Hemolysis as Expression of Nanoparticles-Induced Cytotoxicity in Red Blood Cells

Teodora Mocan, MD, PhD,

Abstract—Nanomedicine has a huge potential to bring benefits in prophylaxis, diagnosis and treatment of various diseases, with increased socio-economic benefits. Since most of the future applications of therapeutic nanoparticles (NPs) are based on intravenous/oral administration, experiments on their interaction with human blood components are of extreme importance. In this context, the knowledge of the degree to which damage on red blood cells following exposure to silver nanoparticles contributes to the overall toxicity of various NPs is still highly needed. The present paper unprecedentedly assesses the hemolytic impact of biomedical nanoparticles on red blood cells (RBCs).

Keywords—cytotoxicity, hemolysis, nanoparticles, erythrocyte, therapy

I. INTRODUCTION

Nanotechnology implies the development of materials and functional structures with at least one characteristic nanometric dimension. Biomedical nanoparticles have interesting electronic properties, based on the unique size-dependent features thus exhibiting highly photothermal effect with surface plasmon resonance and strong optical absorption.[1-4] Their unique chemical stability make these materials suitable for the attachment of biomolecules like peptides, pharmacologically active substances and genes. So far, these nanoparticles have proven several promising effects, such as antitumor[5] gene therapy vector[6], anti-inflammatory[7], antiviral[8] and antibacterial effects[9, 10]. Even though most literature data dedicated to therapeutic nanoparticles refer to in vitro toxicological assessments[11, 12], recent data brings evidences on their in vivo toxicity. Various animal models, including zebrafish[13], Sprague-Dawley rats[14-17], mice[18] showed an increased level of toxicity of these nanoparticles. Following administration, the transit of these nanoparticles through the blood flow constantly occurs independent of administration route.[14-19] Since most of the future applications of therapeutic nanoparticles are based on intravenous/oral administration, experiments on their interaction with human blood components are of extreme importance. In this context, the knowledge of the degree to which damage on red blood cells following exposure to silver nanoparticles contributes to the overall toxicity of various Nps is still highly needed.

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II. HEMOLYSIS

RBC lysis has been demonstrated for amorphous nanomaterials such as silica NPs, thus limiting their rapid application into human trials. The incriminated mechanisms is centered on silanol groups existent on the outer surface of NPs.[20] Also, tricalcium phosphate (TCP) and Hydroxyapatite (HAP) nanoparticles (NPs), highly used in various medical applications, have been proven to increase the hemolysis rate with more than 5 % at 1000µg/ml concentration of RBC exposure. The two types of nanoparticles revealed different outcome, with higher hemolytic effect following TCP exposure. The effect on RBC is accompanied by of reduction in macrophages proliferation rate.[21] This is not the only report on HAP nanoparticles to produce hemolysis. Another research team selected HAP-NPs ranging from 30 to 80 nm in diameter. Direct contact exposure as well as agar overlay techniques were used to analyze cytotoxicity on rat macrophages. Also, hemolysis test was used to assess effects of NPs exposure on RBC.HSP70 identification by means of Western Blot was also performed. Similar to other studies, an increase of over 5% in hemolysis rate was found for concentrations of over 1000 µg/ml. HSP7 was up-regulated by concentrations of 100 µg/ml HAP NPs and inhibition of macrophages’ proliferation could be found at concentrations as low as 20 µg/ml. The study draws a signal of concern regarding the opportunity of selecting HAP NPs for medical applications.[22]

Another interesting study focused on the cytotoxic effects of silver powders with different particulate diameter. Two
micron-sized and two nano-sized powders were prepared for testing by water dispersion, followed by complete characterization of obtained solution (SERS, TEM, and DLS). Although two distinct methods were used for preparation of solution needed for particle-RBC contact, nano-sized silver particles revealed significantly higher hemolytic rates compared to micro-sized silver particles are similar concentration (220 μg/mL) regardless the method used. According to presented data, this could be due to several factors, including: increased silver ion release, increased surface area or mechanical interaction with RBC.[23] Similar data have been reported by another group who showed a decreasing hemolysis rate with increasing NP size for silver NPs exposure. The authors incriminate, similar to other researchers, higher effective surface with consecutive higher silver ions release in smaller particulate size as compared to larger ones.[24] In all this cases the hemolytic effect was considered to be induced by the release of oxidative stress products following exposure. (Figure 1)

