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In vitro blood compatibility and *in vitro* cytotoxicity of amphiphilic poly-N-vinylpyrrolidone nanoparticles

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ABSTRACT

This study focused on defining the *in vitro* behavior of amphiphilic poly-N-vinylpyrrolidone (Amph-PVP) nanoparticles toward whole blood, blood plasma and blood cells in order to assess nanoparticle blood compatibility. In addition, possible effects on endothelium cell growth/viability were evaluated. The Amph-PVP nanoparticles were formed via self-assembling in aqueous media and composed of a hydrophobic alkyl core and a hydrophilic PVP outer shell. Their blood compatibility was evaluated by investigating their effect on red blood cells (RBCs) or erythrocytes, white blood cells (WBCs) or leukocytes, platelets (PLTs) and on complement system activation. Our results clearly demonstrate that the Amph-PVP nanoparticles are stable in presence of blood serum, have no significant effects on the function of RBCs, WBCs, PLTs and complement system activation. The Amph-PVP nanoparticles did not show considerable hemolytic or inflammatory effect, neither influence on platelet aggregation, coagulation process, or complement activation at the tested concentration range of 0.05–0.5 mg/ml. The Amph-PVP nanoparticles did not exhibit any significant effect on HMEC-1 microvascular skin endothelial cells' growth in *in vitro* experiments. The excellent blood compatibility of the Amph-PVP nanoparticles and the lack of effect on endothelium cell growth/viability represent a crucial feature dictating their further study as novel drug delivery systems.

1. Introduction

Nano-dimensional carriers, such as liposomes, micelles, nanospheres, nanocapsules, dendrimers have been extensively studied in the recent years as a basis for novel, effective drug delivery systems and imaging agents (Costa and Torchilin, 2018; Palmerston et al., 2017; Rong et al., 2011; Sercombe et al., 2015; Xu et al., 2013). The application of diverse nanoparticle-based systems is currently particularly popular in research areas involving biomaterials, pharmaceutics, biotechnology as well as biomedicine mainly due to their intrinsic characteristics involving large surface area/volume ratios, the ability to immobilize biologically active substances and to tailor their delivery to specific targets (Leucuta, 2010).

Among numerous classes of drug delivery systems significant interest is placed on the role of polymeric nanoparticles prepared utilizing natural and synthetic polymers in disease treatment strategies.

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Historically, synthetic polymeric nanoparticles for drug delivery were first produced using non-biodegradable polymers such as poly(2-hydroxyethyl methacrylate), poly(methyl methacrylate), polyacrylamide, and polystyrene (Bettencourt and Almeida, 2012; Kreuter and Speiser, 1976; Saini et al., 2012; Wim et al., 2008). Nevertheless, such polymers cannot be considered for systemic use in drug formulations as they possess relatively high side toxicity enhanced by the non-biodegradable nature of the polymer. Thus, biodegradable polymers including poly (lactic-co-glycolic acid), poly-L-glutamic acid, polycyanoacrylate and, polyethylene glycol are currently used in the majority of drug nanocarrier preparations (Couvreur et al., 1982; Locatelli and Comes-Franchini, 2012; Pavot et al., 2014). Such advanced drug delivery systems play a promising role in optimizing biodistribution, improving biocompatibility and bioavailability, as well as minimizing side effects of drugs which impose risks to patients (Alexis et al., 2008; Umerska et al., 2018). Recent scientific reports demonstrate that polymeric nanocarriers can successfully deliver a variety of drugs, including anticancer and anti-neurodegenerative drugs, to specific organs and tissues (such as tumors, brain, liver) (Henrich-Noack et al., 2019; Parveen and Sahoo, 2008; Wilson, 2009). Further development and application of nanoparticles in disease management is crucial and provides immense hope in the treatment of serious diseases such as cancer, AIDS, systemic infections, neurodegenerative and cardiovascular diseases.

Previously, we reported the synthesis of a novel drug delivery system on the basis of nanoparticles prepared from amphiphilic derivatives of poly-N-vinylpyrrolidone consisting of different molecular weight polymeric hydrophilic fragments linked to hydrophobic n-alkyl chains of different lengths (Kuskov et al., 2007, 2010, 2018). We further demonstrated that the self-assembled architectures of such core-shell polymeric nanoparticles in aqueous media, could effectively entrap various hydrophobic drugs, increasing their compatibility, stability and bioavailability in aqueous solutions (Kuskov et al., 2018; Luss et al., 2018). Importantly, the preliminary assessment of the *in vitro* cytotoxicity and *in vivo* acute and subacute toxicity of such amphiphiles and nanocarriers together with their satisfactory biocompatibility (Kuskov et al., 2016, 2017) supported their superior application potential and the need for further investigation as drug delivery systems.

Nano-scaled drug delivery systems can be applied by oral, transdermal, inhalation and injection routes of administration. Upon entering the circulatory system, nanocarriers interact with blood cells and plasma proteins and can potentially induce various pathophysiologic processes. It is therefore crucial to study the biological behavior of nanoparticles aimed for drug delivery in blood and their interaction with blood components. Several studies assessed the effects of various nano-carriers on blood cell hemolysis and on blood clotting parameters (Kim et al., 2005; Laloy et al., 2014; Lee et al., 1995). The major components of blood are plasma and blood cells. Blood cells consist of red blood cells (RBCs) or erythrocytes, white blood cells (WBCs) or leukocytes and platelets (PLTs), also called thrombocytes. The major function of RBCs is the transport of oxygen to body tissues as well as the transport of CO_2 to the lungs. The WBCs have crucial roles in the body immune response, while PLTs actively contribute to the process of creating blood clots in order to prevent excess blood loss due to vessel damage. Plasma components are responsible for maintaining the osmotic pressure and balanced pH, and together with PLTs participate in blood coagulation during the initial clot formation. Taking into account the crucial role of blood components in maintaining body homeostasis, it is imperative to evaluate the effects of polymer nanoparticles on whole blood taking in to account parameters such as hemolysis, thrombosis, coagulation, platelets and complement system activation (BS EN ISO 10993-4, 2017). More specifically, hemolysis is the destruction of RBCs and the release of their components (hemoglobin) (Arzoumanian, 2003), causing anaemia and resulting in renal failure (Dobrovolskaia et al., 2008). The loss of PLTs function, which is pivotal to primary hemostasis, can cause thrombotic or hemorrhagic disorders (Laloy et al., 2014). The blood plasma coagulation cascade is responsible for blood clotting for secondary hemostasis and can be activated by PLTs (Jun et al., 2011). Finally, complement system activation is a body defense mechanism that can be potentially initiated by blood contact with pathological foreign objects including nanoparticles (Krajewski et al., 2013). Endothelial cells, lining blood vessels, are likewise immediately exposed to nanoparticles resulting in possible deleterious consequences as regarding hemostasis and immunological response (Engin et al., 2017; Cao et al., 2017). Importantly, the endothelium barrier consisting of microvascular endothelial cells which are positioned at blood-vessel interface, regulates crucial functions of tissues/organs and enables homeostasis (Mehta and Malik, 2006). Therefore, possible effects of nanoparticles on endothelium cell growth/viability need to be evaluated.

