinity of PEG-RBCs were similar to those of untreated RBCs. Moreover, the A- and AB-shielded erythrocytes presented poor binding of anti-A antibody (anti-Aab) and prevented RBC agglutination (5). Later, Murad et al. found that PEG-derivation did not cause hemolysis, nor did it cause perturbation of the transmembrane transport of normal oxygen, transport of Na+ and K+ cations, or influx of SO_4^- from treated RBCs (8). The permeability of PEG to those molecules indicated that the polymeric shield acted like a sieve, that is, it permitted small molecules to go through the PEG while large molecules such as antibodies did not (8). Although the PEG-derivation did not affect cell structure or functions as well as it blocked antibodies, an undesirable increase in RBC clearance was noticed. The PEG-murine (M) RBCs survived in mice when concentrations below 0.5 mM mPEG (Mw 5,000) were used to treat the cells, but cells were rapidly cleared using 0.6 mM and 0.8 mM mPEG (8). Because poor in vivo survival could result from complement activation and clearance, Scott's team investigated the ability of CmPEG-RBCs to activate the complement system. Bradley et al. noticed that the CmPEG-RBCs (Mw 5,000; 0.6, 1.2, or 2.5 mM concentration) did not activate the complement system when they were incubated with ABO-matched serum but the complement system was triggered when the CmPEG-RBCs contacted ABO-mismatched serum. Interestingly, complement activation was initiated only by the classical pathway that is typically triggered by antibody binding. Such observation contrasts with other works of the Scott team showing blocking of antibodies by CmPEG (6, 8, 9).

Surprisingly, Bradley et al. found that IgM bonded to CmPEG-RBCs and activated complement and cell lysis. In addition to IgM, protein-related complement systems can also bind to the CmPEG layer, such as the case of complement C3 precursor, complement component C9 precursor, complement component 4B preprotein, complement C1r subcomponent precursor, complement C1s subcomponent precursor, C4b-binding protein beta chain precursor, and complement C4b-binding protein alpha chain precursor (9). It is of interest to mention the hypothesis suggested by Bradley et al. to explain complement activation in spite of the presence of CmPEG on the RBCs. First , the exclusion density of covalently grafted PEG is variable and acts as a molecular sieve at the same time that PEG exclusion density is determined by several factors including size of the cell, spatial heterogeneity of the PEG attachment, and size and topography of the antigen (6). Hence, variations in exclusion density may explain why short antigens such as D antigen may be well camouflaged while larger ones such as ABH antigens are not. Consequently, the IgM (A or B antibodies) may contact its corresponding antigens to cause physical disruption of the PEG steric barrier. It may activate the complement and then the complement protein complexes increase the PEG disruption to allow the formation and action of the membrane attack complex (9). Accordingly, the authors hypothesized that both the density of attached PEG and mPEG chain length influenced the efficacy of the immunocamouflage (6).

On the basis of the above evidence, Bradley et al. have explored alternatives to CmPEG, that is, mPEG-benzotriazole carbonate (bifunctional) (BmPEG) (Mw 5,000 and 20,000) (9). Like CmPEG, the BmPEG reacts with amino groups of lysine residues and to a lesser extent with amino-terminal groups in proteins. But the BmPEG attaches the mPEG to the target in the form of a stable amide bond in the form of a triazine ring, such as mPEG does. When the RBCs were treated with BmPEG, they conserved their morphological properties and no cell membrane damage was noted; moreover, the polymer of Mw 20,000 showed normal survival kinetics (~50 days) in mice. It was also observed that increasing the chain length (from Mw 5,000 to 20,000) and concentration (from 0.5 mM to 4 mM), that is, the density of mPEG on the RBCs, blocking of the ABO antibodies improved. Efficiency of the increase was correlated with the topography of the antigen, that is, the relative height of the antigen above the membrane surface. While the Rhc antigen residues close to the lipid

layer were camouflaged by BmPEG (Mw 5,000), the JK^b present well above the membrane were not masked by such PEG. Conversely, the BmPEG 20,000 masked both type of antigens well. The study of Bradley et al. demonstrated that grafted mPEG molecules (polymer concentration), polymer size (Mw), and antigen topography influence on masking (6, 9, 10).

In addition to the Albany team, another group has explored the production of PEGylated RBCs; one of these is from Ohio State University. That research team, headed by S.I. Palmer and A.F. Gundersen who have performed derivatization of bovine RBCs (BRBCs) using the Mw 20,000 succinimidyl ester of methoxypolyethylene glycol propionic acid (SPA-mPEG) (11, 12). This polymer reacts with amino acid residues having amine groups (lysines, for instance) producing a stable amide link free of hydrolysis-prone ester linkages (12). These authors suggested using BRBCs because cows provide a suitable source of RBC donors due to their larger blood volume and easy vein access with the advantage that bRBCs are mechanically robust and have suitable osmotic stability (12). On those cells, SPA-PEG derivatization did not provoke membrane damage (less 1 % rHb); moreover, it did not induce changes in oxygen uptake or oxygen membrane transport (12).
However, when the SPA-mPEG-RBCs were incubated with distinct human plasma samples, an agglutination response was observed, and interestingly, the strength of that event varied between individuals (11). To identify bound antibodies on the SPA-mPEG-RBCs, the PEGylated cells were analyzed with flow cytometry, which showed that plasmatic IgG and IgM antibodies bound to the mPEG layer. Unexpectedly for the investigators, increments in SPA-mPEG density of the RBCs augmented the binding of both those antibodies. However, for those findings, no explanation was given by the authors (11).

Recently a group from The Institute of Blood Transfusion of Beijing also explored PEG-RBCs, showing promising results (7, 13). Tan et al. performed in vivo tests with SPA-mPEG-mice RBCs. The treated cells showed a survival rate of 96.27 % (24 h) similar to the 94.47 % for nontreated RBCs transfused into mice (13). Moreover, a similar half-life was observed for PEGylated (18.4 days) and untreated RBCs (18.1 days). In addition, after

induced bleeding of mice with a decrease of hemoglobin from 131 g/L to 98 g/L, transfused SPA-mPEG-RBCs recovered the hemoglobin value (130 g/L) (13).

Although PEG-RBCs have been investigated by distinct teams, questions remain about why some teams have achieved promising results while others have got conflicting results. For instance, Bradley et al. observed poor in vivo survival of mPEG-RCBs that was correlated with complement activation (6, 9), whereas Yan et al. reported long in vivo survival (13). Comparing results from disparate teams is difficult because different research groups use different tests for evaluating PEGylated RBCs. In this respect, Garraty has tested PEG-RBCs tailored by other research teams in the U.S. (the Albany group and the University of Southern California [USC] group) (3). The PEgylated RBCs from both groups were not agglutinated by commercial anti-D, but the they reacted to the anti-globulin test showing that D antigen was not completely blocked (3). Hence, even though in vitro assays demonstrated antigen masking, when treated cells were subjected to specific agglutination tests, the camouflage was partial and not enough for clinical use (3). Although the development of totally immunologically silent PEGylated RBCs still faces many challenges, camouflaged RBCs are an interesting alternative for transfusion therapy. Moreover, they can be used for other therapeutic purposes.

Armstrong et al. have developed and investigated PEGylated-RBCs for treating diseases characterized by vaso-occlusion or impaired blood flow (e.g., myocardial infarction and sickle cell diseases) because those cells reduce blood viscosity. These investigators compared the viscosity of normal blood, normal blood plus Poloxamer 188 (a commercial polyether surfactant based on two PEO blocks linked by a polypropylene oxyde sequence), and mPEG-RBCs. The latter reduced viscosity about 75% whereas the Poloxamer 188 (5 mg/mL) reduced the viscosity about 30%. Aggregation was also strongly reduced by the PEG-RBCs (14).

Another application of mPEG-RBCs was reported by Blackall et al., who have observed that mPEG blocked the binding of the malarial parasite *Plasmodium falciparum* to RBCs. Accordingly, mPEG-RBCs masked glycophorin A antigen, the binding receptor for *P.*

falciparum (15). Finally, because erythrocytes have uniform circulation times, predictable degradation rates, long history of administration (transfusion), and the possibility of targeting reticuloendothelial system organs, another possible use of PEGylated RBCs is their employment as carriers to deliver therapeutic molecules (16).

3.2.2 Self-assembly of polycationic polymers to camouflage RBC antigens

As described in the introduction, blocking biological recognition by self-assembled functionalized polycations is another strategy for camouflaging blood group antigens. This strategy relies on the binding of the polycation sequence to the negatively charged surface of RBCs, while their PEG sequence(s) provide steric repulsion of antibodies. Elbert and Hubell were the first to report this approach with the adoption of poly-L-lysine-PEG copolymers (PLL-g-PEG) (PLL Mw/graft ratio 20,000/1.75:1 and PLL Mw/graft ratio 350,000/1.75:1 (17).

First, the authors noticed that PLL-PEG inhibited fibroblast spreading on anionic polystyrene surfaces, with inhibition affecting the Mw. These preliminary observations indicated that self-assembly of the copolymer occurred readily on the anionic surface and that the Mw influenced helped the ability of the copolymer in blocking cell spreading (17). After that, rat RBCs were treated with a 0.1% PLL-PEG (either Mw 20,000 or 375,000) solution. When the cells were incubated with wheat germ agglutinin (WGA) (lectin-induced hemagglutination test), they observed that the long copolymer prevented agglutination but the short one did not. Elbert and Hubbel found that the protection was due to the copolymer anchored to the RBCs (17). Although the authors have discontinued exploring PLL-g-PEG for camouflaging RBCs antigens, they have established the basis for using electrostatically bound polymers for developing silent cells (18).

The principle of self-assembly has been applied to perform the method of layer-by-layer (LbL) adsorption to form polyelectrolyte layers (PEL) on RBCs. For instance, Donath et al. grew PEL on human RBCs by alternately adsorbing poly (allyamine hydrochloride) and poly (styrenesulfonate) (19). Then the cells were exposed to a deproteinizer to obtain a

polyelectrolyte capsule mimicking RBC morphology. The authors theorized that polyelectrolyte capsules might be useful for synthesizing and encapsulating macromolecules and inorganic nanoparticles because of their high permeability properties (19), although others have used the idea of growing PEL to camouflage cell surface antigens. Mansouri et al (20) have grown PEL in hRBCs using cationic chitosan with phosphorylcholine (CH-PDCH) and sodium hyaluronate (HA) groups as well as PLL-PEG and sodium alginate (AL). Type A hRBCs were fixed on silica slides pretreated with human fibronectin and covered with CH-PC and HA to form a protective bilayer of CH-PC/HA (P-Shell). This shell brought protection to the cell against the cytotoxic effects of PLL. After that, the cells were covered by PLL-PEG and AL to form a camouflaging bilayer of PLL-PEG/AL (C-shell). The treated cells were protected from anti-A binding because the antigen-antibody–induced hemolysis was poor in the treated cells (3% rHb) but the response was high in the nontreated RBCs (78% rHb). Moreover, the P-shell/C-shell-RBCs exhibited lack of binding fluorescein anti-A, whereas the nontreated cells presented significant fluorescence (20). Having demonstrated that the LbL method was useful in forming a PEL around the RBCs, Mansouri et al. modified the previous method, avoiding fixation of the RBCs and treating the cells in suspension (21). After growth of the PEL shield, no cell aggregation occurred because the shielded RBCs showed an electrophoretical mobility similar to untreated RBCs (−2.5 μm.cm/volts), indicating that the RBCs maintained their repulsion property. Moreover, the treated RBCs had normal profiles of oxygen uptake that prevented anti-A–, anti-B– and anti-D–induced agglutination (21).