Comparison between the observed effects following RBC exposure to different types of nanoparticles has been proposed by other studies. One of them focused on hemolysis, erythrocyte sedimentation rate, membrane topography, hemagglutination, and lipid peroxidation induces by three distinct types of NPs: silver, gold and platinum following their contact with RBC. Similar to the results presented, data suggested silver NPs to be less hemocompatible as compared to platinum and gold NPs. Following silver NPs exposure at 100 μg mL⁻¹, evidences of hemolysis, membrane damage, morphological alterations, and hemagglutination as well as cytoskeleton changes were recorded. Also, RBC membrane presented multiple depressions and pits following exposure. None of these noxious effects were recorded following nano-sized silver NPs, results revealed an increased NP-RBC binding, and a weak tendency of the NPs themselves to form large aggregates. By contrast, zinc oxide NPs recorded high rate of clumping. Such large, micrometer-sized aggregates formed by zinc oxide NPs are very mobile within blood flow and become very toxic to RBCs. Nanosilica NPs, following aggregate formation, has been also said to promote hemolysis. The process follows various steps including shape change from discocytes to spherocytes, loss of membrane’s resiliency and flexibility, swelling and volume increase and finally membrane break.[27] Also, it has been demonstrated that silicon dioxide (SiO2) NPs are able to induce a higher the 5% hemolysis rate following in vivo continuous injection exposure.[28] Comparison between different silica NPs has also been investigated. It has been shown that amorphous silica is able to induce a significantly higher hemolysis rate as compared to MCM-41-type mesoporous silica NPs, thus making the latter a promising candidate for in vivo applications.[29]

Research focusing on TiO2 NPs has brought evidences of negative effects such as hemolysis (in a dose-dependent manner), sedimentation rate, or hemagglutination. The process is, most probably, passing, similar to other particles through several starting with RBC attachment, TiO2 NPs trans-membrane transport and oxidative stress and ending with membrane breakage.[30]

Lipid-based nanoparticles with crystalline structure (LCNPs) have also been intensely investigated. Different chemical compositions have been tested for their safety as potential intravenous drugs components. Results showed an intense hemolytic effect for glycerol monoooleate (GMO)-based LCNPs, thus making such structures incompatible for human intravenous administration. No hemolysis was observed in the case of soy phosphatidylcholine (SPC)/GDO, making them an appropriate candidate for various applications. Also, intermediate hemolysis rate for as long chained molecules, such as sponge phase nanoparticles based on DGMO/GDO/polysorbate 80 (P80). Such molecules should be used with caution in application heading in vivo intravenous administration. All data, however, converge to the conclusion that hemolytic properties of LCNPs are strongly connected to the chemical structure of the monomers in the case.[31] Similar data have been obtained in the case of partially quaternized poly [thio-1-(N,N-diethyl-aminomethyl) ethylene]s, Q-P(T
DAE) NPs. The nanostructures have been demonstrated to induce RBC lipid bilayer disruption with hemolysis, electrostatic attraction followed by hemagglutination. Also, effects have demonstrated to be dependent on blood protein content.[32] A group of researchers focused on synthesis and characterization of surface-modified SLN for adsorptive protein loading. Both the modulation of the emulsifier concentration and the lipid matrix have been performed. As demonstrated by complex techniques, such as X-ray diffraction, thermoanalysis, electron microscopy demonstrated the formation of crystalline and anisometrical particles. Hemolysis test, performed as a measure of toxicity revealed safe rates of RBC lysis of under 5% (1.1 to 4.6%).[33] Microemulsion-based nanoparticles (MEs) has been proposed for in vivo applications. Various toxicological data have been reported. At similar pharmacological concentrations sodium caprylate induced significant hemolysis while MEs and pluronic surfactants seem biocompatible.[34]