Consequently, it is very important for researchers to evaluate the interactions of nano-scaled drug carriers with blood and its components. In this study, we have investigated platelet aggregation, coagulation and the complement system activation after treatment of blood and its components with preparations of self-assembled micelle-like 60–100 nm aggregates made of poly-N-vinylpyrrolidone amphiphilic derivatives, as well as the stability of these nanoparticles in blood serum. The potential hemolytic and inflammatory effects of nanoparticles interacting with red white blood cells were also studied.

2. Materials and methods

2.1. Materials

Amphiphilic poly-N-vinylpyrrolidones (Amph-PVP) consisting of a PVP hydrophilic fragment with molecular weight of 1000, 2000, 4000, 6000, 8000 and 12000 Da and an octadecyl hydrophobic terminal fragment -namely, PVP-OD1000, PVP-OD2000, PVP-OD4000, PVP-OD6000, PVP-OD8000 and PVP-OD12000-were synthesized and characterized as described in previous studies (Kulikov et al., 2017; Kuskov et al., 2018). The main properties of the amphiphilic N-vinylpyrrolidone polymers are presented in Table 1.

Dulbecco's Phosphate-Buffered Saline (DPBS), ethyl acetate, ethanol, dimethylsulfoxide (DMSO), phosphate buffer saline (PBS), (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N'-tetraacetic acid) buffer (EGTA), pyrene and all other chemicals used in this study were purchased from Sigma-Aldrich (USA) unless otherwise specified and used without further purification. Analytical grade preparations were used for all solvents and buffer solution components. The Milli-Q plus

Table 1
Characteristics of the Amph-PVP polymers used in the study.

Amphiphilic polymer	PVP molecular weight (Da)	Hydrophobic group	PDI	CAC, μM/l
PVP-OD 1000	1000	$-C_{18}H_{37}$	1.485 (Kulikov et al., 2017)	4.4 (Kulikov et al., 2017)
PVP-OD 2000	2000	$-C_{18}H_{37}$	1.328	4.2
PVP-OD 4000	4000	$-C_{18}H_{37}$	1.218	5.8
PVP-OD 6000	6000	$-C_{18}H_{37}$	1.368 (Kulikov et al., 2017)	6.5 (Kulikov et al., 2017)
PVP-OD 8000	8000	$-C_{18}H_{37}$	1.204	8.4
PVP-OD 12000	12000	$-C_{18}H_{37}$	1.290 (Kulikov et al., 2017)	9.5 (Kulikov et al., 2017)

PDI - polydispersity index; CAC - critical aggregation concentration.

System (Millipore, USA) was used for the preparation of distilled-deionized water.

2.2. Preparation and characterization of the Amph-PVP nanoparticles

Amph-PVP nanoparticles formed from amphiphilic poly-N-vinylpyrrolidone with hydrophilic PVP fragment of 1000, 2000, 4000, 6000, 8000 and 12000 Da, were prepared and characterized as previously reported (Kuskov et al., 2007, 2018). The critical aggregation concentration (CAC) of the amphiphilic PVP polymers used in this study was measured by spectrometry using pyrene as a fluorescence probe.

The hollow amphiphilic N-vinylpyrrolidone polymeric nanoparticles were prepared via the emulsification method with solvent evaporation. Briefly, each Amph-PVP was dissolved in a minute amount of ethyl acetate to obtain a solution which was then emulsified in an aqueous phase by ultrasonic treatment (Sonoplus HD, 2070; Bandelin, Germany). The colloidal system resulting after removing the organic solvent was concentrated by evaporation using a rotary evaporator Laborota 4010 (Heidolph, Germany). The polymer nanoparticle solution was frozen and lyophilized using an Alpha I-4LD freeze dryer system (Martin Christ GmbH, Germany) to obtain freeze-dried, solid nanoparticle. These nanoparticles were then resuspended in PBS (pH 7.4).

Pyrene-loaded Amph-PVP nanoparticles were prepared following the same method as for hollow Amph-PVP nanoparticles, by adding the appropriate amount of pyrene to the initial polymer solution in ethyl acetate, followed by an oil-in-water (O/W) emulsification, solvent evaporation, freeze-drying and resuspension in PBS.

Dynamic light scattering (Malvern Zetasizer Nano-ZS, Malvern, UK) was used to determine particle size and zeta potential at 37 °C for the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment 2000, 4000 and 8000 Da. The dispersion medium used during the characterization of the nanoparticles was PBS (pH 7.4). The morphology of the Amph-PVP nanoparticles was determined by Transmission Electron Microscopy (TEM), using a JEOL JEM-2100 TEM, USA. Further dynamic light scattering experiments for the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment 1000, 6000 and 12000 Da with nanopure water as the dispersion medium were conducted by means of a light scattering spectrometer (ALV-5000 multiple-τ digital correlator) with vertically polarized incident light of wavelength 532 nm supplied by a diode laser, at 37 °C. The laser beam was linearly polarized in the direction perpendicularly to the scattering plane. In the present study, the full homodyne intensity autocorrelation function was measured at different scattering angles in the range 30–150° with an ALV-5000 multiple-τdigital correlator that covered a dynamic range of about ten decades.

The main characteristics of the prepared Amph-PVP nanoparticles formed from the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment molecular weight 1000, 2000, 4000, 6000, 8000 and 12000 Da are presented in Table 2.

2.3. Study of stability of the Amph-PVP nanoparticles in the presence of blood serum

The Amph-PVP nanoparticles formed from the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment 2000, 4000 and 8000 containing 10–30 μg/ml of solubilized pyrene were incubated in the presence of 50% human blood serum at 37 °C for 24 h under gentle mixing. The fluorescence intensity caused by the release of pyrene at different incubation times was measured at 339 nm excitation wavelength and 385 nm emission wavelength in the presence and in the absence of blood serum. Human serum and pyrene in PBS were used as a reference.

2.4. Hemolysis assay

The effect of the Amph-PVP nanoparticles formed from the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment 2000, 4000 and 8000 on erythrocyte membrane integrity was investigated by an *in vitro* hemolysis assay (Zhu et al., 2007). The release of hemoglobin from RBCs was used as a measure of toxicity of the polymeric nanoparticles. Human blood samples were obtained from healthy donors (age 25–55 years), mixed with heparin and centrifuged at 2000 rpm for 15 min to separate the RBCs. The obtained erythrocytes were washed three times with 5 vol of isotonic phosphate buffered saline (PBS, pH 7.4). After the last washing, the purified RBCs were resuspended in PBS to obtain a 2% (v/v) RBCs suspension which was immediately used for the hemolysis assay. Three (3) ml solutions of the Amph-PVP nanoparticles in PBS (with fixed polymer concentrations of 0.05, 0.1, 0.25 and 0.5 mg/ml) were incubated with 2 ml of the prepared RBC suspension at 37 °C for 1 h under gentle stirring and then centrifuged at 5000 rpm for 5 min.