3.2.3 Other methods of forming polymeric shielding

Winblade et al. have synthetized PLL-g-PEG-phenylboronic acid (PBA) copolymers to modify the surface properties of RBCs (22). These molecules are oligosaccharides that are present within glycoprotein and glycoconjugates as membrane proteins and, because cis-diols exist abundantly on the cell surface, they act as nonspecific ubiquitous targets for binding interaction in steric stabilization (22). Accordingly, it has been observed that a PLL-g-PEG-PBA copolymer binds to RBCs because of PBA but not because of self-

assembly by PLL; the latter is used only as a backbone of the copolymer. Interestingly, PLL-g-PEG-PBA on RBCs has prevented WGA-induced agglutination as well as anti-A induced agglutination in treated A-type RBCs. Although PLL-g-PEG-PBA has been tested for developing camouflage of RBCs (22), no more data have been published in this respect. Winblade et al. have focused their efforts on exploring the ability of PLL-PEG-PBA to block adhesion and spreading of fibroblasts, a strategy useful for medical therapies in which fibroblast proliferation is undesirable for tissue regeneration (22, 23).

3.2.4 Self-assembly of PDMAEMA-based polymers to camouflage RBC antigens

The previous section shows a summary of evidence reported about camouflaging RBCs with polymers. Clearly, polymeric masking of blood group antigens represents a big challenge regardless of the methods and materials employed. To this end, although PEG derivatization has been thoroughly investigated, there are some concerns related to the method and results. On the other hand, the self-assembly method has shown feasibility for camouflaging and it offers an alternative strategy for masking.

Herein we explored PDMAEMA-based polymers to mask antigens; because of their cationic nature, these polymers can self-assemble on the RBC membrane to form a polymer shield. PDMAEMAs have already been evaluated by us in collaboration with the Rosario team (University of Rosario, Argentina). In this respect, Riquelme et al. tested the hemocompatibility of the statistical copolymer PDMAEMA-b-MAPEG (Mw 29,100), a palm-tree copolymer PDMAEMA-b-MAPEG (30,700), and a homopolymer PDMAEMA (Mw 14,600) (24). The polymers were evaluated for their ability to influence the stationary and dynamic rheological parameters of the RBC membrane. The three copolymers (2 μg/mL, 50 μg/mL, and 250 μg/mL) did not alter the membrane's rheological properties whereas the homopolymer, whatever the concentration, significantly altered its rheological properties. On the other hand, the homopolymer and copolymers augmented the size distribution of the RBCs, a response that was correlated with cell aggregation. In this case, the stronger aggregation was observed when the polymers were used at 250 μg/mL. In addition to these observations, Riquelme et al observed that the PDMAEMA copolymers

reduced glycophorin-induced agglutination (24). The results showed that PDMAEMAs are of interest for interacting with RBCs and inducing camouflaging. But the findings need to be more fully explored for a better understanding of PDMAEMA-RBC interactions and to optimize the macromolecular architecture of these polycations to assure camouflaging of the RBC surface.

3.3 Scientific method for the study of camouflaging ability of PDMAEMA based polymers

3.3.1 Aim of the study

3.3.1.1 General aim

The purpose of this study was to evaluate the ability of PDMAEMA based polymers to mask the antigens expressed at the surface of RBC's.

3.3.1.2 Particular aims

To answer to our general aim, this study has focused on the three following particular objectives :

- To evaluate the ability of PDMAEMA homopolymers and copolymers to mask anti-glycophorin A (aGPA)
- To evaluate the influence of the architecture of PDMAEMA based polymers to mask aGPA.
-To evaluate the effect of temperature and structure on the ability of PDMAEMA based polymers to mask aGPA.

3.3.2 Hypotheses

$H_{1:}$ The PDMAEMA-co-MAPEG better masks the glycophorin antigens than the PDMAEMA homopolymers.

H_2: The architecture of the copolymer influences on the masking ability of the PDMAEMA-co-MPAEG.
H_3: Increment in temperature of PDMAEMA treatment will augment the adsorption of the copolymers.

3.3.3 Rationale behind the selection of the technique used to assess the ability of antigen masking of the PDMAEMA based polymers

A first step in the optimization of camouflaging of RBC's is the evaluation of adsorption or anchoring of the polymer on the RBC's. This evaluation can consist in a direct estimation of the density of polymer anchored to the cell membrane but adsorption of polymer can be also tested indirectly with different methods measuring cell changes due to the adsorption of the polymer. This simple approach has been mainly employed in the different studies referred in the introduction and the results have been correlated with the formation of a polymeric shield intended to prevent antibody binding. For that propose, flow cytometry (FACS) is a technique that has been used for measuring blocking of fluorescent antibodies, if polymer-treated erythrocytes block antibodies, the fluorescence will reduce.
FACS is a system for sensing of particles when they move in a liquid flow through a laser light bean, it is useful for making quantitative measurement in single cells or cellular constituents at very short times. FACS measures individual cells and then precise information of each cell can be obtained from among a large population (29). Measurements are made individually within the suspension as contrasted with measuring an average property for the entire population; because single cells are measured, minima debris and aggregation is necessary to ensure a uniform sample and without disrupting particles creating turbulence. Both particle fluorescence and light scatter are measurements that can be made by FACS for the analysis and identification of cells. When cell passes through a laser bean, light is scattered in all directions, hence light scattered in the forward direction at low angles (0.5-10°) from the axis is proportional to the square of the radius of a sphere and it is interpreted as size of the cell. In addition, light enters into the cell and it is reflected and refracted by the nucleus and other contents in the cell; hence, the 90° light

(right-angled, side) scatter is considered proportional to the granularity of the cell (31). To obtain fluorescence from cells, them can be labeled with fluorochrome-linked monoclonal or polyclonal antibodies or can be stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Signals due to fluorescence can be useful to identify membrane receptors, antigens, or particular cell types (31).

Polymer self-assembly has been used to grow polyelectrolytes (PEL) according to a layer-by-layer method (LbL) and to either modify surface of distinct materials or generate nanostructures. When applied on RBC's surface, a polycation-based layer is firstly formed through the interaction with the anionic domains of the membrane. Then the excess of free positive charges is used to adsorb a subsequent anionic-based layer, this process can be repeated to increase the thickness of the PEL coating. This LbL has been performed on RBC's using a double layer made of chitosan-g-phosphorylcholine-sodium hyaluronate and poly-L-lysine (PLL)-g-PEG-sodium alginate (25,26). This methodology has been successful to prevent antigen A recognition. If a multi-layer coating should improve the antigen masking efficiency, it also enhance the risk of altering the membrane properties, in particular their flexibility and diffusion properties. In this respect, a single polymer mono-layer made by one simple one-step method, as proposed by Elbert and Hubell for RBC's, is an attractive alternative. Adopting PLL-g-PEG these authors have reported that self-assembled macromolecules could prevent lectin-induced hemagglutination (27). Herein, we have investigated several poly (2-dimethylamino-ethymethacrylate) (PDMAEMA) based polymers to mask human RBC's antigens. Their PDMAEMA sequence, partially cationized at pH 7.4, is expected to anchor the synthetic macromolecule on the RBC's surface. Their PEG grafts could therefore remain available at the erythrocyte surface to assure a steric barrier which should prevent cell aggregation and promote antibody repulsion. Adopting this polymer family, Riquelme et al. have already reported that PDMAEMA-b-MAPEG copolymers (Mw 29,100 and 30,700) did not alter the morphological and rheological properties of RBC's up to a concentration of polycations of 250 µg/mL (28). In this study the partial masking of antigens A and B and of glycophorin A (GPA) has been also highlighted. We have also evaluated hemocompatibility of PDMAEMA's in whole human blood, showing their safety up to 100 µg/mL (29). Herein, we have assessed the

immunocamouflage ability of PDMAEMA homopolymers differing in Mw. Their properties have been compared with PDMAEMA-PEG copolymers to better understand the correlation between the macromolecular features of these polymers and their functionalities. Immunomasking has been analysed adopting a fluorescent anti-glycophorin A as membrane probe and using FACS to quantify the cell immunoprotection. These studies have been carried out considering different concentrations of the polymers and performing the coating at a temperature ranging between 25 to 37°C.

3.4 Poly (2-dimethylamino ethylmethacrylate)-based polymers to camouflage red blood cell antigens.

Here, we present the experimental section corresponding to evaluation of ability of the PDMAEMAs to masking of antigens. This section presents a structure following an editorial guide for future publication. Introduction for the in preparation-article has been omitted taking into account that an extensive introduction has been already described.

3.4.1 Materials and Methods

3.4.1.1 Materials

Two-(dimethylamino) ethyl methacrylate (DMAEMA) and PEG α-methoxy, ω-methacrylate (MAPEG), Drabkin's reagent (cyanmethemoglobin), Brij 35, and bovine hemoglobin were purchased from Sigma-Aldrich (Bornem, Belgium). |||Fluorescein isothiocyanate (FITC) mouse monoclonal antibody (Mouse IgG1) antihuman glycophorin A (CD235a) (aGPA) was purchased from Invitrogen (Merelbeke, Belgium). Phosphate buffered saline (PBS) pH 7.4 was prepared with KH_2PO_4, 1.4 mM; Na_2HPO_4, 10mM; NaCl, 137 mM; and KCl, 2.7 mM. All other chemicals and reagents were of analytical grade.

3.4.1.2 Synthesis and characterization of PDMAEMA-based polymers

The PDMAEMA-based polymers were synthetized by the solvent-free, atom-transfer radical polymerization (ATRP) method. Synthesis, characterization and purification of polymers were performed as reported elsewhere (25). After purification, each polymer was dissolved in PBS (stock solution 1 mg/mL) and stored at −20°C until their use for the experiments.

3.4.1.3 Blood collection and RBC suspension

Blood was drawn from healthy donors of the Red Cross Blood Transfusion Department in The Central Hospital of the University of Liège. Blood was collected in 3.2% citrated 4.5-mL tubes. This study received the approval of the Ethics Committee of the Medical Faculty of Liège. After collection, blood was centrifuged for 10 min at 3000 rpm to separate the RBCs from the plasma. The erythrocytes were washed 3 times with PBS under the same conditions of centrifugation. Finally, a 5% RBC (~6.8 x 10^5 RBCs/µL) suspension (RBC suspension) was prepared for the experiments. All experiments were done within 2 h of collection.

3.4.1.4 Hemolysis test

The hemolytic property of PDMAEMA-based polymers was evaluated as already reported by us (25). Briefly, in a 2-mL cylindrical microcentrifuge polypropylene tube, 150 µL of the RBC suspension was added to an equal volume of a polycation solution. The cell suspension was mixed immediately afterward using 3 up-and-down movements to avoid any gradient concentration of the polymer. The cell suspension was incubated under lateral agitation (CAT model M5, CAT Ingenieurbüro, Staufen, DE) at a rate of 36 mpm for 1 h at 37°C**.** Samples were then centrifuged for 5 min at 600 rpm. Hemoglobin release in the supernatant was measured with cyanmethemoglobin reagent. Absorbance was measured at 540 nm in a microplate reader (Anthos HT III, type 12600, Anthos, Salzburg, Austria). Hemoglobin concentration was calculated according to a calibration curve established by

the hemoglobin standard (25). Hemolysis was expressed as the percentage of hemoglobin released (% rHb) to the total hemoglobin content. Based on the Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM designation: F 756–00), polymers were classified as nonhemolytic, slightly hemolytic, or hemolytic when the rHb fraction was 0–2%, 2–5%, or >5%, respectively (26). Tests were done in triplicate, each experiment being repeated independently with 2 different batches of blood.

