Antiplatelet effect of differentially charged PEGylated lipid-polymer nanoparticles

Eduardo Fuentes\textsuperscript{a}, Basit Yameen\textsuperscript{b,c}, Soung- Jae Bong\textsuperscript{d,c}, Carolina Salvador-Morales\textsuperscript{f,g}, Ivan Palomo\textsuperscript{a,*}, Cristian Vilos\textsuperscript{d,c,**}

\textsuperscript{a}Platelet Research Laboratory, Department of Clinical Biochemistry and Immunohaematology, Faculty of Health Sciences, Interdisciplinary Excellence Research Program on Healthy Aging (PIEI-ES), Universidad de Talca, Chile

\textsuperscript{b}Laboratory of Nanomedicine and Biomaterials, Department of Anesthesiology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA, USA

\textsuperscript{c}Department of Chemistry, SBA School of Science and Engineering, Lahore University of Management Sciences (LUMS), Lahore, Pakistan

\textsuperscript{d}Laboratory of Nanomedicine and Targeted Delivery, Center for Integrative Medicine and Innovative Science; Faculty of Medicine, and Center for Bioinformatics and Integrative Biology, Faculty of Biological Sciences, Universidad Andres Bello, Santiago, Chile

\textsuperscript{e}Center for the Development of Nanoscience and Nanotechnology (CEDENNA), Universidad de Santiago de Chile, Ecuador 3493, Santiago, Chile

\textsuperscript{f}Bioengineering Department, George Mason University, Virginia, United States

\textsuperscript{g}Krasnow Institute for Advanced Study, George Mason University, Virginia, United States

Received 8 April 2016; accepted 15 October 2016

Abstract

PEGylated nanoparticles have been extensively investigated in different platforms for drug delivery. However, the physiological effects related to platelet activation, and the potential procoagulant activity which could lead to thrombosis and further cardiovascular diseases have not been widely examined. In this work, we studied the effect of differentially charged PEGylated lipid-polymer nanoparticles in the human platelet aggregation and activation by light transmission aggregometry and flow cytometry. PEGylated nanoparticles inhibited the platelet aggregation with a dose dependency (350, 700, and 1400 μg/mL) in both ADP- and collagen-induced platelet aggregation, and P-selectin expression. Charged nanoparticles (anionic and cationic) presented higher inhibitions of the platelet aggregation compared to neutral nanoparticles, and particularly the cationic particles generated a slightly higher effect. The obtained results demonstrated the safety of the differentially charged PEGylated lipid-polymer nanoparticles, and their ability to inhibit the aggregation and activation of human platelets stimulated by two classic platelet activators.

Keywords: Platelet; PEGylated nanoparticle; Lipid polymer; Charged nanoparticle

Recent advances in nanotechnology and nanomedicine have gained a significant momentum as well as an intense scientific research on nanoparticles-based drug delivery systems has proven a broad diversity of applications in several fields of biomedicine and particularly on pharmaceutical technology.\textsuperscript{1,2} Among diverse types and biomaterials of nanotechnology being investigated, the nanoparticles (NPs) coated with polyethylene glycol (PEG) (PEGylation) are of singular interest. Studies have shown that PEGylation of particles increase their time in the bloodstream, decreases their recognition by macrophages and reduces the opsonization significantly which all strongly supports the use of PEG in the development of drug delivery platforms.\textsuperscript{3,4} PEG is highly hydrophilic, non-cytotoxic and has become the most widely used NPs surface coating material approved by regulatory authorities including the US Food and Drug Administration (FDA). Studies have demonstrated that the PEG density on the nanoparticle surface is a critical factor that modulates nanoparticle circulation times, crossing through the blood–brain barrier and cellular uptake.\textsuperscript{5–7} Furthermore, the level of hydrophilicity/hydrophobicity and the surface charge of NPs are the major properties that govern the interaction of NPs and cells. Although hydrophobicity is required to enhance the interaction of nanoparticles with the cellular membrane to stimulate the uptake into cells, hydrophilicity is also required to...
obtain a good dispersion into the blood and to prevent aggregation of NPs. Moreover, due to the negative charge of cellular membranes, cationic NPs exhibit better affinity and display high levels of endocytic cellular uptake. However, highly cationic NPs can also be cytotoxic generating disruption of the cellular membrane and changes in the intracellular concentration of calcium. Several authors credit the proper charge of NPs for achieving a suitable drug delivery in terms of circulation time cell internalization, blood clearance kinetics or biodistribution. However, the effect of surface charges of PEGylated polymeric nanoparticles on the platelet function has not been widely investigated. The function of the platelets is closely associated with the etiology of thrombus formation and cardiovascular diseases (CVD). CVD are the leading cause of death worldwide and cover diverse pathologies including coronary heart disease, cerebrovascular disease, deep vein thrombosis and pulmonary embolism, among others.

Figure 1. (A) Schematic illustration of PLA formulations DSPE-PEG(2000) carboxylic acid–terminated (anionic), methoxy–terminated (neutral), and amino–terminated groups (cationic). (B) Size of the nanoparticles in distilled water and PBS measured by DLS. (C) Image of nanoparticle formulations after 72 h at room temperature. (D) Zeta potential variation of different nanoparticles used in this study.