The rate of hemolysis was estimated by measuring the absorbance of the hemoglobin released into the supernatant at 540 nm. Normal PBS without polymeric nanoparticles was used as a negative control for 0% hemolysis, and ultrapure water was used as a positive control for 100% hemolysis. All obtained hemolysis data were evaluated as a percentage of the complete hemolysis, which was calculated using following equation:

$$\text{hemolysis } \% = \frac{\text{Abs (nanoparticle sample)} - \text{Abs (pure PBS)}}{\text{Abs (distilled water)} - \text{Abs (pure PBS)}} \times 100\%$$

2.5. Evaluation of Amph-PVP nanoparticle effects on human whole blood monocyte activation

Amph-PVP nanoparticles formed from the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment 2000, 4000 and 8000 were incubated with blood at 37 °C overnight and the blood samples were consequently centrifuged at 3000 g for 15 min.

The supernatant plasma was used to measure the response of human monocytes and macrophages to blood interaction with Amph-PVP

Table 2
Characteristics of Amph-PVP and pyrene-loaded Amph-PVP polymeric nanoparticles (mean ± S D, n = 3) in PBS at 37 °C.

Amphiphilic polymer	Amp-PVP nanoparticles			Pyrene-loaded Amph-PVP nanoparticles			
	Particle size, nm	PDI	Zeta potential, mV	Pyrene content, %	Particle size, nm	PDI	Zeta potential, mV
PVP-OD1000[a]	142 ± 4	–	–	–	–	–	–
PVP-OD2000	58.2 ± 3.9	0.132 ± 0.019	−6.52 ± 0.37	9.8	51.1 ± 4.2	0.142 ± 0.032	−4.94 ± 0.26
PVP-OD4000	76.4 ± 6.1	0.145 ± 0.022	−8.02 ± 0.41	9.5	68.7 ± 5.1	0.151 ± 0.041	−6.21 ± 0.42
PVP-OD6000[a]	178 ± 10	–	–	–	–	–	–
PVP-OD8000	95.8 ± 5.7	0.164 ± 0.023	−9.22 ± 0.44	8.2	83.4 ± 5.3	0.166 ± 0.038	−7.14 ± 0.39
PVP-OD12000[a]	194 ± 10	–	–	–	–	–	–

PDI – Polydispersity index; Amph-PVP concentration – 0.5 mg/ml.
[a] Dispersion medium water, 37 °C.

nanoparticles *in vitro* in terms of production of an inflammatory cytokine (IL-1β). IL-1β released from monocytes was quantified by an ELISA assay (Human pro-IL-1β/IL-1F2 Quantikine ELISA kit) according to the manufacturer's instructions (R&D Systems).

The monocyte activation test (MAT) is widely used for the detection of pyrogens in medical devices and pharmaceutical preparations (Perdomo-Morales et al., 2011). The principle of the MAT is that pyrogens, through the induction of monocytes via Toll-like receptors (TLRs), stimulate cytokines production (for example, IL-1β). This method is therefore reliable when it is necessary to assess the potential of nanoparticles to induce a "pyrogen-like" effect.

2.6. Evaluation of Amph-PVP nanoparticle influence on platelet aggregation and coagulattion

Platelet aggregation was investigated using an aggregometer Helena AggRAM (Great Britain) through a previously described method (Ungerer et al., 2011). Platelet-rich plasma was extracted by centrifugation at 200*g* at room temperature for 10 min 50 μl of Amph-PVP nanoparticles' suspension diluted by PBS and 1.0 mg/ml collagen (Sigma-Aldrich, USA) as positive control or PBS as negative control were respectively mixed with 200 μl of platelet-rich plasma. Subsequently, all samples were incubated for 60 min at 37 °C. The maximum aggregation rate was calculated via the device software.

For the platelet coagulation investigation (Alexandre et al., 2015), platelet-poor plasma was collected through the centrifugation of fresh human whole blood for 10 min at 2500 g and room temperature. 50 μl of Amph-PVP suspensions diluted by PBS and pure PBS as negative control were mixed with 500 μl of platelet-poor plasma. All samples were incubated for 60 min at 37 °C, and then analyzed by an automatic coagulation analyzer (Siemens Healthcare Diagnostics, Germany) for prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT).

2.7. Effects of Amph-PVP nanoparticles on complement system activation

Amph-PVP nanoparticles formed from the amphiphilic N-vinylpyrrolidone polymers with hydrophilic fragment 2000, 4000 and 8000 were incubated for 1 h with human blood serum. For the evaluation of complement activation via the classic pathway (CP) parameters, 0.1 ml of the control sample (without Amph-PVP nanoparticles) and the corresponding Amph-PVP nanoparticle sample in serum was placed into cuvettes. 0.6 ml of PBS was added to the samples which were consequently incubated at 37 °C for 3min, whereupon 0.1 ml of the RBCs suspension was added to each cuvette. Optical density was registered every 5 s at 800 nm using an Immunochem 2100 spectrophotometer (USA).

When the lysis of the first portion of RBCs was completed, the same portion of RBCs was added into the cuvette and the process of their lysis was registered until the exhaustion of the complement system was achieved, which was exhibited as a termination of hemolysis. The time between addition of erythrocytes and occurrence of hemolysis (lag-period or lag-t) value was determined; the rate of complement-dependent RBCs lysis (V of lysis by classic pathway) was determined by the decrease in absorbance; hemolytic capacity of the complement (HCC) was determined by the number of standard portions of erythrocyte suspension that were completely lysed in the blood serum sample (Raepple et al., 1976; Ramadori et al., 1984).

For the evaluation of the complement activation via the alternative pathway (AP) parameters, 0.4 ml of EGTA-buffer and 0.2 ml of PBS were added to 0.1 ml of control (without Amph-PVP nanoparticles) or the corresponding Amph-PVP nanoparticle blood serum samples. After incubation for 3 min, 0.1 ml of the standard erythrocyte suspension was added to the incubation mixture. The time between introduction of erythrocytes and occurrence of hemolysis (lag-period or lag-t) and the rate of complement dependent hemolysis by alternative pathway was determined. The results were expressed as percentage to the control level.

2.8. The effects of Amph-PVP polymeric nanoparticles with hydrophilic fragment 1000, 4000, 6000 and 8000 on endothelial cell growth

Human microvascular endothelial HMEC-1 growing cells from non-confluent cultures were harvested and seeded in black 96-well plates (Corning; 3603) at a density of 1.000 cells per well in 200 μl of DMEM (10% FBS). The cell density number was chosen from optimization experiments. The cells were allowed to rest overnight. Treatments were added for the next 48 h at 37 °C and 5% CO_2 in 0% FBS. The cells were then lysed and their number was calculated using the CyQUANT fluorometric assay (Thermo Scientific; C7026) according to the manufacturer's instructions. Fluorescence was measured in a Fluorometer (Biotek) using the proposed excitation (485 nm) and emission (528 nm) filters. A separate standard curve was used to convert fluorescence units to cell numbers. All experiments were performed in triplicate.