3.4.1.5 Polymeric coating of the RBCs and evaluation by FACS

The RBCs were treated with the PDMAEMA homopolymers as described in section 2.4 (first mixing with a pipette and a second mixing with lateral agitation). However, the second mixing of the homopolymers was performed at room temperature (RT°; 25°C) whereas for the copolymers the second mixing was done at 3 distinct temperatures (25°C, 30°C, and 37°C). After that, 1 mL PBS was added to the treated RBCs to wash and centrifuge them (Mini Spin Plus Microcentrifuge, Eppendorf) for 10 min at 600 rpm. This step was repeated twice (3 washing steps in total). Then the RBCs were resuspended at 5% concentration.

After adding 5 µL aGPA (4.5 µg/mL) to 50 µL in a conic Eppendorf tube (1.5 mL) of either treated or nontreated erythrocyte suspension (2% RBC concentration), the PDMAEMA RBCs and native RBCs (nontreated) were incubated for 30 min at 4°C (lateral agitation, 300 rpm). After incubation, the cells were gently washed with 1 mL PBS and centrifuged at 1200 rpm for 5 min at RT° (Mini Spin Plus Microcentrifuge). Because we used an FITC-conjugated antibody, only a washing step was performed (Ref). Samples were analyzed 30 min after being stored in darkness at RT°. All samples were analyzed with a FACSCalibur Flow Cytometer at RT° (FSC Volt 800; SSC volt 447; FL1 615, FL2 550, FL3 650) (Becton Dickinson, Erembodegem, Belgium). Collected data were analyzed with WinMDI 2.8 software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA). Native RBCs were used as a sample reference for size distribution and granularity and the native RBCs incubated with aGPA were used as sample reference for

fluorescence (mean fluorescence intensity [MFI]). The experiments were performed in triplicate and with at least 2 repetitions. A 1-way ANOVA and Tukey-Kramer HSD were performed to identify possible significant statistical differences between MFI of PDMAEMA-RBCs and nontreated RBCs.

3.4.1.6 Evaluation of RBCs by optical microscope

When required, the treated RBCs were observed by an optic microscope as reported by Cerda-Cristerna et al (25). Briefly, 10 µL PDMAEMA-RBCs (5% concentration) was diluted in 990 µL PBS in a 1.5-mL Eppendorf tube. An aliquot (40 µL) of that suspension was dropped into a flat-bottom, 96-well multiplate, then the samples were studied with an inverted microscope (Inverso-TC, CETI, Kontich-Antwerpen, Belgium) at ×25 magnification and imaged with a digital camera (VisiCam 5.0, VWR, Leuven, Belgium). Two repetitions were performed using distinct polymer batches.

3.4.2 Results and discussion

3.4.2.1 Synthesis and characterization of PDMAEMA

We were able to synthesize the PDMAEMAs of a low-Mw and polydispersity and to block copolymers such as PDMAEMA-co-MAPEG. It should be stressed that ATRP was advantageous in tailoring well-defined PDMAEMA-based polymers for evaluating their coating properties. Control of the molecular features of these macromolecules is particularly important because their hemoreactivity can be significantly affected by their Mw, according to their polydispersity. Fig. 3.1 and Table 3.1 show the characteristics of the PDMAEMA-based polymers considered in this study. Two linear PDMAEMA homopolymers (coded P1 and P2), 2 palm-tree PDMAEMA-co-MAPEG copolymers (coded P3 and P4), and a random PDMAEMA-co-MAPEG copolymer (coded P5) have been investigated. The 2 homopolymers are distinct in Mw; hence, we were interested in the influence of that feature on the masking ability of PDMAEMA on RBCs. In the case of the copolymers, both P3 and P4 have palm-tree architecture, but P4 has higher PEG content

and longer PEG chains than P3; on the other hand, the random P5 has statistically distributed PEG. Accordingly, we synthesized and used these 3 copolymers to evaluate the influence of architecture on their antigen masking ability. To avoid any side reactivity of the blood with monomer, catalyst, or initiator residues, special attention was paid to extensively purify these polymers, having been submitted to 3 successive and complementary extraction steps. As reported by us, after purification, Cu residue, determined by ICP/MS, was found to be 158 ± 17 ppm. Monomer residue, estimated from 1H.NMR analysis, was below 0.1% (25).

Polymer	Code	Mw (SEC) *	PDI	PDMAEMA % (Molar %) **	PEG %
PDMAEMA	P1	10,000	1.19	100	-
PDMAEMA	P2	26,400	1.17	100	-
PDMAEMA-co-MAPEG	P3	30,000	2.20	85	15
PDMAEMA-co-MAPEG	P4	30,700	1.18	75	25
PDMAEMA-co-MAPEG	P5	29,100	1.52	51	49

Table 3.1 Characteristics of the PDMAEMA homopolymers and copolymers. Relative average Mw (Mn and Mw) was determined by size exclusion chromatography inTHF/triethylamine (TEA) (2.5%) against polystyrene standards. ** Molar % determined by ^{1}H.NMR in $CDCl_3$

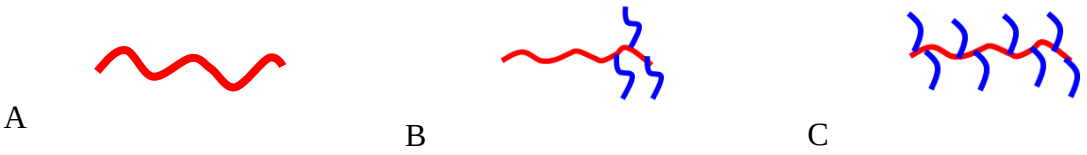


Fig. 3.1 Structure of homopolymers and copolymers employed in this study. The PDMAEMA backbone is represented in red and the PEG moieties are in blue. (A) PDMAEMA homopolymer (P1 and P2), (B) palm-tree PDMAEMA-co-MAPEG copolymer (P3 and P4), and (C) statistic-distributed PDMAEMA-co-MAPEG copolymer (P5).

3.4.2.2 Hemolysis test

It is well-known that polycations can perturb the cell membrane (27) and that they can induce exposition of phospathidylserine, resulting in leakage of the membrane and consequent cell death (28, 29). Because of this cytotoxic property when contacting RBCs, cationic polymers can provoke hemolysis (30, 31). Hence, before exploring the camouflaging ability of PDMAEMAs, we implemented a hemolysis test to evaluate a possible alteration in the RBCs' plasma membrane due to the employed polymers. The RBCs were incubated with either 20 μg/mL or 200 μg/mL each of polymer (P1–P5), after which all PDMAEMAs showed nonhemolytic properties (% rHB < 2) (Fig. 3.2).

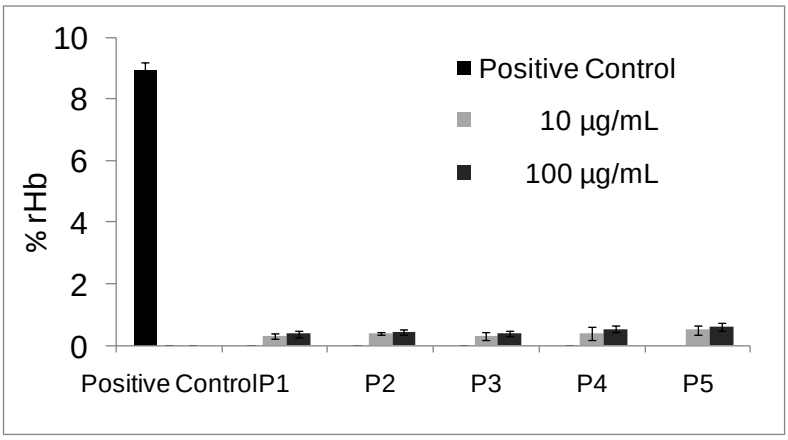
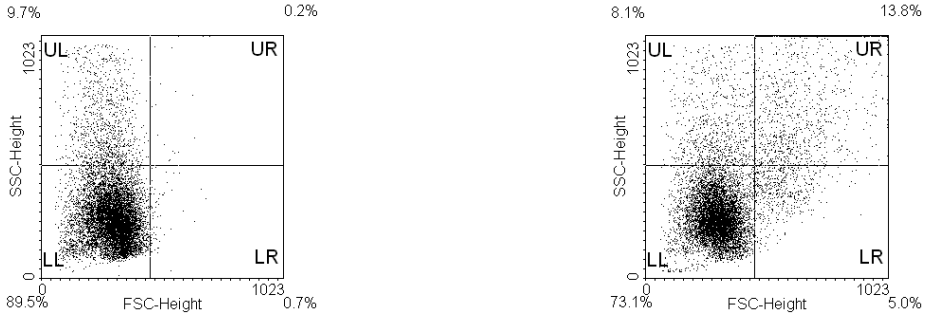


Fig. 3.2 Graphic showing the percentage rHb on the cells that contacted the 5 PDMAEMA-based polymers.

These results agree with our previous findings; nonhemolytic properties were observed for 10–1000 μg/mL PDMAEMA (Mw 10,000; 26,400; 40,000) and PDMAEMA-co-MAPEG (Mw 30,700; the same polymer as that of P4) that contacted washed RBCs for 1 h.(25) We wish to stress that, compared with our former study, we used here a 5% RBC concentration instead of 40% (25). Even though a lower RBC concentration could induce hemolysis due to the increase of free polycation molecules exposed per unit of erythrocyte membrane area, the evaluated polymers were innocuous for the cells under the experimental conditions employed to form the polymeric shield.

3.4.2.3 Effect of PDMAEMA homopolymers on RBCs

After we ruled out the PDMAEMA-induced cell membrane damage, we evaluated by FACS the effect of homopolymers on 3 parameters: size distribution, granularity, and mean fluorescence intensity that should be correlated with the immunocamouflage of RBCs. Antigen glycophorin A was selected to evaluate camouflaging because it furnishes most of negative charges of the RBC membrane and it presents a short variation in density compared with the ABO antigens (32). Before using the copolymers, we were curious to test the effect of homopolymers on masking ability, allowing us to explore the effect of only the PDMAEMA polymer for camouflaging and compare it with the copolymers. For that purpose, we assessed coating the RBCs with either P1 or P2 (P1-RBCs and P2-RBCs, respectively), considering 2 polycation concentrations: 2 µg/mL and 10 µg/mL. In addition, aGPA-incubated RBCs as well as native RBCs were adopted as controls (see representative dot plots in Fig. 3.3). The latter presented size distribution and granularity on the left side of the dot plots (upper left and lower left quadrant) (Fig. 3.3A), the same as the PDMAEMA-RBCs. Surprisingly, when the P2-RBCs were incubated with aGPA, both size distribution and granularity were incremented. This augmentation was noticed on the right side of the dot-plots (upper right and lower right quadrants) (Fig. 3.3B), typically occurring because of cell aggregation.



A B

Fig. 3.3 Dot plots, granularity vs size distribution (x-axis vs y-axis, respectively) resulting from the analysis of (A) native RBCs and (B) P2 10 µg/mL-RBCs-aGPA. The RBCs incubated with only aGPA or with only P1 and P2 presented a RBCs distribution similar to A.