Figure 2. Representative transmission electron microscopy (TEM) images of the nanoparticles with carboxylic acid–terminated (A), methoxy–terminated (B), and amino–terminated groups (C). The scale bar is 100 nm.
processes are characterized by lesions in the vascular endothelium, impaired blood flow and changes in coagulation (hemostasis). Studies in patients with ischemic disease blood have shown that platelets play an essential role in the pathogenesis of arterial occlusive disorders due to the state of hyperaggregability of patients. The hyperaggregability state also has been described in patients with advanced cancer, which the platelet activation occurs in consequence of the release of pro-inflammatory cytokines and the expression of procoagulant molecules in the cell membranes of tumor cells. Platelets are anucleate cells with discoid shape, and size between 1.5 and 3 microns produced from megakaryocytes from bone marrow. Platelets contain granules loaded with growth factors (platelet-derived growth factor, platelet factor 4, etc.), ions (calcium, magnesium, etc.) and small molecules (ATP, ADP and serotonin). The platelet aggregation is initiated with binding of specific agonists (collagen, ADP and arachidonic acid, among others) under physiological conditions to certain receptors/glycoproteins located on the surface of the platelet. This binding triggers a series of signals inside platelet (mobilization of Ca²⁺, protein phosphorylation, synthesis of thromboxane and phosphatidylinositol), which induce cytoskeletal changes, reorganization of surface molecules and release of the substances contained in the granules.

Understanding the physiological interaction of nanoparticles intended for diagnosis and treatment of diseases is a pivotal factor in the success of modern nanomedicine. The first level of interaction of nanoparticles is the blood, and their safety in the immune cells and complement activation have been previously addressed. However, it is well known that cardiovascular diseases are the most frequent cause of death worldwide and that CVD are closely associated with processes of platelet activation and thrombosis. In this context, as the platelet activation involves the interaction of its proteins localized on the surface of the cellular membrane with proteins of blood and endothelial cells, the potential contact with NPs could induce platelet activation with a consequent thrombotic effect. To analyze the effect of the PEGylated nanoparticles with a different surface charge on the platelet function, we synthesized three PLA formulations with DSPE-PEG (2000) methoxy–terminated, carboxylic acid–terminated, and amino–terminated groups (Figure 1, A).

**Results**

Nanoparticles with a diameter ~ 100 nm have shown potential therapeutic applications due to their improved cell uptake by clathrin-dependent endocytosis, clathrin-independent endocytosis,
and caveolae-dependent endocytosis. In this work, the differentially charged PEGylated lipid-polymer nanoparticles were formulated using the nanoprecipitation method because this technique presents high reproducibility, and generates particles with homogeneous size. The diameter of the nanoparticles was determined by dynamic light scattering (DLS) in samples suspended in distilled water, and phosphate buffered saline (PBS) (see Figure 1, B). The anionic nanoparticles (PLA-peg@COOH) examined in distilled water exhibited a slightly smaller average diameter than neutral (PLA-peg@CH₃) and cationic (PLA-peg@NH₂) formulations (77.1 ± 4.6 nm versus 105.6 ± 3.1 and 98.4 ± 6.6 nm respectively). In contrast, the measurements of the size in PBS were consistent throughout three different formulations with diameters of 110.8 ± 3.0 nm, 116.4 ± 3.7 nm, and 113.2 ± 2.9 nm for anionic, neutral, and cationic nanoparticles respectively. In contrast, the measurements of the size in PBS were consistent throughout three different formulations with diameters of 110.8 ± 3.0 nm, 116.4 ± 3.7 nm, and 113.2 ± 2.9 nm for anionic, neutral, and cationic nanoparticles respectively. The differences between the size obtained from the same samples suspended in distilled water and PBS were expected because the presence of charges (ions) in the PBS solution decreases the temporal motion fluctuations of nanoparticles. On the other hand, the nanoparticles exhibited a suitable stability because each formulation remained transparent without signs of precipitation or agglomeration after 72 h at room temperature (Figure 1, C). This is only a qualitative assessment; however, all experiments were performed with fresh samples prepared on the day of each experiment. The surface charge is one of the major properties that provide the stability of nanoparticles. However, our recent studies by molecular simulations have shown a significant effect on the molecular arrangements of PEG chains in the surface of nanoparticles, and its interaction with the hydrophilic environment. Despite the neutral charge of PEGylated nanoparticles, the presence of that polyethylene glycol allows to create a proper interface between the nanoparticle core and the hydrophilic environment. Therefore, the stability was adequate even after 72 h at room temperature. Also, the stability of nanoparticles responds to the molecular weight of the polymer. In our case, the PLA core was prepared with a high molecular weight, generating a dense polymeric core that needs a longer period for hydrolysis process. As we expected, DLS measurements confirmed the anionic, neutral and cationic surface charge of nanoparticles with values of -23 ± 7, -1 ± 6, and 18 ± 7 mV, see Figure 1, D. The analysis of samples by transmission electron microscopy (TEM) exhibited spherical shape nanoparticles, with a uniform diameter, and high electron density on the surface of nanoparticles due to the presence of DSPE-PEG groups that absorbs a higher amount of staining agent (Figure 2). The central motivation of this study was to analyze the safety of PEGylated nanoparticles differentially charged on its potential platelet proaggregant activity able to cause thrombosis by the study of the human platelet function (platelet
aggregation model). Doses of 350, 700, and 1400 μg/mL of nanoparticles were analyzed, and interestingly the platelet aggregation was inhibited by the PEGylated nanoparticles with a dose dependency in both ADP- and collagen-induced platelet aggregation (Figures 3 and 4). To corroborate the antiaggregant activity of the nanoparticles, we studied the inhibitory activity on ADP or collagen-induced platelet P-selectin expression by flow cytometry since platelet P-selectin plays a major role in arterial thrombogenesis. As shown in Figure 5, ADP 8 μmol/L or collagen 1.5 μg/mL induced platelet P-selectin expression was inhibited by different concentrations of the nanoparticles. Thus P-selectin expression was inhibited by positive, negative and neutral nanoparticles. Interestingly, charged nanoparticles (anionic and cationic) produced a higher inhibition of the platelet aggregation compared to neutral nanoparticles, and particularly the cationic particles due to a slightly greater effect. Despite the fact that the antplatelet effect was significant at those higher concentrations evaluated (700 and 1400 μg/mL), the safety of the differentially charged PEGylated lipid-polymer nanoparticles regarding potential platelet proaggregant activity was demonstrated.