2.9. Statistical analysis

Investigations results were expressed as means ± SD. The statistical analysis was performed using IBM SPSS Statistics 19.0 (IBM Corporation, USA).

The statistical significance of differences between several parallel experiments or between experimental groups was analyzed by the independent Student's t-test for paired samples and ANOVA. For the statistical significance a level of confidence of $P < 0.05$ was applied.

3. Results and discussion

Due to their ability to entrap hydrophobic drugs and increase their bioavaibility, and pharmaceutical efficiency while decreasing their side toxicity, polymeric nanoparticles have been widely investigated and applied in biomedical areas, especially for creation of novel drug delivery systems (Chan et al., 2010). Despite the global use of different nano-scaled polymeric drug carriers for research, more detailed information concerning their biological effects, toxicity and safety are still required (Dhawan and Sharma, 2010; Jones and Grainger, 2009).

Taking into account the need of evaluating the interactions of nanoparticle drug carriers with blood and its components, in this study we investigated *in vitro* hemolysis, monocyte activation, platelet aggregation, coagulation and activation of complement system after treatment with nanoparticles produced from several amphiphilic poly-N-vinylpyrrolidone derivatives with different molecular weights. Through analyzing the obtained experimental data, we were able to deeply comprehend the blood compatibility of Amph-PVP nanoparticles which are promising candidates for efficient drug delivery in the organism.

3.1. Characterization of Amp-PVP nanoparticles

In this study, nano-scaled micelle-like particles were prepared from PVP-OD2000, PVP-OD4000 and PVP-OD8000 amphiphilic polymers via self-assembling these amphiphilic polymers at concentrations higher than their critical aggregation concentration (CAC). The CACs of the three newly synthesized polymers were in the micromolar range, which was judged to be low enough to maintain nanoparticles stable in their suspensions during investigations (Table 1). The PDIs and CACs of the Amph-PVP nanoparticles prepared from PVP-OD1000, PVP-OD6000 and PVP-OD12000 had been determined in a previously conducted study (Kulikov et al., 2017). The Amph-PVP nanoparticles were prepared using a previously developed and optimized emulsification and solvent evaporation technique and used to investigate the interaction with blood components and to assess hemacompatibility.

For characterization freeze-dried nanoparticles were resuspended in PBS. The sizes and zeta-potentials of polymer nanoparticles prepared

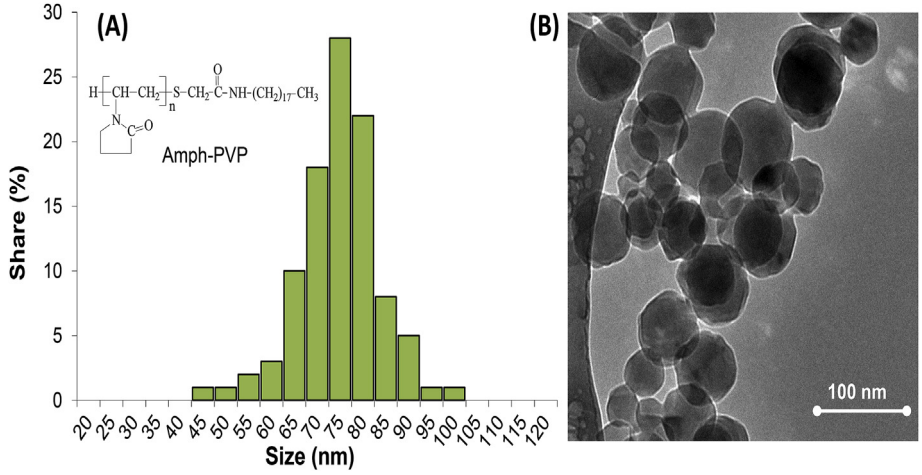

Fig. 1. Size distribution (A) and morphology (B) of Amph-PVP polymeric nanoparticles (PVP-OD4000, PBS, 0.5 mg/ml).

from the above mentioned PVP amphiphilic derivatives were measured by dynamic light-scattering and are shown in Table 2 while a representative size distribution for PVP-OD4000 nanoparticles is shown in Fig. 1A. The particle sizes of the PVP-OD2000, PVP-OD4000 and PVP-OD8000 were 60, 80 and 100 nm, respectively.

The size of the nanoparticles slightly increased from PVP-OD2000 to PVP-OD8000, which is judged to reflect the influence of PVP hydrophilic fragment molecular weight on the size of the resulting nanoparticles. For all three new polymer samples the size distribution of their corresponding self-assembled nanoparticles was rather narrow and monomodal in nature. The polydispersity index (PDI) of the nanoparticles was below 0.20 while their zeta potential in PBS was slightly negative and did not strongly depend on polymer molecular weight. The representative morphology of these nano-sized carriers as observed by TEM is shown in Fig. 1B. The particles were of spherical shape, well dispersed and with sizes that were in accordance with the results of the dynamic light scattering measurements.

The data obtained from these measurements therefore confirm the formation of nanoscaled, spherical-shaped, self-assembling aggregates from the Amph-PVP polymers of different molecular weight which are sufficiently stable in aqueous media, The absence of the PBS in the medium results in greater particle sizes for the Amph-PVP polymers as evidenced by the corresponding light scattering correlation functions which further revealed a bimodal distribution for all samples as characteristically shown for PVP-OD12000 in Fig. 2. These results are in line with previous published results (Kuskov et al., 2007). The lower mean average size for particles in PBS solution as compared to that in water can be explained by the strengthening of the hydrophobic interactions in the solution with the higher ionic force, that leads to the formation of more compact structures.

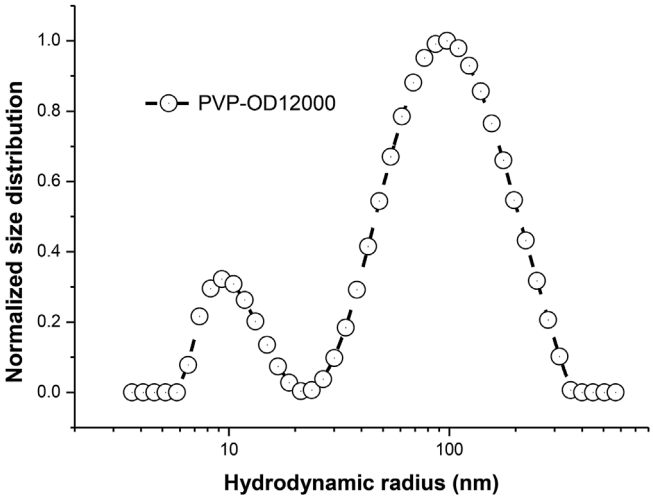

Fig. 2. Size distribution of Amph-PVP (PVP-OD12000) polymeric nanoparticles in nanopure water at 37 °C.