In view to understand the origin of cell aggregation mediated by aGPA, we included 2 additional controls. The first relied on incubating PDMAEMA-RBCs with bovine serum albumin (BSA), a protein present at 4 mg/mL concentration in the reagent solution of the aGPA. Hence, BSA could act as a cross-linkage between the RBCs because of its size and global negative charge at neutral pH. If PDMAEMA adsorbed to the RBC membrane, we would anticipate that the polycation will express positively charged domains outward that could interact with the anionic sites of BSA. We also included another control consisting of PDMAEMA-RBCs with a nonspecific RBC antibody, anti-CD45 (aCD45). This mouse IgG1 antibody, although having macromolecular features similar to aGPA (unlike BSA), is not likely to interact with human RBCs. Therefore, if a positive answer occurred, any response would result from nonspecific interaction and should proceed via FC fragment. For a better and more quantitative interpretation of the results, the percentage of events on the right side of each dot plot was subtracted from the percentage of events in the left side. Accordingly, if cell aggregation occurs, the percentage of RBCs (% RBCs) on the left side will decrease (Fig. 3.4). Interestingly, the results demonstrated that neither BSA nor aCD45 affected the % RBCs; this evidence supports the selectivity of RBC aggregation due to specific antigen-antibody recognition. This immune-cell agglutination was corroborated by optical microscopy; a comparison of the images confirms the specific RBC agglutination observed only under the P2-aGPA-RBC conditions (Fig. 3.5).

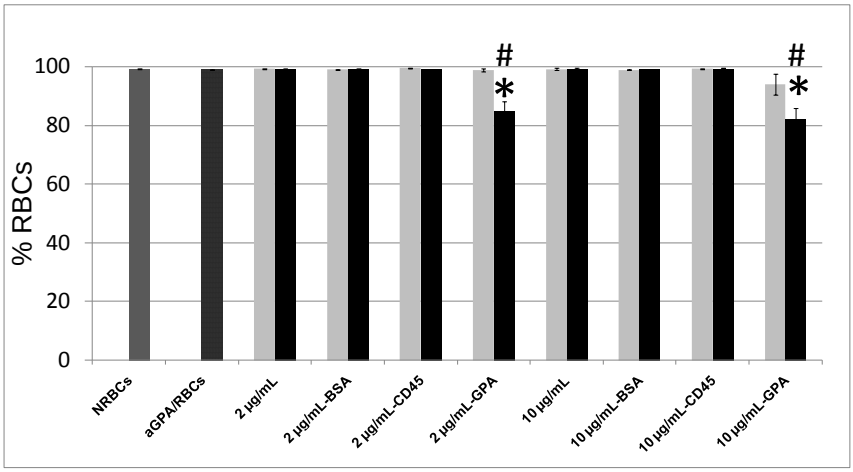


Fig. 3.4 Percentage of RBCs of the samples treated with PDMAEMA and incubated with aGPA, BSA, or aGPA. Gray bar: native RBCs; dotted dark gray bar: aGPA-RBCs; light gray bar: P1-treated RBCs; black bar: P2-treated RBCs. NRBCs: native RBCs; aGPA/RBCs: RBCs incubated with aGPA. Bars with (*): statistically significant differences between that sample and native RBC. Bars with (#): statistically significant differences between P2 and its corresponding P1. % RBCs of each bar is the average of at least 2 distinct replicas.

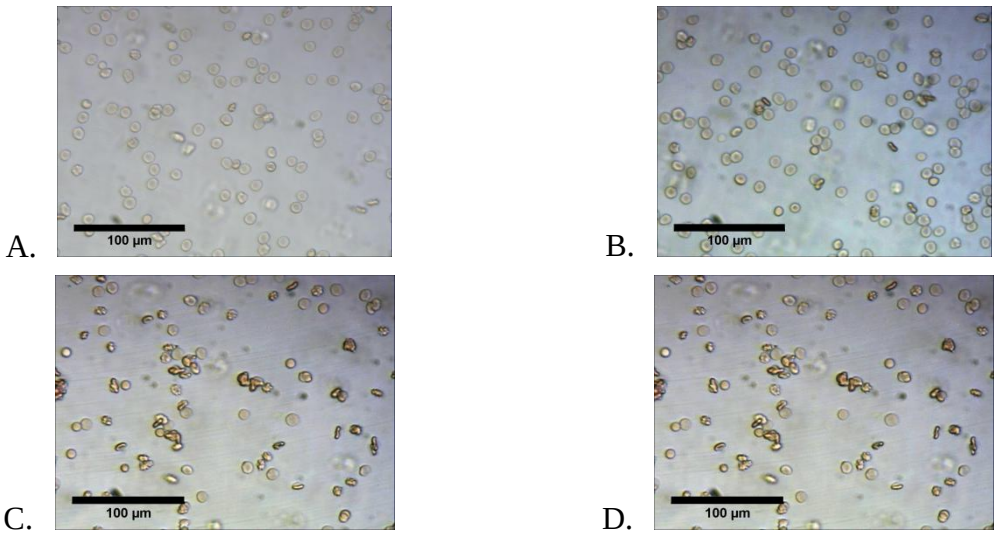


Fig. 3.5 Images obtained with optical microscope (×25). (A) Control sample, no aggregation; similar image was observed for all samples treated only with P1 or P2. (B) RBCs treated with aGPA, no aggregation is observed. (C) P1 10 µg/mL with aGPA, aggregated cells are observed; (D) P2 10 µg/mL with aGPA, aggregation bodies are observed. Bar represents 100 µm.

These observations showed that PDMAMEAs above Mw 10,000 and at concentrations of at least 2 µg/mL selectively enhance cell agglutination following immune recognition of the GPA antigens on the RBC surface. These unexpected results might be explained because the polycations neutralizes the electrical charges of the RBCs, (33, 34) an event that promotes RBC aggregation (30, 35).

However, in the range of PDMAEMA concentration and Mw considered in this section, we are far from totally neutralizing the RBCs. We have observed that P1 at 10 µg/mL reduces the RBCs' electrophoretic mobility from −2.85 to −2.50 µm.cm/volts without inducing cell aggregation (data not published); a similar effect of P2 can evidently be expected. In addition to the effect of PDMAEMA on the negative nature of RBCs, the aGPA could also participate in the reduction of zeta potential. GPA is a sialoglycoprotein composed of an intracellular, a transmembrane, and an extracellular domain (36). Because the latter domain contains a high sialic acid content, GPA is the main source of negative charges representing ~60% of the RBCs' negative surface charge (36). Accordingly, the principal function of GPA is induction of cell-to-cell repulsion, avoiding RBC aggregation (32). Taking into

account that aGPA is an IgG1 antibody with an isoelectric point in the range of 6.4 to 9.0 (37), its binding to GPA should contribute to neutralization of the RBC membrane under the conditions adopted within our experiment. Moreover, antibody binding may affect the electrical charges of the antigen (38). Another possible mechanism may rely on the double valency of aGPA-IgG, taking into account that reduction of RBCs zeta potential should promote cell bridging to facilitate interaction of the 2 binding sites of the IgG molecules with antigens expressed by the 2 adjacent cells. Therefore, aGPA molecules might act as a cell cross-linkage to promote RBC aggregation. It has been reported that other polycations, such as protamine and polybrene, also promote RBC antibody detection because of their ability to reduce intercellular distance (38, 39). Although these assumptions will need experimentaltions to support them, it would be valuable to valorize these observations as a means of enhancing immunoreaction for diagnostic purposes.

We also assessed the ability of homopolymers to camouflage the cells. The native RBCs incubated with aGPA presented a homogenous cell population showing a signal of fluorescence that was used as a reference point to determine the percentage of camouflaged RBCs. Surprisingly, PDMAEMA-RBCs presented a cell population with distinct regions of fluorescence. To normalize this deconvolution, we determined 4 clockwise regions (Fig. 3.6) based on the reference point of fluorescence (aGPA/RBCs): Region 1 (R1); events located there were exclusive of the analysis because they represented cell aggregates without value for the interpretation of other data; Region 2 (R2) corresponded to a higher MFI. If PDMAEMA-RBCs were located there, they were considered sensitized RBCs (sRBCs); Region 3 (R3) presented cells with MFI similar to the control RBCs, therefore PDMAEMA-RBCs located there were considered to be noncamouflaged RBCs (ncfRBCs); and Region 4 (R4); if PDMAEMA-RBCs were present, they were considered partially camouflaged RBCs. The percentage of R2, R3, and R4 cfRBCs was determined by the number of events located in each of these regions. For discussion purposes only, we described the sRBCs, ncfRBCs, and pcfRBCs as subpopulations.

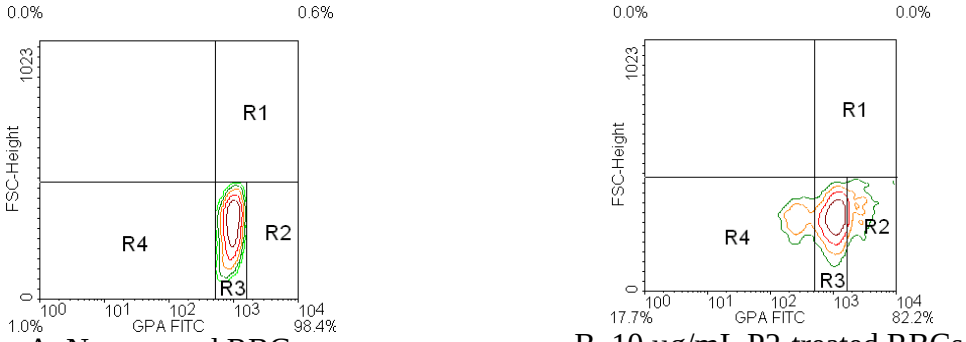


Fig. 3.6 Contour plots showing localization of R1, R2, R3 and R4. The y-axis measures fluorescence and the x-axis indicates size distribution. (A) Native RBCs incubated with aGPA, a homogenous cell population is located in R3. (B) P2-treated RBCs, a nonhomogenous population is observed; all cells located in R4 present partial camouflage.

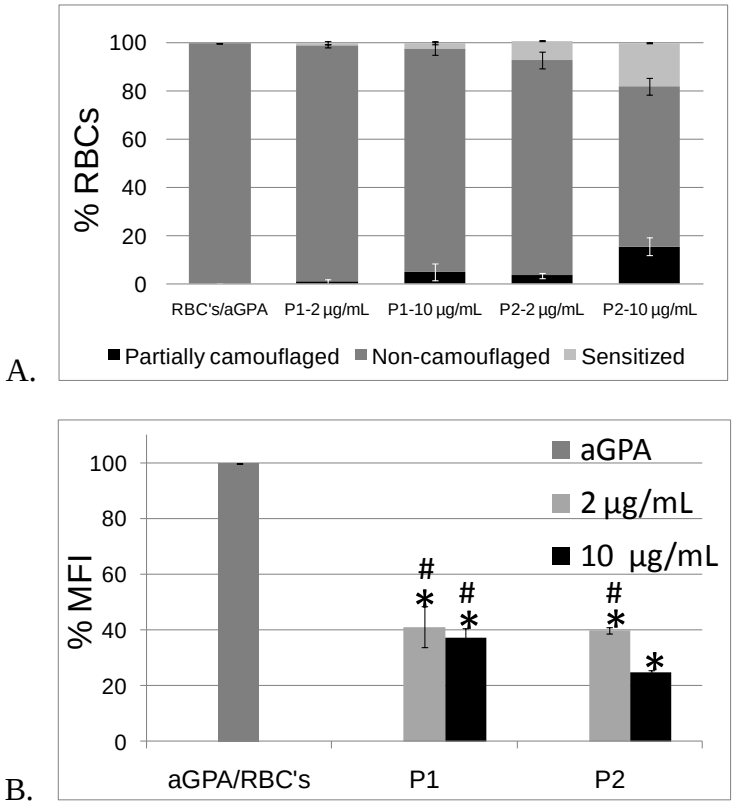


Fig. 3.7 (A) Distribution of percentage of partially camouflaged, noncamouflaged and sensitized RBCs in a PDMAEMA-RBC population represented by each bar. (B) % MFI Bars with (*): significantly statistical differences between that sample and native RBCs incubated with aGPA. Bars with (#): significantly statistical differences between those samples and P2 at 10 µg/mL. A bar is the average of at least 2 distinct replicas.