Other types of nanoparticles, like for example E78NPs were found to absolutely harmless to RBC membrane integrity, especially at low concentrations (1mg/mL), thus making the suitable for blood-delivered applications regardless the surface alteration (PEG-coated or non-PEGylated E78 NPs).[35] Similarly, de novo synthesized NPs, such as heparin-immobilized polyethersulfone (PES) has passed hemolysis test, demonstrating RBC lysis of less than 5%.[36] Likewise, a study testing the effect of RBC exposure to calix-arene based Solid Lipid Nanoparticles (SLNs) demonstrated no hemolytic effect.[37] Crystalline carbon nanoparticles(CNPs) with intense fluorescent properties obtained by means of addition of different organic tags, such as α-naphthylamine, fluorescein and rhodamine B. were synthesized. Erythrocyte exposure to these type of nanoparticles induced no signs of toxicity.[38] Minimal cytotoxicity and RBC toxicity was observed for polymethacrylate nanoparticles (NP), a type of NPs designed for applications of oligodeoxynucleotides delivery.[39] Cross-linked gelatin nanoparticles with the encapsulation of cycloheximine, an already demonstrated inhibitor for protein synthesis have also been designed. Data obtained from blood compatibility testing experiments revealed minimal or no signs of hemolysis.[40] An increasing interest has been generated by layered double hydroxides (LDHs) for cell delivery of bio-active molecules, drugs and genes. However, only limited studies have focused on testing their biocompatibility testing. One of these studies tested several chemical compositions (MgAl-LDH as well as ZnAl-LDL). In vitro testing was performed on carcinoma cells, normal human cells and RBCs. Quantification of possible noxious effects was done by measuring cell proliferation, membrane damage, cell proliferation and hemolytic effect. None of the tested chemical structures was able to induce significant hemolysis. A slightly higher toxicity was recorded for ZnAl-LDH as compared to MgAl-LDH, detectable as increased hemolysis and membrane damage. The authors conclude that LDH could represent a promising substrate for future in vivo drug delivery applications.[41] By means of emulsion/solvent evaporation technique, a study imagined the method to generate a distinct type of NPs, made of a conjugate of poly(D,L-lactide-co-glycolide) with alendronate (PLGA−ALE NPs). Following administration, lysis of erythrocytes was studied. No hemolysis was detected following PLGA−ALE NPs exposure, the rate being similar to that recorded for PBS exposure.[42] Another research group studied nanoparticles based on complex polymeric structures, such as poly(3-hydroxybutyrate)–poly(ethylene glycol)–poly(3-hydroxyl butyrate) (PHB-PEG-PHB). They have been proposed for drug delivery applications, therefore cytotoxicity and hemolysis test were performed. No signs of cytotoxicity and hemotoxicity were recorded, even for concentrations as high as 120µg/mL. These results sustain the designed NPs’ potential for the intended applications.[43] Other types of NPs, like N-acyl chitosan nanoparticles, prepared by the tripolyphosphate anions reactions, have been designed and proposed for several applications. The testing experiments revealed good hemocompatibility, as quantified by thromboelastography and hemolysis.[44]

