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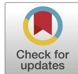

Fluorosurfactants for medical nanoemulsions, their surface-active and biological properties

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ABSTRACT

Nano- and microemulsions have found various applications in pharmaceutical and medical areas both in research field as well as in applied solutions for drug delivery or diagnostic agents. However, production of stable and bio-/ hemocompatible nanoemulsions are still challenging. New group of ionic surfactants have been synthesized with perfluorohexyl- or perfluorooctyl-groups as hydrophobic tail. The CMC and the parametres of the O/W emulsion (the particle size distribution and the zeta-potential) were determined. The influence of the surfactants on *in vitro* proliferation of human endothelial cell lines HMEC-1, murine fibroblasts L929 and hemolysis were investigated as characteristic for biocompatibility. Three candidates of surfactants were selected for pre-clinical tests on a small animal model (adult Sprague Dawley rats) on the basis of preliminary studies. This allowed to obtain nanoemulsions with narrow droplets size (average droplet diameter 141–147 nm with PDI index 0.059 – 0.065) and showed better stability over time in comparison to the commercially available surfactants. Neither cytotoxic nor hemolytic potential were observed during incubation of obtained fluorosurfactans with model cell lines L929 and HMEC-1 (average cell viability above 85 % after incubation with 1% solutions) and erythrocytes (hemolysis rate below 3.1 % for all 0.5 % solutions). During acute toxicity test on rat model, it was found that all three tested surfactant solutions showed no significant differences in controlled parameters and survival rate with control group ($p > 0.05$). Presented surfactants are dedicated but not limited to emulsification of organic fluorocompounds.

1. Introduction

An important challenge in nanomedicine is the possibility of obtaining stable and biocompatible nanoemulsions (NEs), that can be applied for drug delivery, cancer imaging and treatment, [1] vaccine carriers, antiviral therapy carriers against HIV and Ebola virus, genetic and biological agents delivery, *etc* [2]. NEs found also applications as a multifunctional targeting and diagnostic agents for ultrasound, MRI (Magnetic resonance imaging) and NIRF imaging (Near-infrared fluorescence) [3]. Contemporary nanomedicine studies verify many promising types of NEs for normothermic organ perfusion [4] or total parenteral nutrition [5]. It was also proposed to use NEs in food industry, cosmetics, agriculture, microfluidics and building blocks chemistry [6]. Specific group of NEs are perfluorocarbon emulsions which have the ability to dissolve and transport respiratory gases: oxygen and carbon dioxide (synthetic oxygen carrier systems) [7–9] and were used for oxygen delivery in blood substitutes [10], sensitizers in photodynamic cancer therapy [11,12] and as ultrasound imaging agents [13]. Perfluorocarbons (PFCs) are derivatives of aliphatic, cyclic or polycyclic hydrocarbons in which all of the hydrogen atoms have been replaced by fluorine atoms, the most electronegative element in the periodic table. These compounds are both hydrophobic and lipophobic, so they can enter the bloodstream only in an emulsified form. Moreover, PFCs effectively reduce the surface tension of aqueous solutions and show biologic inertness and low toxicity even at high doses [14]. The selection of a suitable surfactant is therefore a key element in obtaining a PFC nanoemulsions.

Although wide range of potential application of NEs', their stability

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remains significant limitation for further development. Small size of the droplets makes NEs instable in time due to the Ostwald ripening phenomenon. [15] Production of stable nanoemulsion requires high concentrations of surfactants due to the large interfacial area, however surfactants in high concentrations show significant cytotoxicity [16], which is unacceptable when considering NEs medical applications. For this reason, the development of new biocompatible and effective surfactants seems crucial for future exploration of NEs' potential and the one of the promising group are fluorosurfactants.

The synthesis of fluorosurfactants with potential biomedical application was already reported, [17–19] however, there is a lack of sufficient data regarding physicochemical and biological properties. In this paper, the synthesis and the *in vitro* biocompatibility tests for novel fluorosurfactants are described, including hemolysis and cell lines toxicity research. Properties of synthesized surfactants were compered to commercially available surface-active agents: ionic sodium dodecyl sulfate (SDS) and hexadecyl(trimethyl)ammonium bromide (CTAB)- which give highly stable emulsions but at the same time show inacceptable toxicity; zwitterionic Soybean Lecithin- commonly used as a model biocompatible emulsifier for pharmaceutical formulations [20] and nonionic Pluronic F-68 and Pluronic F-127- reported as no toxic trough oral, topical, intranasal, vaginal, rectal, ocular, and parenteral administration, which make them suitable for medical applications [21, 22].