Although percentage camouflage efficacy remained low for all the conditions assessed, some dependence was observed in the function of PDMAEMA concentration and Mw (Fig 3.7A). Both increase in polycation concentration and increase in Mw augmented the antigen camouflage because the most effective protection was noticed (15.4 ± 3.7 % cfRBCs) in the presence of P2 at 10 µg/mL. To better evaluate the camouflage, we compared the percentage of mean fluorescence intensity (% MFI) of the protected cells (calculated by reference to the MFI of the native incubated with aGPA and considered as 100%) (Fig 3.7B). This parameter indicated that although most of the blood cells (up to 95%) kept GPA antigens accessible to the antibody, the protected cells demonstrated a strong reduction in % MFI (up to 80% reduction compared with the control cells) (Fig. 3.7B).

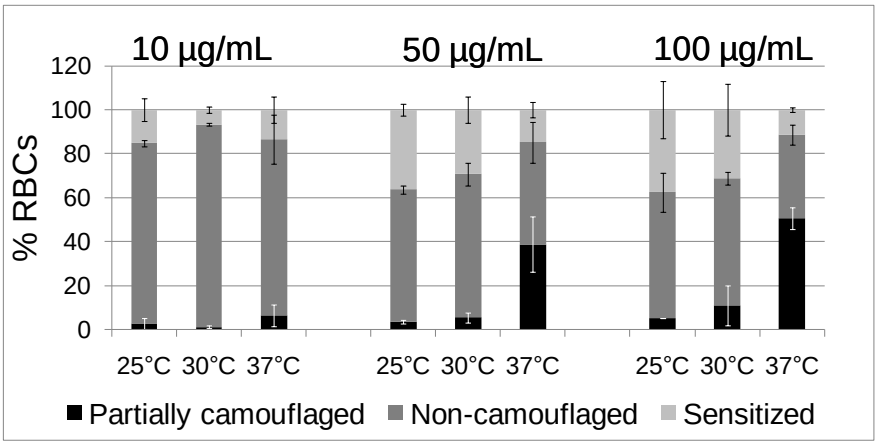
Although no total camouflaging was observed, this series of experiments highlights interesting findings on the heterogeneity in reactivity of the RBC population to the PDMAEMA's macromolecules. But before discussing these points, we estimated whether the number of polycation molecules added to the RBCs was enough to cover the entire surface of the cells. For that calculation, we started from 2 approximations: (1) 1 Mw 10,000–20,000 PDMAEMA molecule should cover 80 nm^2 of the cell surface (based on the hydrodynamic diameter of PDMAEMA in a solution of 10 nm), and (2) the area of an RBC is 135 μm^2 (40). On the basis of these data, full coverage of the RBCs would need about 1.7 million polycation molecules if they were to form a regular monolayer at the plasma membrane surface. Considering the concentration of the RBC suspension used for the coating (2.5% when diluted with the polycation solution), we can readily calculate that the 2 polymer concentrations used (ie, 2 and 10 µg/mL) should correspond to 50% and 250% of the total theoretical surface exposed by the RBCs. Although this calculation is limited by the exact conformation of the polycation macromolecules adsorbed to the membrane and of their compaction/surface density, the estimate provides an approximate picture of the polyelectrolyte molecules used to cover the cell membrane. In the present case, if this assumption does not take us too far from reality, our experimental conditions should permit completely coating the RBCs' surface with a 100 µg/mL PDMAEMA concentration, considering an association constant favorable for their migration to the surface. In contrast

to the apparent lack of reactivity of PDMAEMA to the RBC, the existence of an RBC with an increased percentage of FMI is astonishing. With respect to PDMAEMA-induced sensitization, it is well known that polycations such as protamine and polybrene enhance RBCs' antibody detection because of reduction in intercellular distance facilitating agglutination (38, 41). Thus, polycations act on the second stage of agglutination, that is, agglutination by itself, but they do not influence the first stage, sensitization (38). Surprisingly, taking into account that sensitized cells did not increase their size distribution (ie, cells located at Region 2), PDMAEMA-induced sensitization was not due to cell aggregation. RBC sensitization could have occurred because the polymer facilitated attraction of aGPA, something that is explained on the basis of antigen-antibody–binding interactions. When an antigen and an antibody randomly located at a nanometric distance from each other, they are attracted by the long-range forces of ionic and hydrophobic bonds. Then, these forces and bonds overcome the hydration energy (38). As result, the expulsion of water molecules from the antigen and antibody facilitates antibody binding (38). Therefore, considering that PDMAEMA may participate in increasing ionic bonds on the RBC surface, the named polycation could facilitate aGPA attraction. Additionally, because PDMAEMA has a 7.4 pKa—indicating partial protonation (50% of dimethylamino groups protonated) (42) — the polymer backbone can present both hydrophobic and hydrophilic areas. Possibly the hydrophobic ones promote physical removal of water molecules, also faciliting aGPA binding.

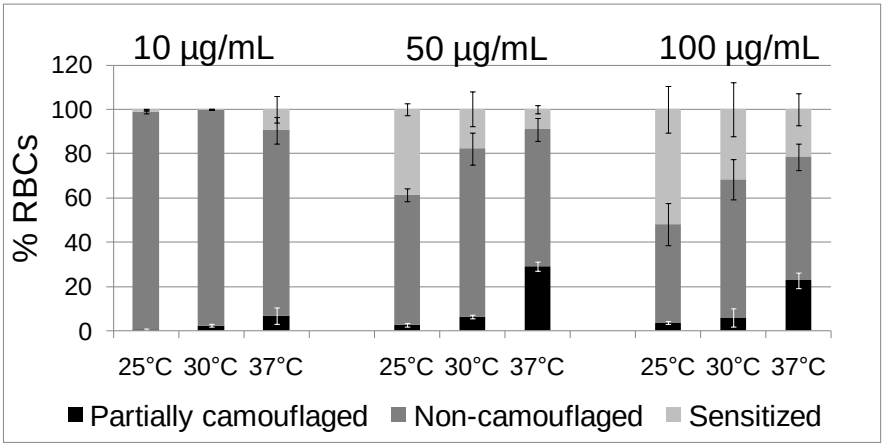
3.4.2.4 Effect of PDMAEMA copolymers on RBCs

Three distinct PDMAEMA-co-MAPEG copolymers (see Fig. 3.1 and Table 3.1) were used to assess the efficiency of the PEG sequence to provide the steric repulsion expected to increase the immunomasking action. The comparison should allow us to explore the influence of both the length of the PEG segments (oligomers of Mw 480 or 3050; comparing P3 and P4, respectively) and the PEG distribution along the PDMAEMA backbone (palm-tree vs. random distribution; comparing P3 and P4 vs P5, respectively).

A.



B.



C.

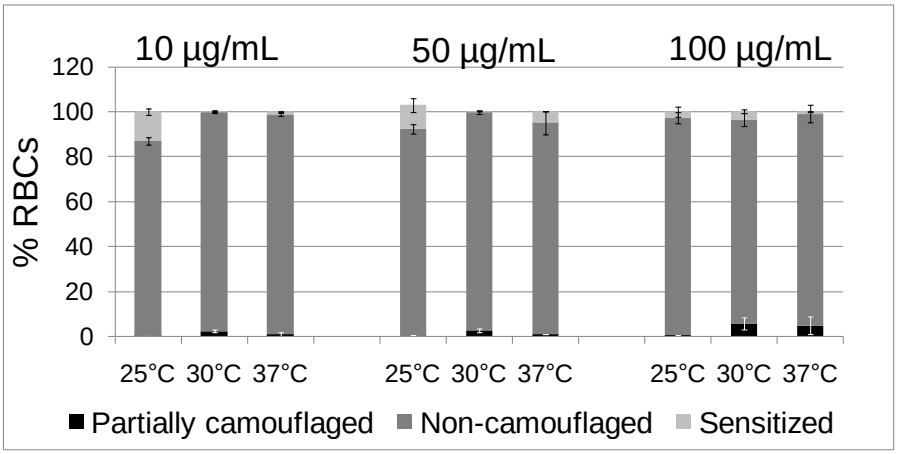


Fig. 3.8 Effect of temperature on percentage of RBCs. Distribution of percentage of pcfRBCs, ncRBCs, and sRBCs in a PDMAEMA-RBC population represented by each bar. (A) P3-treated samples, (B) P4-treated samples, and (C) P5-treated samples.

Riquelme et al. noticed that those copolymers affected the RBCs and induced aggregation at 250 µg/mL (24), thus we decided to use concentrations below 100 µg/mL to not only avoid those concerns but also because according to the above calculation (see paragraphs above), the concentration of 100 µg/mL should be enough to totally cover the RBC surface. Additionally, taking into account the thermoresponsive properties of PDMAEMA-based polymers (43), we compared the effect of 3 temperatures (25°C, 30°C, and 37°C) on immunomasking. To facilitate comparing hemoreactivity of the polycations, we integrated within Fig. 3.8 the evolution of the percentages of pcfRBCs, ncfRBCs, and sRBCs as a function of the 3 variables, that is, the nature and concentration of the copolymers and temperature.

From the data, 2 main observations can be noticed. First, P5 has a behavior different from the other copolymers, but also compared with the homopolymers. Indeed, whatever the conditions adopted, P5 did not significantly affect the FACS signals measured from the coated RBCs. Hence, although of short length, the PEG in P5 played a decisive role in the lack of cell reactivity. We expect that a statistical distribution of the PEG grafts along the PDMAEMA backbone hampers the ionic interaction of the copolymer on the RBC surface. This hindering process can be explained by the simple model of polymer adsorption schematized in Fig. 3.9. On the other hand, in the case of polymers P3 and P4, their PEG moieties are sufficiently separated from the polycationic sequence; therefore, that pseudo-block copolymer structure is more favorable to anchor the PDMAEMA to the cell plasma membrane while leaving the PEG extremity free. Clearly, architecture plays a major role in the adsorption of polymer and camouflage of RBCs. The second main observation that emerges from comparing these FACS data is the fact that when a pcfRBC population is observed, sRBCs also appear almost automatically. Moreover, when comparing the respective contribution of these 2 cell subpopulations in the various experimental conditions adopted, the fraction of sRBCs clearly surpasses the masked erythrocytes. For example, when incubated with P4 at 100 µg/mL at 25°C, this sRBC population accounts for more than 50% of the cell events compared with the less than 10% for the pcfRBCs. Also interesting is the evolution of the proportion of these 2 cell fractions with the temperature adopted to perform the coating. Whereas a reduction in sRBCs is readily noticed when

raising the coating temperature, the opposite is observed when done for the pcfRBCs. It is even more surprising that these changes in hemoreactivity occur within a very narrow temperature range.

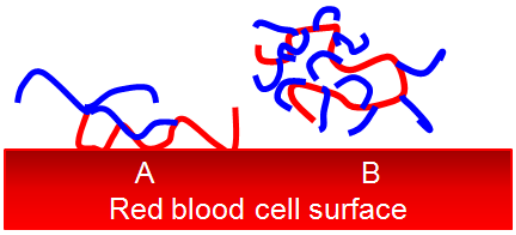


Fig. 3.9 Model of PDMAEMA-RBCs interaction. (A) Model for palm-tree copolymers adsorption, (B) model for statistical copolymer. Red lines represent PDMAEMA backbone whereas blue lines represent PEG chains.

