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

Bernardino Isaac Cerda-Cristerna [a,b], Héctor Flores [b], Amaury Pozos-Guillén [b], Elías Pérez [c], Chantal Sevrin [a], Christian Grandfils [a,*]

[a] Interfacultary Research Center of Biomaterials, University of Liège, Institute of Chemistry, Building B6C, Sart-Tillman (Liège), Liège (ZIP code 4000), Belgium
[b] Laboratory of Basic Sciences, University of San Luis Potosí, Faculty of Dentistry, Avenida Dr. Manuel Nava No. 2, San Luis Potosí, (ZIP code 78290), San Luis Potosí, México
[c] Laboratory of Polymers, University of San Luis Potosí, México, Institute of Physics, Avenida Dr. Manuel Nava No. 6, San Luis Potosí, (ZIP code 78290), San Luis Potosí, México

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ABSTRACT

Poly(2-dimethylamino-ethylmethacrylate) (PDMAEMA), a cationic polymer, has been widely reported as a nonviral carrier. Despite the fact that the cytotoxicity of this polymer has been extensively studied, there is a lack of information about its blood compatibility. Hence, this work evaluates the hemocompatibility of free-form PDMAEMA homopolymers differing in molecular weight (Mw) with or without a poly(ethylene glycol) (PEG) sequence in the form of a palm tree-like structure. Poly(ethylenimine) (PEI) was used as a reference in order to compare its hemoreactivity. Hemagglutination, hemolysis, platelet number, blood coagulation, and the complement systems were assessed in normal human whole blood according to the ISO 10993–4. Results showed that Mw, concentration, and incubation time strongly affected the hemocompatibility of the polymers evaluated. Our in vitro observations highlight that PDMAEMA homopolymers interacted strongly with the surface of the red blood cells but not with the inner structure of the membrane, while PEI behaved in the opposite way. No clear correlation has been evidenced between PDMAEMA-induced hemagglutination, PEI-induced hemagglutination, and hemolysis. Interestingly, if these polyelectrolytes strongly affect the platelets and blood coagulation cascades in a dose dependent way, none of them significantly affects the complement system. Our work reveals new knowledge on the toxicology of 2 families of polycations largely explored for gene delivery and on their mechanisms of cellular and humoral interactions.

1. Introduction

Polycations have been widely explored as nonviral vectors (polyplexes) for gene delivery [1]. Poly(amidoamine) (PAMAM), protamine sulfate, poly(L-lysine) (PLL), chitosan derivates, poly(ethylenimine) (PEI), and poly(2-dimethylamino-ethylmethacrylate) (PDMAEMA) are among the most common cationic polymers employed for such purpose. However, although they have shown suitable properties as gene carriers, at the same time they can cause in vivo or in vitro toxicity [2–4]. Toxicity requires major attention when using cationic carriers for IV administration because polycations can interact electrostatically with various negatively charged domains as found on blood elements such as red blood cells (RBCs) or plasma proteins [5]. It has already been reported that polycation–RBC interactions can provoke in vitro cell aggregation (hemagglutination) [5–7] or hemolysis [4–7], while polycation–plasma protein interactions can promote complement system activation [8,9] or delayed blood coagulation [10].

Evidently the hemoreactivity of polycations is governed not only by their inherent cationic charges but also by other molecular factors such as architecture of the polymer, molecular weight (Mw), or local polymer concentration. For instance, Fischer et al. observed that branched (Br) PEI and linear PLL showed higher hemolytic activity than globular PAMAM [5]. Domurado et al. reported that the Mw of PLL influenced on hemolysis and cell aggregation of RBCs [6]. Planck et al. noticed the same trend in the influence of Mw on the activation of the complement system, that is, longer PLL chains being more reactive than shorter ones [8]. During injection, not only the polymer concentration but also the local concentration reached within the blood was determined, among other things, by the injection flow rate, homogenization speed, and hemoreactivity of the polycations. Moreau et al. observed that a 5-fold increment of partially quaternized poly(thio-1- (N,N-diethyl-aminoethyl)ethylene) (Q-P(TDAE)) significantly enhanced the percentage of released hemoglobin (% rHb) as well as hemagglutination [7]. Clearly, polycations can induce several blood responses, affecting both the cells and the biological cascades regulated by plasma proteins.