It has been already known that cytostatic therapies have hematological toxicity as a common adverse effect. Various methods have been proposed for diminishing these effects. One of them was to encapsulate the drug into biocompatible materials. One such treatment agent is Paclitaxel, a drug known to present a low stability in aqueous media and to present various adverse effects induced by the solubilizer used in preparation. One group of researchers developed a new formulation of amphiphilic cyclodextrin nanoparticles by encapsulation of Paclitaxel. Hemolysis and cytotoxicity experiments were performed to test the biocompatibility of the newly synthesized NPs. Data were compared to a common vehicle: cremophor; ethanol (50:50 v/v). Significantly reduced hemolysis was obtained for the encapsulated nanoparticles, as it could be inferred by SEM- imaging.[45] Another cytostatic agent of interest was Doxorubicin. For the encapsulation purpose, spherical self-assembled nanoparticles based on oleoyl-chitosan (OCH) have been developed. The efficiency of Doxorubicin loading was 52.6%. Drug releasing was complete and rapid at PH =3.8. At PH 7.4 releasing process had two distinct phases: a first burst release, followed by a sustained release. Drug efficiency, quantified by the death rates following in vitro exposure of various cancer cell lines, was significantly higher than Doxorubicin solution alone. However, before proposing any particular applications, the newly synthesized NPs have been tested by means of MTT assay and hemolysis test. No cytotoxicity signs were found on embryo fibroblasts. All hemolysis experiments, performed under distinct conditions resulted in acceptable hemolysis rates (under 5%). All data suggest a good potential for the developed NPs towards human anticancer applications.[46] Similarly, self-assembled Fe3O4 magnetic nanoparticles (MNP)s were developed for encapsulation of daunorubicin (DNR). By contrast with DNR alone, the so-loaded capsule presented a good hemocompatibility, with no hemolysis.[47] Similarly, research has been developed for reducing the hemotoxicity of camptothecin, a potent anticancer drug. Lipid nanoparticles
based on different lipid cores were designed for its encapsulation. The final NPs, holding camptothecin content revealed a hemolysis rate of under 5%, thus placing the RBC adverse reaction within acceptable limits.[48] Not only the cytostatic agents raised the interest of researchers for encapsulation. Anti-malarial drugs, such as artemether (ARM) were loaded in lipid mass. For this purpose, a film of thin-film hydration method was used. The lipid structures were glyceryl soybean oil and trimyristate (solid lipid). Particles were further loaded with the ARM 10%. Nanoparticulate surface was optimized by means of non-ionic, cationic and anionic surfactants. Testing of the newly developed NPs revealed a level of hemolysis of under 7% and a significantly enhanced in vivo anti-malarial activity as compared to the regular drug solution, currently on the market.[49]

Interesting results were brought by experiments testing NPs of mixed chemical structure. High biocompatibility, including low hemolytic rates were found for monodisperse Au-silicate nanoparticles (10.7 ± 1.6 nm in diameter).[50] Similar data have been reported following RBC exposure to core–shell Fe3O4@ Au composite magnetic nanoparticles (MNP). All concentrations used induces hemolysis rates of under 5%[51], the already established cut-off for hemolytic effect.[52] Also, a complex method has been imagined for superparamagnetic Fe3O4@nanoparticles synthesis using nickel-nitritoltriacetate (Ni-NTA) structure modification. The opportunity of their application for vivo imaging has been demonstrated by the lack of hemolysis after RBC exposure.[53]

A very recent report demonstrates the modulation of hemolytic properties of nanoparticles by the protein content of plasma. Data shown demonstrate that RBCs exposure to polystyrene nanoparticles (PS-NP) in protein-free medium results in hemolysis (in a particle). It has also been reported that PH can represent another modulator factor for the hemolytic process. The authors focused on protein-encapsulated hydrophobic γ-PGA (γ-hPGA) nanoparticles. Due to a conformational change of γ-hPGA NPs, hemolysis appears under PH conditions ranging from 7 downward to 5.5, but never at physiological PH. Hydrophobicity, increased by the PH-induced conformational change, might be the mechanism responsible for membrane disruption. The authors conclude that the designed NPs could find their utility into DNA, protein, drug delivery or other systems based on endosome-disruptive NPs.[54]

These findings generated the idea to reduce the hemolysis rate by different methods. One of them consists of using special types of nanoparticles (such as magnet-controlled sorbent NPs: MCS-B) for extracorporeal processing of blood. A group of researchers performed complex experiments towards three different aims. The first one was to detect the existence of a relationship between the timing of hemolysis process debut and the intensity of MCS-B exposure. The second objective was to investigate specific metabolic pathways in exposed RBC: ATP transport, Na-K and Ca-Mg ATPases. Thirdly, the group oriented their investigation towards standardizing and optimizing the MCS-B protocol, so to obtain the best anti-hemolytic effect. Results established that an optimal desired effect can be best obtained by means of 1-2 sequences of extracorporeal blood processing with MCS-B NPs. The treatment decreased the Ca-Mg ATPases without altering the Na-K ATPases.[55]