These antagonistic behaviors should definitely find their origin in a modification in affinity for those copolymers having cell membranes affected by the temperature. It is known that the 2 polymer sequences in our polycations are thermoresponsive to a lower critical solution temperature in an aqueous solution of 30 to 40°C and 100 to 150°C for PDMAEMA and PEO, respectively, depending on their Mw and pH (43). For example, in the case of PDMAEMA, its full protonation at low pH does not allow its phase transition, which is observed only at either neutral or basic pH. temperatures, these polymers become insoluble in water, a typical behavior for the polymers forming hydrogen bonds with water. As a consequence, within this critical temperature zone, the physicochemical state of the macromolecules changes drastically with as a first step the conformational change of the molecules, which undergo coil-to-globule transition before achieving within a second step the cloud point, which can be observed macroscopically. Through this dehydration process wherein polycations result from breakage of the hydrogen bond, a definite decrease in polarity of PDMAEMA should be observed with a temperature increase and should promote their adsorption to the RBC membrane.

The temperature of phase transition of our copolymers should be increased by the presence of the PEG grafts compared with the homopolymer of DMAEMA. For example, Ward et al. have reported a cloud point of 72°C for PEGMA-DMAEMA copolymer (with theoretical sequence lengths of 2920–6580, respectively) when dissolved in an aqueous

medium at pH 7.8 (43). However, Jean et al. found that the cloud point corresponds to the macroscopic phase separation of thermoresponsive polymers that have already undergone molecular conformational changes at lower temperatures (44). In addition to this explanation, which needs support by a physicochemical characterization of our polycation solutions, we cannot rule out a kinetics contribution. Hence, the effect of temperature is clarified because if that factor increases, the Brownian movement of molecules will also increases, promoting contact between RBCs and PDMAEMAs and resulting in higher polymer adsorption. Considering that temperature increment has been reported as a factor enhancing polycation adsorption, it might occur.

After we evaluated the effect of temperature, the percentage MFI was calculated, but only for samples treated with 100 µg/mL, because the highest percentage of pcfRBCs was noticed (Fig. 3.10). Interestingly, the copolymers reduced the %MFI to that of the homopolymers. This fact shows that homopolymers by themselves can induce some grade of protection to the cell and that PEG was useless for masking. In this respect, the PEG in the PDMAEMA backbone might not have had the physical properties necessary for masking, especially considering the size of the PEG chains.

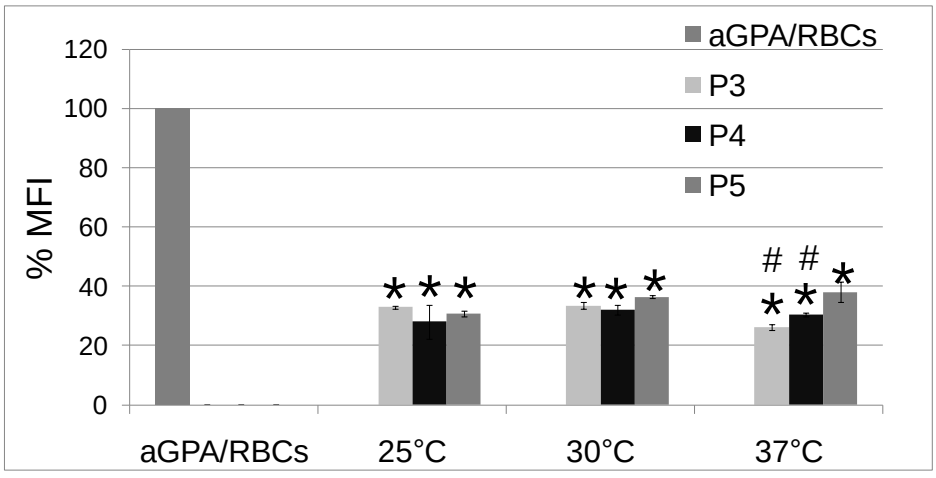


Fig. 3.10 % MFI of samples treated with 100 µg/mL. Samples with (*) showed significant statistically differences respect to RBC's/aGPA MFI %. Samples with (#) showed significant statistically differences respect to P5 at 37°C.

As reported by Bradley et al., antigen size and Mw of polymer are important in the efficacy of immunocamouflage. For antigenic determinants of Jka/Jkb blood groups farther out on from the cell surface, the Mw 5,000 PEG was inefficient for masking; but when Mw (20,000) and density increased, the PEG covered those antigenic sites (6). In our case, it was expected that PDMAEMAs exhibit enough volume to cover the GPA extracellular antigenic domain having a Mw of 22,000 (45) . But once adsorbed, the polycation molecule may change to conform to a more restricted area of protection. Moreover, on inert surfaces, polycation adsorption may take the form of trains or loops, depending on the difference in segment length between both the cationic groups on the polycation backbone and the anionic sites expressed at the surface (44, 46). If a similar adsorption behavior occurs on the RBC membrane, we should take into account that the RCB membrane is a living system wherein the lateral mobility of proteins could significantly influence charge redistribution (47). Thus, polycation adsorption on the RBC surface is not as predictable as on a static surface. This unpredictable behavior is well represented in our study when compared with the protection efficacy of the 3 copolymers. Although P4 presented the longest PEG chains (Mw 3050), it did not induce as low a percentage of MFI as might be expected according to the effect of the PEG Mw on camouflaging, as discussed above. Conversely, P5, having the lowest Mw PEG (Mw 455) induced the highest protection but had the lowest adsorption on the RBC population. Thus, the influence of molecular features of each copolymer herein employed is contradictory. In this respect, in addition to the already explained complexity of polymer-RBC interaction affecting polycation activity, we cannot ignore that differences between possible hemoreactivities caused by subject-to-subject variations or even by variations cell-to-cell. These latter, for instance, have already been suggested as factors influencing polycation-RBC adsorption and the polycation-induced effect on RBCs (48).

3.4.3 Conclusions

Among the 3 main strategies proposed in the literature to mask erythrocyte surface antigens, enzymatic cleavage, chemical grafting of PEG segments, and physical deposition of a polyelectrolyte multi- or monolayer, we have investigated the latter. Because of its simple application, this procedure should be the easiest to upscale for routine purposes in the clinic. If the final objective of this research was to achieve an efficient immunocamouflaging of human RBCs, we were from the beginning aware that it might be a challenging and perhaps unrealistic goal. For this reason, considering other possible applications of our polymers (in particular for diagnostic or drug delivery purposes), we selected as a second main aim of our research a better understanding of the hemoreactivity of tailored polycations. In a former study we reported on the hemocompatibility of 4 PDMAEMA-based polymers differing in Mw with or without a PEG sequence in the form of a palm tree–like structure (25). That study, performed according to the 5 categories of tests recommended in the ISO 10993–4, also stressed the need to implement additional biochemical and biophysical methodologies recommended to highlight the intimate molecular and cellular events involved in polymer cell interaction.

Herein, adopting a 1-step method to investigate a single polymer monolayer at the surface of the RBC, using either PDMAEMA or PDMAEMA-co-MAPEG copolymers, we have observed that none of the materials were able to fully protect the GPA binding sites from their antibodies. If there is any benefit of the PEG sequences of the palm-tree architecture being highlighted, that is when they are separated from the polycationic backbone to give rise to improved stability of the RBCs and a higher efficiency in immunomasking (up to 40 %). We expected more extreme differences in action between the homopolymer and copolymers as well as a higher advantage offered by the polyether sequences. Several possible explanations can be suggested for the lack of efficiency of our research strategy. Among them the simplest is a partial covering of the RBCs by the polycations due to a possible heterogeneity in repartition of the negative charge domains present on the RBC's plasma membrane. This argument has already been offered by Elbert and Hubbell to justify the immunoreactivity of the PLL-PEG that they have assessed to achieve full antigen

protection from the immune system; it is mandatory to have a homogeneous and total coating of the RBC surface (17). The other cause of poor protection is related to the PEG length as, discussed above.

In addition to complexities of polycation-RBC interaction, it should be noted that subject-to-subject variations may affect polycation adsorption and their subsequent effects on the RBC membrane. Even the heterogeneity of a cell population coming from the same blood donor can affect the interaction between RBCs and polycations. For instance, Sovadinova et al. have recently observed that the effects of amphiphilic methacrylate copolymers containing primary amino groups (Mw of copolymers: 2300–2800) on RBCs varied because of cell-to-cell differences (48). A major finding of that study was that the polycations induced nano-porosity in a heterogeneous pattern from cell to cell. The researchers interpreted their observation as a possible consequence of a variation in polymer adsorption profile among cells due to an erythrocyte-to-erythrocyte variation in sensitivity to pore formation (48). In the same context, polymer-induced blood response variations have also been reported by Armstrong et al., in this case regarding PEG- and dextran-induced RBC aggregation (49). In addition to this interindividual variability, the distribution of erythrocyte ages within a given a given blood sample with associated biochemical and cellular age-dependent variations has been used to explain the unpredictable polymer-induced blood response (49). Accordingly, Neu et al observed that RBCs glycoalix presented changes related to age and they affected on the RBCs-polymer interaction and cell aggregation (50).

Although outside our field of interest, the different polycations also induced sensitization to aGPA for a given fraction of the RBCs. Other than differences in cell reactivity within an RBC population and the observation that the process is temperature dependent, we have no direct experimental evidence to explain this finding. To our knowledge, no other authors have reported this evidence; it should be explored in the future. As a matter of fact, if our study has provided significant insights into understanding the PDMAEMA-RBC interaction—showing the complexities of the interplay between synthetic macromolecules and cell membrane components—it has also posed more questions on cell reactivity. The

cell sensitization process and the heterogeneity in cell response are 2 topics that should receive more attention in the future.

3.5 References

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4. GENERAL CONCLUSION

In the frame of this PhD, we have assessed the hemocompatibility and camouflaging antigen ability of PDMAEMA based polymers. Surprisingly, even though PDMAEMAs have widely been studied to tailor nanoparticles for biomedical use, their hemocompatibility properties have not been deeply investigated. Accordingly the first section of our thesis has been devoted to the better understanding of PDMAEMA-whole blood interactions with their consequences on blood. Based on this in vitro study we have selected the most appropriate functional PDMAEMA for RBC's antigen camouflaging. The camouflaging strategy relies on the fact that the polycation sequence of PDMAEMA should bind to negatively charged surface of RBC's while their PEG sequences should contribute to provide steric repulsion against antibodies. This polymer self-assembly coating, originally suggested by Elbert and Hubell (1998), has been recently investigated by some of us As a follow-up to these works we have extended the preliminary testing to better understand the PDMAEMA-antigen masking ability. In addition to the masking approach, this study of camouflaging ability of PDMAEMA has allowed to better understand red blood cell-PDMAEMA interactions. Hence, on the basis of our results, we have been able to drawn the following conclusions.

Hemocompatibility of PDMAEMAs

- As a first motivation to our hemocompatibility study, it is worthwhile to stress that hemocompatibility should be analyzed on a multi-factorial basis. Usually, hemocompatibility of xenobiotics is typically evaluated only by hemolysis and hemagglutination tests. However, hemocompatibility testing for biomaterials should include several test categories, such as recommended by the ISO 10993-4. As showed in our work, PDMAEMAs can indeed affect several blood elements including platelets, red blood cells, and blood coagulation.

-Although PDMAEMA-induced toxicity has been reported elsewhere, we observed that their PDMAEMA-induced hemotoxicity can be avoided when these polycations fit to particular chemical properties, i.e, Mw and polymer composition. Others factors such as blood exposition time and polymer concentration also influence their hemoreactivity. Our

results should be considered for the selection of appropriate polymers for tailoring nanoparticles designed for drug delivery purposes. For instance, the adoption of low Mw PDMAEMA (Mw 10,000) to prepare nanoparticles is definitely recommended to prevent any undesirable blood response. If higher Mw are needed, i.e. in the range of 25,000-40,000, the final polymer concentration to expose to the blood should be lower than 10 µg/mL to avoid undesirable blood responses.