Therefore, hemocompatibility studies of these polymers should include sufficient tests to allow evaluating the main families of blood responses. In this respect, *ISO 10993–4: Biological evaluation of medical*

* Corresponding author.
E-mail address: C.Grandfils@ulg.ac.be (C. Grandfils).

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devices-Part 4: Selection of test for interactions with blood has clearly identified 5 categories for the testing implant devices intended to be in contact with blood: (1) thrombosis, (2) coagulation, (3) platelet behavior, (4) hematology, and (5) immunology [11]. We therefore recommended that, even at the prescreening level, hemocompatibility studies for polycationic carriers include these 5 categories of testing. Surprisingly, this has not been the case for 1 of the most common polycations employed as a nonviral vector—PDMAEMA.

PDMAEMA has gained popularity because it has shown similar transfection efficiency but lower cytotoxicity than the gold standard of nonviral vectors—PEI [12–14]. Despite the fact that the cytotoxicity of PDMAEMA has been widely addressed in several works [12–15], most hemocompatibility studies have been restricted to hemolysis and hemagglutination tests. Moreover, these studies have focused on polyplexes [14,16–19]. Consequently, the literature contains little information on the hemocompatibility of noncomplexed PDMAEMA. In blood, free-form (ff) polycations can be released from the polyplexes due to the increase in ionic strength and their encountering various plurianionic sites which can compete and destabilize the preformed polyelectrolyte complexes, thereby potentially increasing their blood reactivity [20]. These ionic complexes ultimately dissociate, thus enabling the pharmacological activity of the carried molecules. It should also be noted that PDMAEMAs are bioexcretable but not biodegradable. Any interaction of these synthetic polymers with blood elements can therefore modify their excretion, among other things, by modifying the Mw of the protein clusters that they could build (in case of association with soluble proteins) or by their interaction with various cell membranes (including the kidney glomeruli).

In an effort to reinforce our knowledge of the hemocompatibility of the ff PDMAEMA, we undertook the present study to evaluate the hemocompatibility of 3 distinct PDMAEMA homopolymers varying in Mw between 10,000 and 40,000. A copolymer with a P(DMAEMA-b-methacrylate) end functionalized polyethylene glycol macromonomer (MAPEG) palm tree-like structure was also evaluated. This type of copolymer has already shown suitable hemocompatibility properties due to steric shielding of PEG moieties [19].

The rationale behind the selection of the range of Mw represents a compromise between body clearance of the polymers, ability to generate polyelectrolyte complexes and hemoreactivity. Taking into account that PDMAEMA are non-degradable polymers, their use for parenteral applications require therefore selecting Mw which could facilitate their elimination, in particular through the kidney route. Keeping in mind this important specification, if higher Mw PDMAEMA have been reported promote gene transfection [14,21,22], (i.e. with Mw 100,000 up to 915,000), with formation of stable polyelectrolyte complexes, a Mw of 40 kDa has been considered as the highest limit to facilitate kidney elimination. Additionally, it has been already highlighted in the literature that high Mw PDMAEMA based polyplexes, when injected in the bloodstream, induce hemolysis and hemagglutination [14]. These adverse responses could be avoided using either low Mw (LMW) PDMAEMA or their pegylated copolymers as we have assessed in our current study. A BrPEI was adopted as also used by others as a reference material [13,14]. The hemocompatibility studies were based on ISO 10993–4. Extensively purified and well-characterized polymers were used in the experiments.

2. Materials and methods

2.1. 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.

2.2. Synthesis and characterization of PDMAEMA

PDMAEMA and P(DMAEMA-b-p(MAPEG) were synthesized by solvent-free, atom-transfer radical polymerization (ATRP) [19]. After polymerization, the polymers were purified in 3 successive steps consisting of chromatography realized on alumina support, precipitation in heptane, and dialysis against MilliQ (1 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 $CDCl_3$. 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 [23].

2.3. 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.

2.4. RBC aggregation

Briefly, in micro Eppendorf tubes (200 μL), 1 vol of polymer solution was diluted in 9 vol 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 s) 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 [24]. Two independent experiments were performed to support our results. Two distinct polymers batches were used to perform the tests.

2.5. Hemolysis test

The hemolysis test was adapted from Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM designation: F 756–00) [25]. Polymer solutions and blood were prepared and incubated as described in Section 2.4. After incubation, the samples were centrifuged for 5 minutes at 600g 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