Discussion

Last years have been a steady increase in commercially available nanoparticles-employed drugs and a massive influx in some researches exploiting a variety of nanoparticle platforms across the globe. Most of these researches focus on the therapeutic outcome of nanoparticles, and there has been a limited study on unforeseen physiological effects related to platelet activity induced by nanoparticles. CVD, the leading cause of death worldwide, is often caused by platelet activation and aggregation leading to thrombosis. In recent years, some light has been shed on how variations in physicochemical properties of nanoparticles such as surface charge and size might affect platelet function although the exact mechanism is still yet to be defined. There have been multiple studies indicating that charged nanoparticles of different materials induce platelet activation and aggregation. Particularly in a study by Geys et al, negatively charged nanoparticles triggered coagulation leading to fibrin formation and simultaneous platelet activation by thrombin while positively charged amine nanoparticles enhanced the ADP-induced platelet aggregation. In another study by Dobrovolskaia et al, different formulations of PAMAM dendrimers varying in size and surface charges were investigated and demonstrated that cationic, but not anionic or neutral, particles induce platelet aggregation in vitro. While Dobrovolskaia et al also concluded, after an extensive study on the blood contact properties of the particles, that colloidal gold nanoparticles induced no platelet activation and thrombosis. In an investigation conducted by Balakrishnan et al, aggregation and adhesion of platelets were observed when human whole blood was exposed to polyvinyl chloride resin particles, however, when the particles were coated with PEG, the particles did not induce to platelet aggregation and coagulation.

Conclusions

In the present study, the differentially charged PEGylated lipid-polymer nanoparticles (anionic, neutral and cationic) induced inhibition of the human platelets aggregation and activation stimulated by two classic platelet activators (ADP and collagen).
collagen) with a dose dependency (350, 700, and 1400 µg/mL).

Charged nanoparticles showed enhanced inhibition compared to the neutral particles. The obtained results demonstrated the safety of the differentially charged PEGylated lipid-polymer nanoparticles related with the platelet activation, and supports the use of polyethylene glycol (PEG) in the development of platforms for systemic drug delivery.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.nano.2016.10.010.

References


Graphical Abstract

Eduardo Fuentesa, Basit Yameenb,c, Soung- Jae Bongd,e, Carolina Salvador-Moralesd,g, Ivan Palomoa,⁎, Cristian Vilosd,e,⁎⁎

aPlatelet Research Laboratory, Department of Clinical Biochemistry and Immunohaematology, Faculty of Health Sciences, Interdisciplinary Excellence Research Program on Healthy Aging (PIEI-ES), Universidad de Talca, Chile
bLaboratory of Nanomedicine and Biomaterials, Department of Anesthesiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
cDepartment of Chemistry, SBA School of Science and Engineering, Lahore University of Management Sciences (LUMS), Lahore, Pakistan
dLaboratory of Nanomedicine and Targeted Delivery, Center for Integrative Medicine and Innovative Science, Faculty of Medicine, and Center for Bioinformatics and Integrative Biology, Faculty of Biological Sciences, Universidad Andres Bello, Santiago, Chile
eCenter for the Development of Nanoscience and Nanotechnology (CEDENNA), Universidad de Santiago de Chile, Ecuador 3493, Santiago, Chile
fBioengineering Department, George Mason University, Virginia, United States
gKrasnow Institute for Advanced Study, George Mason University, Virginia, United States

Scheme of differentially charged (anionic, neutral and cationic) PEGylated lipid-polymer nanoparticles, their morphological structure evaluated by TEM. Effect of cationic PEGylated nanoparticles on the platelet aggregation by light transmission aggregometry and flow cytometry.