- Presence of PEG moieties on PDMAEMA backbone partially improves the hemocompatibility. This protection effect was mainly noticed for hemagglutination, while the reactivity of the humoral components involved in hemostasis were not significantly reduced by the addition of these hydrophilic steric layer afforded within the copolymers. Indeed when blood coagulation was evaluated, both copolymer and homopolymers affect as same both the intrinsic or extrinsic pathways within the experimental conditions adopted in and adopting polymer concentration in a range of 10 and 100 µg/mL.

-Interestingly most of these polycations do no elicit complement activation. This contrast in hemoreactivity with coagulation can be explained due to specific routes adopted by each biological pathway and by a possible competitive interplay between polycations and the different proteins involved either in coagulation or complement activation. These concurrent interactions could be affected by the size, nature, structure and concentration of the proteins participating in each cascade.

- Hemocompatibility of PDMAEMAs may depend on subject-to-subject variations. This point has been in particular discussed for complement activation with an unexpected reactivity induced by polymer P2. Taking into account that this activation was not dose-effect related, we concluded that other factors assigned to subject-to-subject variations could explain these contradictory results. Moreover, subject-to-subject and even cell-to-cell variations are reported as factors influencing polymer-blood interactions.

Camouflaging effect of PDMAEMAs

The principle of polymer self-assembly has been applied to perform a polyelectrolyte layer (PEL) on RBC's. This self-assembly process of polymer to the cell plasma membrane surface should occur readily through the predominance of anionic sites present at the glycocalyx surface of the RBC's. Herein, we have assessed the immunomasking ability of PDMAEMA homopolymers differing in Mw. Their properties have been compared with PDMAEMA-co-PEG copolymers in view to better understand the correlation between the macromolecular features of these polymers and their intended functionalities. Immunomasking has been analysed adopting a fluorescent anti-glycophorin A as membrane probe and using FACS to quantify the cell immunoprotection. These comparative studies have allowed us to state the main findings outlined below.

-Only a partial cell immunoprotection has been observed adopting the PDMAEMAs, either as copolymers or homopolymers. At this point, although we obtained valuable findings, the PDMAEMAs based polymers are not suitable for masking antibodies. That scientific evidence, that have been already discussed, encourages thinking about the real possibility of totally camouflaging the RBCs antigens.

-PDMAEMA distribution on the RBCs population is heterogeneous. Possibly, this non-heterogeneous distribution occurs because of cell-to-cell variations. Age of cells, membrane protein mobility or charge distribution on the RBC's membrane are factors that can vary in the RBCs, hence, they can influence on the cell surface density of negative charges interacting with the PDMAEMA.

-PDMAEMAs induced RBCs sensitization for the anti-GPA antibody. Unexpectedly, but very interestingly, the PDMAEMA-base polymers causes a sensitization of the cells to their recognition by the aGPA antibody. This process has been shown to be temperature dependent and affected by the composition of the polycations. Future investigations would be requested to understand the origin and specificity in the enhancement of this biological

interaction. If it is applicable to other antigen-antibody interactions, the PDMAEMA will represent a valuable novel sensitizer agent in the optimization of diagnostic systems.

-PDMAEMA-RBCs interaction is a complex event depending on several factors. Our results has highlighted that the binding of PDMAEMA to RBCs membrane is significantly affected by several experimental parameters, among others, polymer concentration, cell-to-cell variations, polymer architecture, temperature and polycation molecular weight. Several hypotheses have been proposed to explain how these factors influence on the adsorption of PDMAEMA. Future investigations would be however needed to ascertain our assumptions. Moreover, little information has been published about polycation-RBCs membrane interaction occurring at molecular level. Insights about these interactions will help to better understand polycation adsorption on RBCs cell membrane. Accordingly, exploratory experiments have been performed using electrophoretic mobility analysis, scanning of RBCs membrane by atomic force microscope and confocal microscopy to evaluate the effects of PDMAEMA on cell membrane. Although preliminary, our results contribute with information supporting the explanation of PDMAEMA adsorption event, correlated to some modifications in erythrocyte membrane morphology.

As already mentioned, PDMAEMAs are valuable polymers for biomedical applications, especially for the tailoring of nanoparticles for drug or gene delivery. If the nanoparticles are injected in blood, they will contact several blood elements that can interact with the PDMAEMA. Accordingly hemocompatibility testing is an important step in the prescreening phase of these new vectors. Although performed on human blood, thus closer from the clinical reality, our study remains outside from the in vivo complex reality. Future studies in vivo will be requested to evaluate the safety character of these synthetic polymers and the effect of molecular weight which allows the kidney clearance.

On the other hand, although we have realized the great challenge existing behind our masking antigen strategy, the exploration approach has offered valuable results supporting the understanding of the PDMAEMA-RBCs interaction. Among these results, the unexpected sensitization effect remains as surprise. Definitely this observation would merit

additional assessment in order to better understand such event. This evidence could also open a new door for the application of these polycations in the biomedical area.

5. APPENDIX

Appendix 1. Effect of PDMAEMA on electrophoretical mobility of RBCs

We evaluated the effect of PDMAEMA Mw 10,000 on the EM of RBCs. We used PDMAEMA Mw 10,000 to avoid RBC's aggregation and obtain reliable results. The measuring was performed as following. A DELSA 440SX (Coulter-Beckman,) equipment was used to analyze the scattered light coming from the planar sheet of the cell, at four different angles simultaneously: 8.6, 17.1, 25.6 y 34.2°. Standard beads were used as reference for conductivity and mobility. The measuring was performed in standard operating conditions, adopting a 10 V/cm electric field, pH of 7.4 at 25°C, an on – off time of 2.5 and 0.5 sec respectively, a frequency range of 500 Hz and a frequency shift of 250 Hz. The analysis of the EM for the non-treated and PDMAEMA-treated RBCs (see chapter 3; 3.4.1.5 Polymeric coating of the RBCs and evaluation by FACS) was carried out on mobility analysis solution (NaCl 5 mM; HEPES 1 mM; sucrose 28 mM; pH 7.4) (~ 1700-1800 cells/µL). A one way-ANOVA test was used to obtain possible statistical differences between control (non-treated) and treated RBCs.

Results showed a correlation between polymer concentration and reduction of EM: the higher the concentration, the lower the EM (Figure App.1). Effect of polycation concentration on EM of RBCs has been observed also by Grandfils et al (1). They observed that EM decreased when spermine (Mw 348) concentration augmented (10 µM, 28 µM, and 40 µM) (1). Accordingly, increment in polymer concentration facilitates the availability of free PDMAEMA and more cationic molecules adsorbed onto the cell surface membrane. Our experiment demonstrates that PDMAEMA affects the electrical net charge of the RBCs, this event might occur only by adsorption of the polycation on the RBCs surface. Evidencing of adsorption is important because it should be the first step for the binding of the polycation intending camouflaging of antigens.

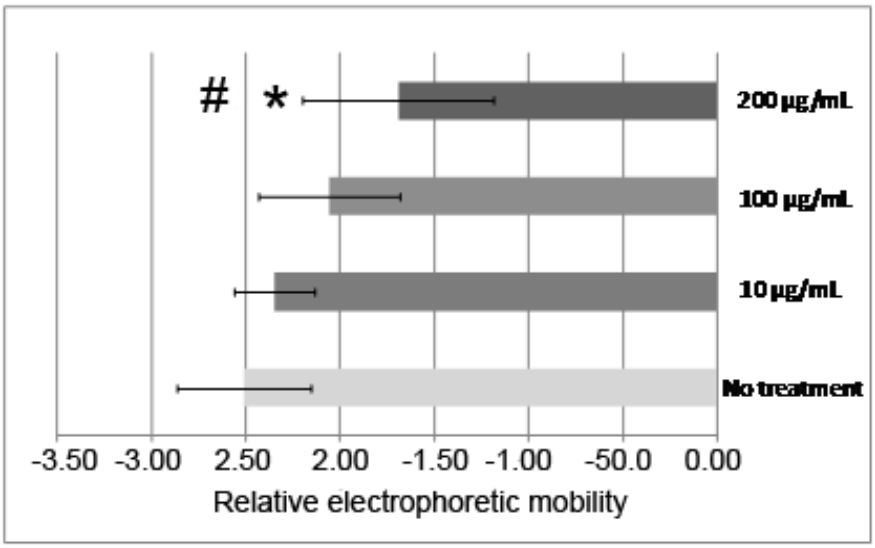

Fig. App. 1 Relative electrophoretic mobility (µm.cm/volts.s) of non-treated RBC's and treated with 3 distinct concentrations of PDMAEMA. * $p < 0.1$ represents the comparison with the control. # $p < 0.1$ represents the comparison with 10 µg/mL

The series of experiments were done with participation of Professor Elías Pérez and pre-grade student Mayela Romero. They both visited the CEIB (July, 2009 and December, 2009, respectively) in the frame of collaboration between the CEIB (ULG, Belgium) and the Laboratory of Polymers on the Physics Institute of the UASLP (México).

1.2 References

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Appendix 2. Effect of PDMAEMA on roughness of RBC cell membrane surface

The atomic force microscope is a valuable tool for scanning the cell membrane surface and it has been used for measuring the roughness of RBCs. Girasole et al. have described that roughness is a significant value to study changes on the RBC's membrane (17, 18). Roughness can be measured as mean roughness value (R rms), this value is associated to the width of the Gaussian distribution of the height of the points composing the AFM image of the scanned surface (1). R rms is a reliable parameter of evaluation because it is a constant value on the membrane of a single RBCss and on the membranes of a RBCs population (1, 2). Accordingly, we employed the AFM to evaluate the effect of PDMAEMA on the R rms of RBCs.

Briefly, the method was performed as following. Glass slides (2 cm x 2 cm) were treated during 20 minutes in a UV lamp chamber. This chamber was filled with O_2 in order to produce ozone to eliminate any organic traces in the glass slide. Then, each glass slide was submerged into 500 µL 1% poly ethyleneimine (PEI) solution to modify the negative glass surface onto positive. After it, the glass slide was washed with PBS to remove excess of PEI. After that, 15 µL of each experimental sample (RBCs treated with 10 µg/mL PDMAEMA Mw 10,000; or with Mw 40,000, or not treated [see chapter 3; 3.4.1.5 polymeric coating of the RBCs and evaluation by FACS]) were dropped in the center of the glass slide. Immediately, 50 µL of 1% glutaraldehyde were dropped on the cells. Samples were dried at room temperature during 15 minutes. The samples were washed with PBS to remove the excess of glutaraldehyde. Finally, samples were dried at room temperature during 20 minutes. A Nanoscope II AFM (Digital Instruments, Santa. Barbara, CA, USA) equipment was used to image the samples. Tapping mode was applied for the scanning. Three independent experiments were performed. Twenty RBC's were measured to get the diameter. Three single cells were analyzed to get the R rms. The analysis of samples was done with the software WxSM (Nanotec Electronica S.L., Madrid, SP).