3.2. Stability of the Amph-PVP nanoparticles in the presence of blood serum

Since the properties of medical drug delivery systems are mostly determined by their interaction with different blood components, our first task was to analyze the stability of Amph-PVP polymeric self-assembled nanostructures in the presence of human blood serum. As previously shown (Kuskov et al., 2007, 2018), Amph-PVP aggregates are able to entrap the hydrophobic dye pyrene in their inner core. The change in fluorescence intensity of the encapsulated pyrene probe was used to determine the degree of the destabilizing effect of human blood serum on polymeric nanoparticles.

The *in vitro* stability of the nanoparticles was studied in the presence of human blood serum, using pyrene-loaded Amph-PVP nanoparticles. More specifically, pyrene was entrapped in the nanoparticle hydrophobic core using the emulsion/solvent evaporation method as described above. The characteristics determined for the pyrene-loaded nanoparticles are presented in Table 2.

The change in pyrene fluorescence intensity at 339 nm with time was used to compare the stability of the nanoparticles obtained from the different Amph-PVPs in the presence of blood serum. The results, shown in Fig. 3, demonstrate that the PVP-OD2000 nanoparticles have higher stability in human blood serum, probably due to an optimal hydrophilic/hydrophobic balance. PVP-OD2000 and PVP-OD4000 nanoparticles preserve more than 50% of pyrene intact for more than 12 h.

This approximate half-life in human blood serum makes PVP-OD2000 and PVP-OD4000 good candidates as carriers for drug delivery by intravenous injection administration. The PVP-OD8000 nanoparticles demonstrated a less than optimal behavior in contact with blood, which could be attributed to weaker hydrophobic interactions with the encapsulated within their core hydrophobic dye.

3.3. Effects of the Amph-PVP nanoparticles on red blood cells

All drug delivery systems entering blood circulation interact with RBCs. For polymeric drug carriers it was previously shown that amphiphilic polymers are able to interact with phospholipids or solubilize lipids (Savic et al., 2003). Thus, the toxic effect of such nanoparticles on blood components must be excluded prior to the investigation of their drug delivery action. *In vitro* erythrocyte-induced hemolysis is

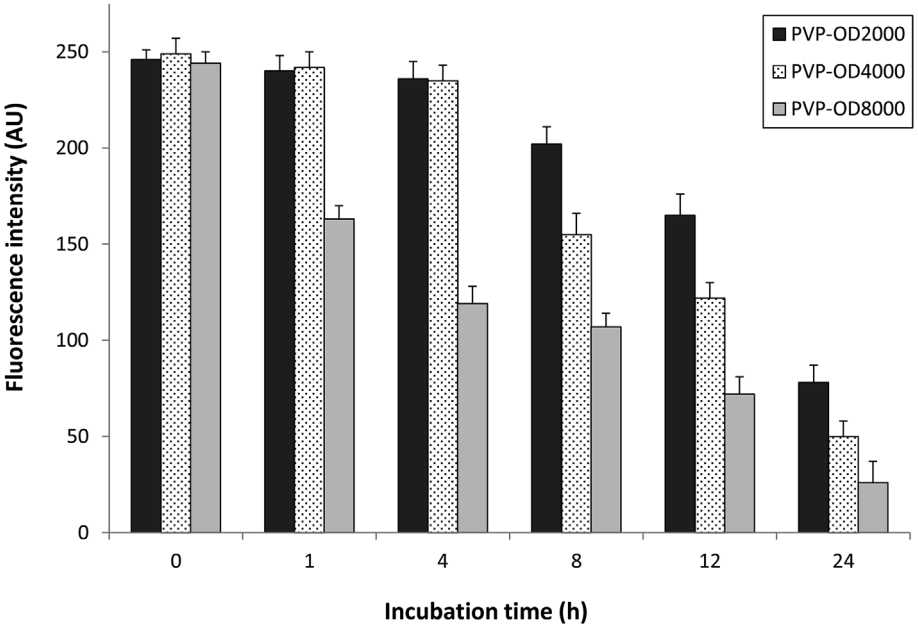

Fig. 3. Amph-PVP nanoparticle stability in the presence of human blood serum during a period of 24 h measured by pyrene fluorescence intensity.

considered to be a simple and reliable measuring method for estimating blood compatibility of polymeric nano-scaled carriers.

In this study, the hemolytic assay results (Fig. 4) demonstrated that even at relatively high concentrations of Amph-PVP polymers (up to 0.5 mg/ml) the resulting nanoparticles did not display significant hemolytic activity on human erythrocytes (RBCs) (p = NS). The degree of hemolysis was estimated to be slightly dependent on polymer nanoparticle concentration in the tested range. In *in vitro* studies the hemolysis degree can be characterized as "not significant" when it varies from 5 to 25% (Amin and Dannenfelser, 2006). In our experiments the hemolytic degree for three polymer types was found to be lower than 10% in the concentration range tested, indicating that the Amph-PVP nanoparticles meet the above-mentioned safety criteria. It should be stressed that, hemolytic activity of the PVP-OD2000 nanoparticles was found to be slightly higher than that of the PVP-OD4000 and PVP-OD8000. The higher hydrophobicity of PVP-OD2000 probably dictates the formation of more stable nanoparticles, leading at the same time to stronger interactions with lipids at high concentrations. In conclusion the Amph-PVP nanoparticles did not display any significant hemolytic effect on the human RBCs demonstrating a satisfactory hemacompatibility which supports their suitability for as drug carriers for intravenous administration.

3.4. Effects of the Amph-PVP nanoparticles on white blood cells

It is well established that an inflammatory reaction is often the first response induced from the interaction of administered nanoparticles with WBCs. Several recent investigations demonstrated that nano-scaled systems can provoke inflammatory reactions in the body by the activation of the inflammasome multimolecular complexes which promote caspase-1-dependent maturation of the IL-1 family proinflammatory cytokines (Chen et al., 2018; Wang et al., 2014). It was also found that among these cytokines, interleukin-1β (IL-1β) is crucial for host-defense responses to infection and injury (Dinarello, 2011). Thus,

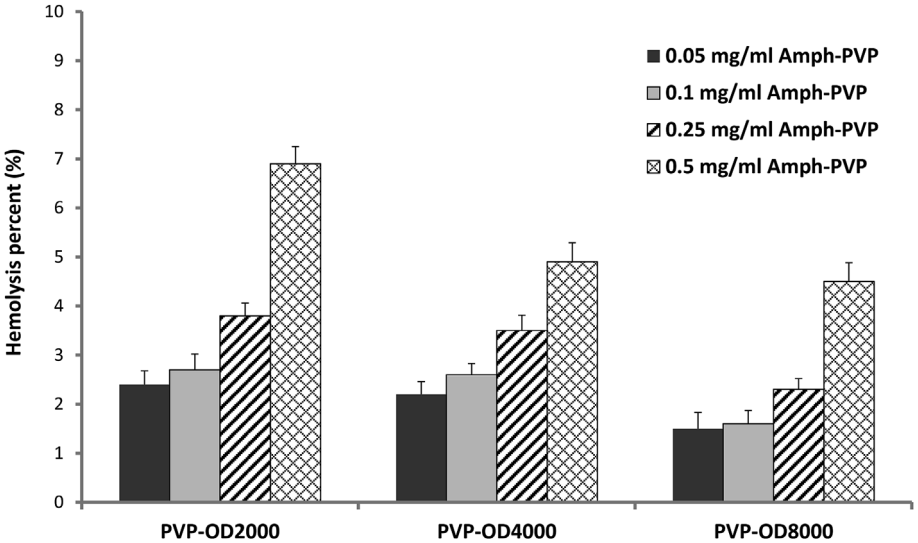

Fig. 4. Effects of the Amph-PVP nanoparticles on hemolysis of human erythrocytes (RBCs). Error bars indicate standard deviations of triplicate determinations (n = 3).