Another interesting research direction was to alter the surface of NPs in order to reduce their hemolytic effects. One such approach was that of gold NPs synthesized by a biocompatible polymer (1900-DAPEG) which allowed surface conformational alterations. Compatibility testing of the newly synthesized NPs, performed by comparison with surfactant stabilized gold-NPs (which served as control) revealed decreased hemolytic rate. Also, other helpful properties were observed, such as being significantly lower platelet activators as their compared to their counterparts.[56] Significantly, it has been also demonstrated that the rate of hemolysis is significantly different between raw PLGA, PEGylated PLGA and surfactant stabilized PLGA.[57] Observations and experiments were carried out for obtaining an optimal PEG-attachment at the surface of the nanoparticles. One such study focused on mesoporous silica NPs (MCM-41) with diameter of 150±20nm. PEGxk chains of various chain densities and molecular weights were covalently linked to the surface of the nanoparticles. PEG10k variant of newly synthesized NPs showed a reduced hemolysis rate( 0.9%) as compared to the rest of tested NPs.[58] It has also been hypothesized that chitosan/pDNA-linked NPs could benefit from a higher blood compatibility as compared to their raw counterparts. Although an increased interaction was detected between different particles, most probably coming from positively charged surface or free polymer residues, the results showed negative or neutral zeta potential. None of the so designed NPs induced significant hemolysis.[59] The study does not represent the only research focused on chitosan-based NPs. Another report described the method for development of Chitosan-N-trimethylaminomethacrylate chloride–PEG (CS-TM–PEG) copolymers, meant to reduce the toxicity of chitosan. Results showed a hemolysis rate from10.3% to 13.6% for the non-PEGylated copolymer and from 4.76% to 7.05% for the PEGylated designed NP. The concentrations of the copolymer used were 250-2000 μg/ml concentrated. The study concludes that these 200-400nm wide nanoparticles, showing an efficiency of 90% could be made more hemocompatible by PEG-alteration of their surface.[60]

Another approach was described by a team of researchers who performed de novo gold NPs synthesis using a natural extract (Zingiber officinale). The extract used is known to present double valence: both reducing and stabilizing. The newly developed nanomaterials have demonstrated reduced complement and platelet activating properties as well as low hemolytic activity.[61] The auto fluorescence levels, represents a strong indicator of erythrocytes health status. When we tested the auto fluorescence changes following administration of colloidal gold nanoparticles (20 nm) found no signs of toxicity among the tested Au solutions. (Figure 2)
None of the above aforementioned studies, however, included NPs themselves as controls for the purpose of identifying interferences with the assay they could come from the specificity of the NPs.[62] The need for more complex investigation techniques brought recent techniques into consideration and usage into experimental studies, such as light scattering by RBCs based on T-matrix formalism.[63] He-Ne laser light scattering was also investigated by means of a rouleau of n (2 ≤ n ≤ 8) RBCs of regular size. The rouleau was formed by the regular agglomeration of RBC along the axis of a vessel when low blood flow conditions are assured. The RBC-RBC interactions were analyzed numerically by means of fast Fourier transform methodology.[64] Development of another method called tracking velocimetry (based on cell magnetotheories), was also reported. The technique enables researchers to quantify the migration of RBCs (both deoxygenated and containing methb). The unequal particles contained in the four hem groups (deoxygen and methb) offers the mentioned molecules magnetic properties, very different from the diamagnetic properties of oxyhemoglobin. These findings offer the theoretical backgrounds for differential migration of the two classes of molecules when exposed to a strong magnetic field. Such a method could be an useful tool for biochemical analysis in cell characterization and separation based on magnetic characteristics of self-molecules.[65]

REFERENCES