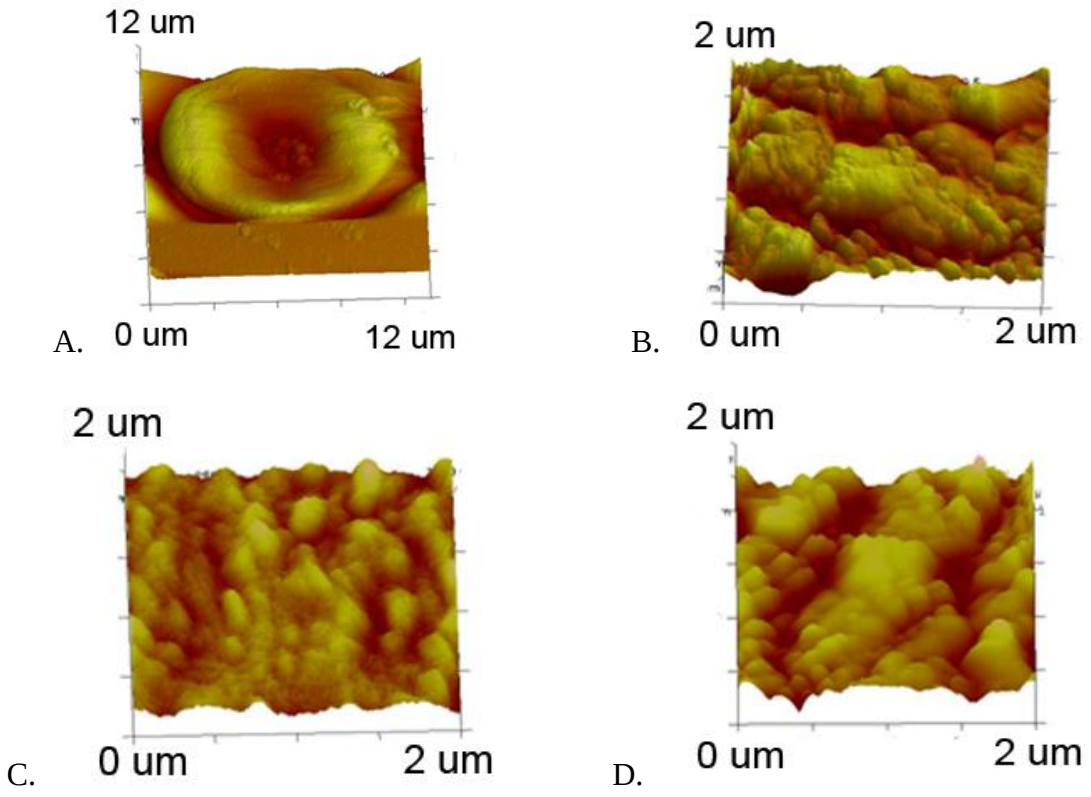

Fig. App.2 (A) Non-treated RBC with typical cell morphology. (B) Cell membrane surface of non-treated cell (R rms 3.29 nm), (C) Cell membrane surface of Mw 10,000-treated RBC (R rms 4.16 nm), and (D) Cell membrane surface of Mw 40,000-treated RBC (16.3 nm).

After analyzing the acquired images, we founded that RBC diameter was 10.6 µm (SD 0.9 µm), 10.6 µm (SD 0.8 µm), and 10.7 (SD 0.9 µm) for non-treated RBC's, PDMAEMA Mw 10,000-treated RBCs and PDMAEMA Mw 40,000-treated RBCs, respectively. The R rms value was 3.29 nm, 4.16 nm and 16.3 nm respectively for the non-treated RBC, the Mw 10,000-treated RBCs and the Mw 40,000-treated RBC (Figure App.2). The R rms of non-treated RBCs was similar to that reported for Girasole et al (R rms 3.14 nm) (1). On the other hand, we noticed that R rms incremented when PDMAEMA contacted the RBCs. Because polycations can induce phosphatidylserine migration from inner to outer membrane leaf (3, 4) and flip-flop effect on lipid membranes (5, 6), the change of R rms might occur because adsorbed PDMAEMA induces ultra-structural perturbations on the RBC membrane. Although limited on number of samples, our experiment demonstrated

that a topographical parameter, the R rms, was affected by the PDMAEMA. However, it will be necessary future evaluations for better support our observations.

This series of experiments were made in collaboration with the Professor Jorge Menchaca, in the Université Paris Sud 11, Laboratory of Physic of Solids.

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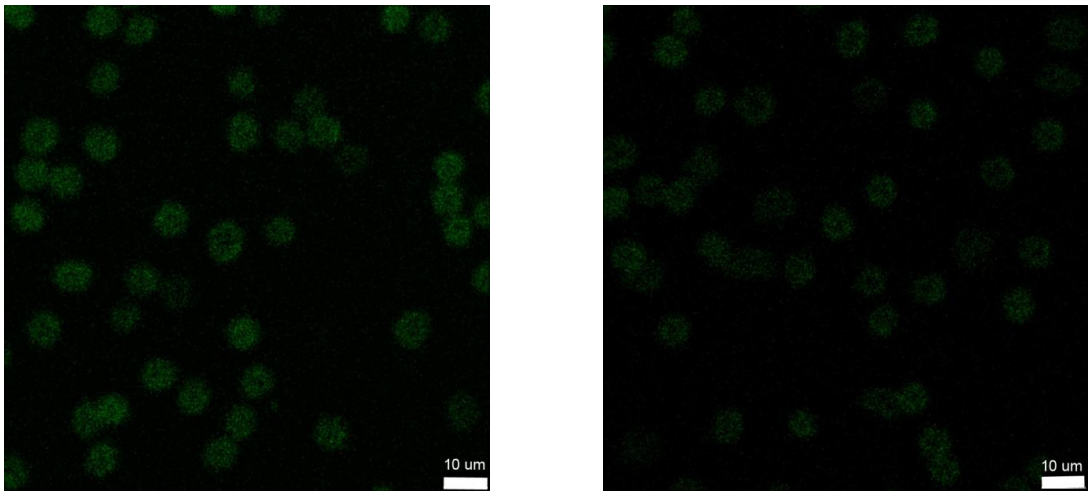
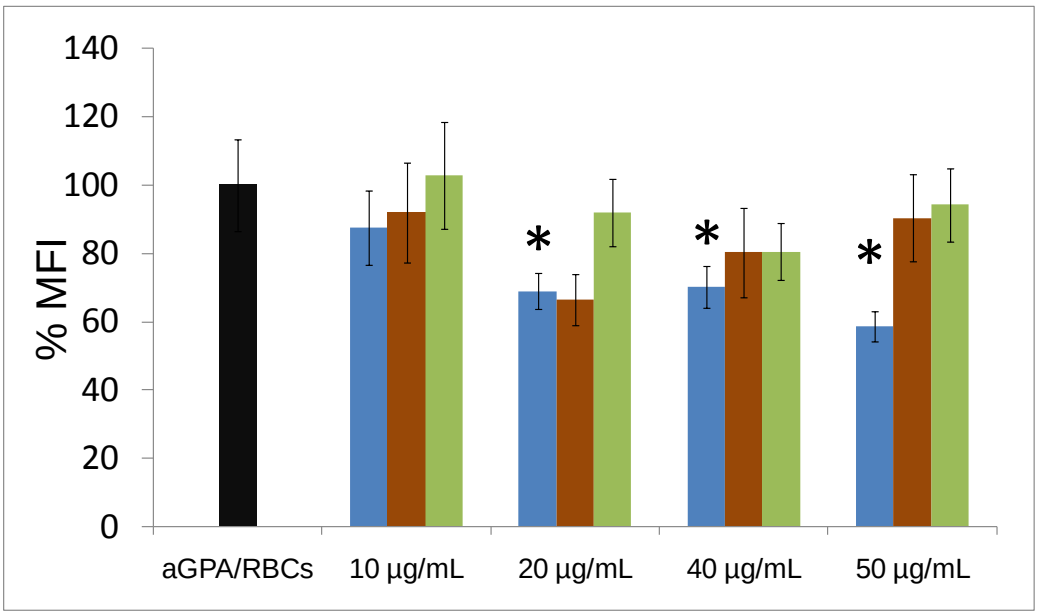
Appendix 3. Evaluation of PDMAEMA camouflaging efficiency by Confocal microscope

We evaluated the camouflaging ability of 2 different PDMAEMA-co-MAPEG of palm-tree architecture and 1 copolymer of statistical architecture (P3, P4, and P5 copolymers, see Chapter 3, Fig. 3.1). Previously, Riquelme et al noticed that PDMAEMA-co-MAPEG copolymers blocked partially anti-glycophorin A antibodies. They evaluated immuno-camouflaging by measuring agglutinated shape parameter and fluorescence density by confocal microscope (1). Accordingly, we used a confocal microscope (Confocal Leica DMI4000B) to measure the % MFI of copolymer-treated RBCs but we adopted particular conditions to avoid cell aggregation. Thus, a 5 % RBCs suspension was treated with 10-50 µg/mL of the copolymers whereas Riquelme et al. used a 12 % RBCs suspension and polymer concentrations ranging 100-250 µg/mL. Here, RBCs were treated with the copolymers and incubated with anti-glycophorin A (aGPA) as reported in section 3.4.1.5. The fluorescence intensity was measured on a region of interest representing a single cell. At least 2 replicas of experiments were performed and each time we measured the fluorescence intensity of 100 RBCs. If doublets or triplets of cells appeared, they were excluded from the measuring. A One-Way ANOVA and Tukey-Kramer HSD were performed to identify possible significant statistical differences between the native RBCs incubated with only aGPA (aGPA/RBCs) and treated-RBCs incubated with aGPA.

Results showed that all polymer concentrations of P3 reduced partially the % MFI while some concentrations of P4 and P5 reduced also the fluorescence but some others augmented this value (Fig. App. 3). Consequently, no polymer concentration dependency was noticed for P4 and P5 hemoreactivity.



A. B.



C.

Fig. App. 3. (A) Representative image of aGPA/RBC's; no aggregation was observed. (B) Representative image of 10 µg/mL/P5-treated RBCs. Compared with the RBCs in image A, the RBCs in image B have a weaker intensity of green color, i.e. fluorescence intensity. (C). Graphic representing the % MFI of aGPA/RBCs and PDMAEMA-co-MPAEG treated RBCs. Blue bar is for P3-treated RBCs, the brown bar is for P4-treated RBCs and the green bar for P5-treated RBCs. Bars with (*) show statistically significant differences in comparison to the blood control ($P < 0.05$).

These results support two major observations that have been noticed by evaluating immune-camouflage by FACS. First, the PDMAEMA-co-MAPEGs have shown ability for blocking partially the aGPA antibody, but at same time they induced sensitization of RBCs to the antibody. This sensitization was not correlated to cell aggregation. And second, taking into account that camouflaging and sensitization were not affected in a polymer concentration manner, the hemoreactivity of the copolymers might strongly depend on cell-to-cell variations. This assumption can be supported by the evidence reported by Neu et al (2). They founded that age of RBCs affects on the width of glycocalyx and this phenomenon is reflected on polymer-RBCs interactions (2). Evidently, considering that PDMAEMA-RBCs interaction is governed by electrostatic charges, the adsorption of the polymer might be heterogeneous on the RBCs surface because of variation on negative charge distribution. This event should be investigated in the future.

This series of experiments were performed in the Laboratory of Basic Sciences and Tissue Engineering of the UASLP (México).

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2. Neu B, Sowemimo-Coker SO, Meiselman HJ. Cell-cell affinity of senescent human erythrocytes. Biophysical Journal 2003;85(1):75-84.

Appendix 4. Published articles

Hemocompatibility assessment of poly(2-dimethylamino ethylmethacrylate) (PDMAEMA)-based polymers. **Cerda-Cristerna** BI, Flores H, Pozos-Guillén A, Pérez E, Sevrin C, Grandfils C. J Control Release. 2011 Aug 10;153(3):269-77. Epub 2011 Apr 28. PMID: 21550368

Increment in molecular weight of poly (dimethylamino-ethylmethacrylate) based polymers cause strong red blood cell aggregation but not hemolytic response.**Cerda-Cristerna** BI, Flores H, Pozos A, Perez E, Sevrin Ch, Grandfils Ch. J Control Release. 2010 Nov 20;148(1):e30-1. No