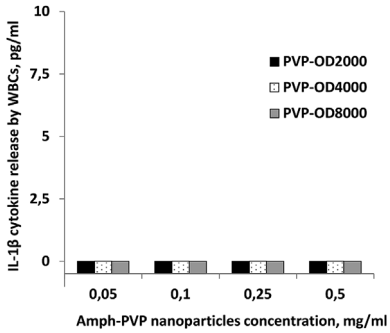

Fig. 5. Effects of the Amph-PVP nanoparticles on IL-1β cytokine release from WBCs.

this specific activation and secretion of human blood phagocytic cells, can be utilized for reliable assessment of putative inflammatory effects of nano-scaled drug carriers.

In this study, we determined the *in vitro* concentration of IL-1β cytokine in whole human blood after incubation with Amph-PVP nanoparticles. The results, shown in Fig. 5, demonstrate that there was no significant difference in the amount of IL-1β cytokine released by white blood cells incubated with Amph-PVP nanoparticles of various molecular weights and concentrations, as compared to non-treated controls. Thus, we can conclude that Amph-PVP nanoparticles do not initiate WBCs inflammatory response.

3.5. Effects of the Amph-PVP nanoparticles on platelets

In order to characterize the *in vitro* hemacompatility of Amph-PVP nanoparticles we also studied their effect on PLTs aggregation and coagulation. To measure the effect of Amph-PVP nanoparticles on PLT aggregation, PLTs count was carried out after 60 min of incubating platelet-rich plasma with various concentrations of polymer preparations (Fig. 6). The results of the experiment are presented on Fig. 6. The Collagen solution (1.0 mg/ml) was used as positive control and induced PLTs aggregation up to 80%. At the same time the three tested Amph-PVP nanoparticles did not significantly accelerate platelet aggregation at the concentrations from 0.05 to 0.5 mg/ml, as their effect did not exceed an induction of 20%, which fits well into the adjusted assay threshold (Neun and Dobrovolskaia, 2011a). In comparison to negative control (0 mg/ml), it was found that treatment of PLTs with 0.05–0.5 mg/ml of Amph-PVP nanoparticles slightly increased PLTs maximum aggregation, and this effect was more pronounced for Amph-PVP 2000 nanoparticles ($p < 0.01$). These data demonstrate a superior

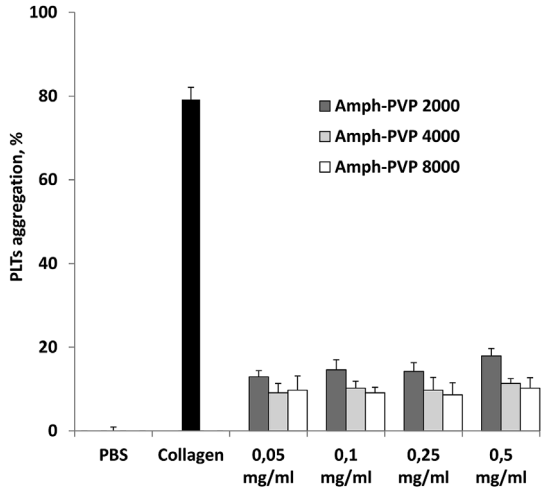

Fig. 6. Effects of the Amph-PVP nanoparticles on platelet aggregation.

biocompatibility of Amph-PVP 4000 and Amph-PVP 8000 in regard to PLTs.

To study the effects of the Amph-PVP nanoparticles on PLTs coagulation, the time of the three major coagulation pathways (final common, extrinsic and intrinsic pathways) can be estimated by measuring, respectively, thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) (Neun and Dobrovolskaia, 2011a). Based on the available literature the normal physiological values for TT, PT and APTT are 10.3–16.6 s, 9.4–12.5 s, and 25.1–36.5 s, respectively (Deb et al., 2012).

After incubation with Amph-PVP nanoparticles at different concentrations for 60 min, platelet-poor plasma was used for determination of the clotting time. The obtained results demonstrate that, in the studied range from rather low (0.05 mg/ml) to high concentrations (0.5 mg/ml), the three types of Amph-PVP nanoparticles did not induce any significant effects (p = NS) on platelet-poor plasma coagulation time regardless of the pathway studied (Fig. 7). These results clearly demonstrate that the use of Amph-PVP nanoparticles exhibits low risk of causing thrombosis due to the activation of coagulation cascade after administration and establishes the need of their further study as drug delivery systems.

3.6. Effects of the Amph-PVP nanoparticles on complement system activation

The human complement system consists of about 35–40 proteins in the blood plasma and on cell surface and represents a biochemical cascade that serves as part of the overall immune defense system clearing pathogens from the body (Brandt, 1985). For most of the administered drugs, complement system activation is undesirable because it can provoke allergic reactions (hypersensitivity) or even anaphylaxis which is a life-threatening condition (Neun and Dobrovolskaia, 2011b). The mechanisms of complement system activation by different nano-scaled drug carriers are still not fully investigated and understood, but it is established that one of the major factors influencing these processes is the surface charge of nanoparticles. Nanoparticles carrying significant positive or negative charge activate the complement system more than neutral particles (Bartlett and Davis, 2007; Nagayama et al., 2007; Reddy et al., 2007). Non-ionic polymer coatings (e.g., polyethylene glycol (PEG)), which to a large extent neutralize surface charge, were found to decrease complement activation initiated by nanoparticles administration (Vonarbourg et al., 2006). It is thus crucial to investigate nanoparticles intended for systemic administration as drug delivery system for their tendency to activate the complement system. In this study the influence of Amph-PVP nanoparticles on the activation of the complement system via the classical pathway (CP) and alternative pathway (AP) was investigated. The complement classical pathway is activated by immune (antigen-antibody) complexes. Activation of the alternative pathway is antibody independent and is promoted by complement factors B and D (Neun and Dobrovolskaia, 2011b).

First, the activation of the complement system by CP and AP was studied in terms of measuring lag-t, which is the time from the moment of red blood cells introduction into the tested serum until the initiation of the lysis process. This parameter characterizes the rate of the limited proteolysis reaction cascade and formation of a membrane attack complex. As the second parameter, the value of lysis rate (V) was estimated. The lysis rate was determined by measuring the decrease in opacity of red blood cells suspension reflecting on the “action” of the membrane attack complex in cells lysis. As seen from the data in Table 3, none of the investigated Amph-PVP nanoparticles samples had any significant influence on the activation of complement both by classical and by alternative pathways (p = NS). The absence of influence of the Amph-PVP nanoparticles on the hemolytic capacity of the complement (HCC), also established in this experiment, once again confirmed that the investigated preparations do not initiate the

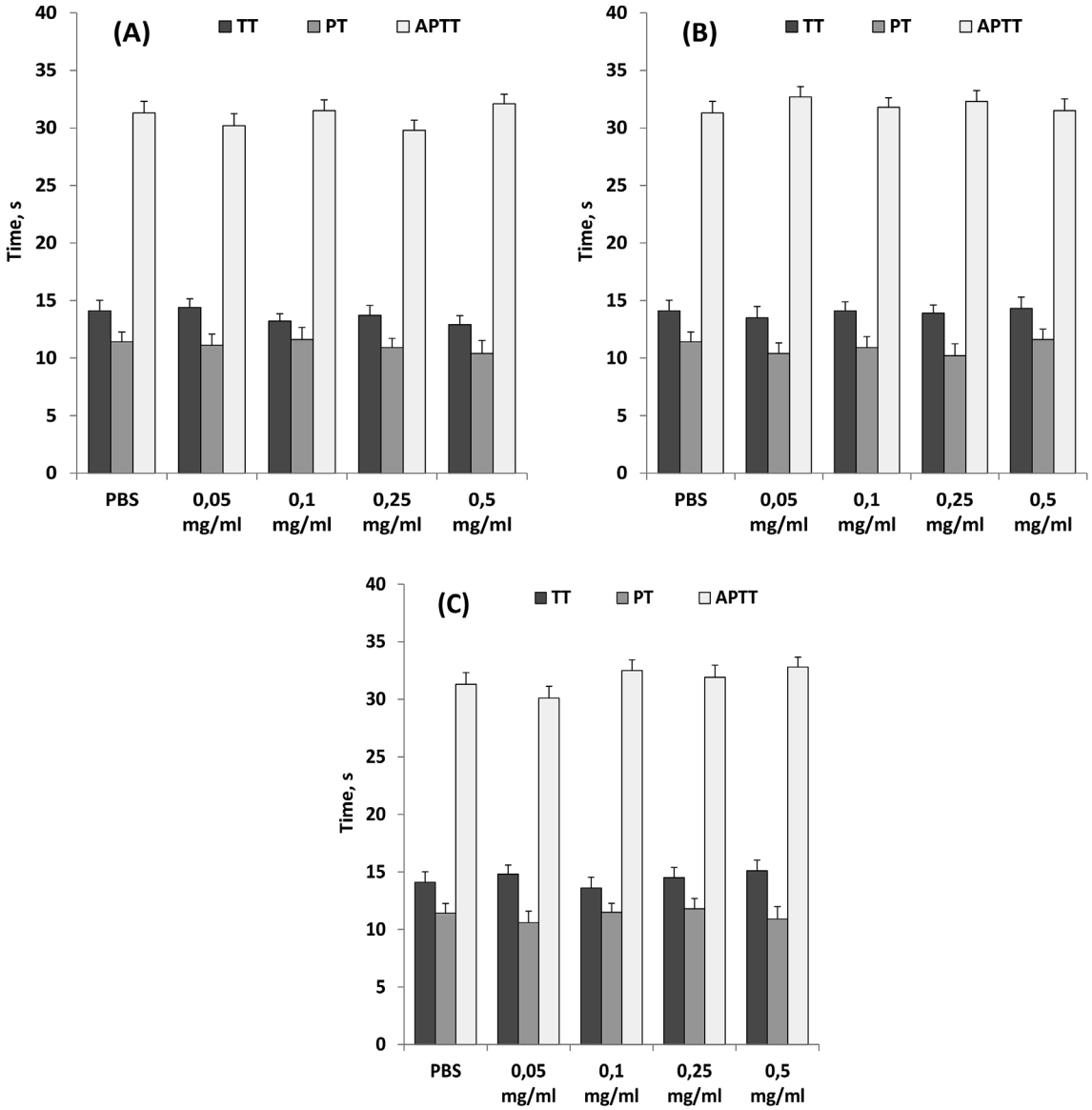

Fig. 7. Effects of the Amph-PVP 2000 (A), Amph-PVP 4000 (B) and Amph-PVP 8000 (C) nanoparticles on platelet coagulation.

Table 3
Effects of the Amph-PVP nanoparticles on the complement system activation via classical (CP) and alternative (AP) pathways. The results are presented in % of control (n = 3).

Amph-PVP (0.5 mg/ml)	lag-t (CP)	V (CP)	lag-t (AP)	V (AP)	HCC
Amph-PVP 2000	98 ± 3	96 ± 4	89 ± 4	87 ± 5	100 ± 4
Amph-PVP 4000	95 ± 5	97 ± 5	94 ± 3	96 ± 4	99 ± 5
Amph-PVP 8000	97 ± 4	99 ± 3	92 ± 4	102 ± 5	99 ± 4

complement cascades and do not decrease the lytic potential of the system (Table 3).

To summarize, the investigation of complement system activation together with the data on Amph-PVP nanoparticles interaction with different blood components demonstrated that all tested nano-carriers are very weak activators. This can be attributed to the intrinsic nanoparticle properties including a slightly negative surface charge (−5 to −10 mV), small sizes (less 100 nm) and hydrophilicity of the PVP outer shell. The low negative zeta potential of the Amph-PVP nanoparticles presumably contributes to very weak complement system activation and is in full agreement with earlier studies suggesting that a neutral or slightly positive or negative charge of the nanoparticle surface can minimize interactions with proteins (Devine et al., 1994).

The method of preparation of Amph-PVP nanoparticles used for this study allowed for the formation of rather small particles with sizes ranging between 60 and 100 nm. Such small sizes are expected to decrease the possibility of complement system activation, as complement cascade is the result of the reaction involving many complement protein components and needs adequate local space for formation (Moghimi and Hunter, 2001). Finally, another important parameter which, we assume, can play a major role in the low complement system activation by Amph-PVP nanoparticles, is the hydrophilic surface of nanoparticle outer shell consisting, in our case, of PVP chains. The presence of a hydrophilic coating on the nanoparticle surface is known to reduce the opsonization process occurring through hydrophobic interactions (Jeon et al., 1991). The Amph-PVP nanoparticles consist of a hydrophobic core formed by the octadecyl aliphatic moiety and a hydrophilic outer shell of PVP chains with molecular weight 2000, 4000 or 8000 Da, serving as a coating and, probably, creating a steric repulsion able to exceed the range of van der Waals attraction forces between Amph-PVP nanoparticles and blood proteins.

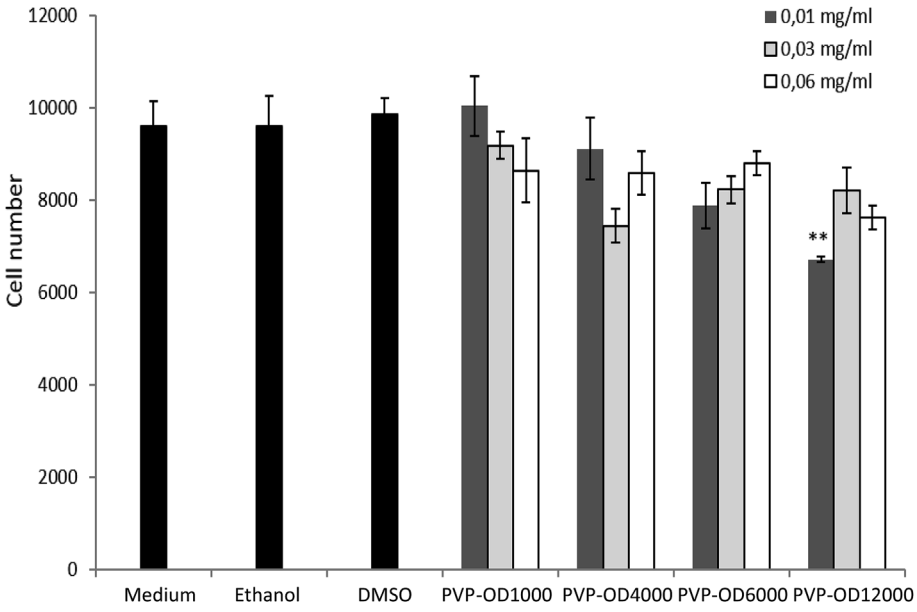

Fig. 8. The effect of Amph-PVPs (1000, 4000, 6000 and 12000) nanoparticles at different concentrations, on HMEC-1 endothelial cell growth. Results represent the average of three separate experiments (n = 6 for each experiment). Means ± S.E.M were plotted. Statistical significance, using the one way Anova with Tukey post-test, ** = $p < 0.01$ compared to controls (Medium and DMSO).

3.7. Effects of the Amph-PVP nanoparticles on endothelial cell growth viability

Treating HMEC-1 microvascular skin endothelial cells with nanoparticles constructed from Amph-PVPs of increasing molecular weight (1000, 4000, 6000 and 12000), at 0.01, 0.03 and 0.06 mg/ml during 48 h induced a trend of decrease of these cell growth compared to the controls ($p < 0.01$) (Fig. 8), as probed by the one way Anova with Tukey post-test. When the effect of each treatment on cell viability was compared separately utilizing Students t-test, statistical significance was not reached (data not shown). These results are well in agreement with previous studies on HepG2 and EBF-H9 cell viability in the presence of the PVP-OD2000, PVP-OD4000, and PVPOD8000 nanoparticles which likewise demonstrated, excellent biocompatibility even at the highest polymer concentration (Kuskov et al., 2018).

4. Conclusion

Understanding the interactions of drug delivery nanocarriers with blood is crucially important for their utilization in targeting a wide range of biomedical applications. After administration, a drug-loaded nanoparticle can be drastically impaired by blood cells or plasma causing the delivery and therapeutical effect to be disrupted. On the other side, nanoparticles themselves can influence blood cells and other blood components, altering their properties and functions in the body. This can result in the initiation of processes including blood cell lysis, aggregation, coagulation or activation of the complement system as immune response. In some cases, blood components can also be adsorbed on nanoparticles surface, resulting in a change of their properties and relative concentrations (Neagu et al., 2017; Shishatskaya et al., 2016).

Considering the above we have investigated the *in vitro* potential biological effects of Amph-PVP nanoparticles on blood components and vessel lining endothelial cells. It was shown that three amphiphilic poly-N-vinylpyrrolidone derivatives with PVP molecular weight 2000, 4000, 8000 Da at specific concentrations in aqueous media self-assemble into core-shell type, spherical nanoparticles with size range of 60–100 nm and slightly negative surface charge. These nanoparticles possess relatively high stability in the presence of blood serum. In the present study we demonstrated that the Amph-PVP nanocarriers have no significant effects on the function of RBCs, WBCs, PLTs and the complement system. For red blood cells, Amph-PVP 2000, Amph-PVP 4000 and Amph-PVP 8000 at concentrations up to 0.5 mg/ml did not cause significant hemolysis (hemolysis rate was determined to be lower than 5%). None of the nanoparticles studied showed any considerable effect on platelet aggregation and coagulation, as well as on the complement system activation at concentrations up to 0.5 mg/ml. In summary, our investigations provide a comparative overview of Amph-PVP nanoparticle *in vitro* blood biocompatibility. Treating HMEC-1 microvascular skin endothelial cells with Amph-PVP nanoparticles of increasing molecular weight induced a decrease in their growth rates. However, when comparing individual treatment with controls the changes in growth rates did not reach statistical significance. Our study provided specific conditions of hemacompatibility and safety of Amph-PVP nanoparticles utilization as an option for their further investigation as drug delivery systems for various biomedical applications.

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Abbreviations

PVP: poly-N-vinylpyrrolidone
Amph-PVP: amphiphilic N-vinylpyrrolidonepolymer
RBC: red blood cell
WBC: white blood cell
PLT: platelet

DPBS: Dulbecco's phosphate buffer saline
PBS: phosphate buffer saline
EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N'-tetraacetic acid
PVP-OD1000: amphiphilic N-vinylpyrrolidone polymer with molecular weight of hydrophilic polymer fragment 1000 Da and one hydrophobic octadecyl group
PVP-OD2000: amphiphilic N-vinylpyrrolidone polymer with molecular weight of hydrophilic polymer fragment 2000 Da and one hydrophobic octadecyl group
PVP-OD4000: amphiphilic N-vinylpyrrolidone polymer with molecular weight of hydrophilic polymer fragment 4000 Da and one hydrophobic octadecyl group
PVP-OD6000: amphiphilic N-vinylpyrrolidone polymer with molecular weight of hydrophilic polymer fragment 6000 Da and one hydrophobic octadecyl group
PVP-OD8000: amphiphilic N-vinylpyrrolidone polymer with molecular weight of hydrophilic polymer fragment 8000 Da and one hydrophobic octadecyl group
PVP-OD12000: amphiphilic N-vinylpyrrolidone polymer with molecular weight of hydrophilic polymer fragment 12000 Da and one hydrophobic octadecyl group
CAC: critical aggregation concentration
TEM: transmission electronic microscopy
DLS: dynamic light-scattering
TT: thrombin time
PT: prothrombin time
APTT: activated partial thromboplastin time