2. Materials and methods

2.1. Materials

Perfluorodecalin (Abcr GmbH, Germany), 1*H*,1*H*,2*H*,2*H*-Perfluoro-1-octanol, 1*H*,1*H*,2*H*,2*H*-Perfluoro-1-decanol, 1*H*,1*H*,2*H*,2*H*-Perfluoro-1-decanethiol, 1,1,3,3-tetramethylguanidine (TMG), hexadecyltrimethylammonium bromide (CTAB), Pluronic F-127 and Pluronic F-68 (Kolliphor P188) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soybean Lecithin was purchased from Serva Electrophoresis GmbH (Germany). Sodium dodecyl sulfate (SDS) was purchased from Thermo Scientific (USA). 6% Voluven solution (Hydroxyethyl starch; HES 130/0.4, in isotonic sodium chloride solution) was purchased from Fresenius Kabi (Germany). Commercially available reagents and dry solvents were used as received. Analytical and preparative TLC were performed on Silica Gel 60 F254 (Merck). Milli-Q water was used throughout all experiments.

2.2. Synthesis of ionic fluorosurfactants

A series of fluorinated acids were obtained (**1**-**14**, Table 1) from commercially available thiols and alcohols with different fluorine chain lengths ($R_f = C_6F_{13}$ or C_8F_{17}).

First attempt (Fig. 1; c): The anhydride ring opening reaction was performed according to the Yoshikawa and Masaru method. [23] Briefly, a appropriate alcohol (1H,1H,2H,2H-Perfluoro-1-octanol or 1H, 1H,2H,2H-Perfluoro-1-decanol,; 1 eq.), anhydride (1.1 eq.) and DMAP (0.2 eq.) were dissolved in THF (C = 2.7 M) and stirred at 100 °C for 120 min. Then, the mixture was cooled to 30 °C, 100 mL of water was added, and the mixture was further cooled to 15 °C. The white precipitated crystals were collected by filtration to obtain suitable carboxylic acid in a very good yield.

Second attempt (Fig. 1; a, b): Highly efficient synthesis was performed in one or two steps following by the procedure of *S*-alkylation in the presence of TMG described by Włostowski [24] (see the Supporting Information for details).

2.3. Chemical characterization

The structure of all obtained compounds was confirmed by NMR (Varian VNMRS, 500 MHz ^{1}H, 126 MHz ^{13}C, 470 MHz ^{19}F), MS (API 3000 triple quadrupole mass spectrometer, Applied Biosystem MDS SCIEX), FTIR (Nicolet 6700 FT-IR, Thermo Scientific) and elemental analysis assays (Elementar UNIcube analyzer).

2.4. Critical micelle concentration (CMC) procedure description

Measurements of critical concentration of micellization (CMC) of surfactant solutions were evaluated by the conductometric method using Mettler Toledo SevenMulti equipment with conductivity expansion. CMC parameter was determined based on the change in the

Table 1
Structure and numbering of the obtained surfactant.

Acid ($R_{f1} = C_6F_{13}$, $R_{f2} = C_8F_{17}$) \ Amine	a	b	c	d	e	f	g
R_{f1} (1)	-	1b	1c	1d	1e	1f	-
R_{f2} (2)	2a	2b	2c	2d	2e	2f	-
R_{f1} (3)	-	3b	3c	3d	3e	3f	3g
R_{f2} (4)	4a	4b	4c	4d	4e	4f	4g
R_{f1} (5)	-	5b	5c	5d	-	-	5g
R_{f2} (6)	-	6b	6c	6d	-	-	6g
R_{f1} (7)	-	7b	7c	7d	7e	7f	-
R_{f2} (8)	8a	8b	8c	8d	8e	8f	8g
R_{f1} (9)	-	9b	9c	9d	-	-	-
R_{f2} (10)	-	10b	10c	10d	-	-	-
R_{f1} (11)	-	11b	11c	-	-	-	-
R_{f2} (12)	-	12b	12c	12d	12e	-	-
R_{f2} (13)	-	13b	13c	-	-	-	-
R_{f2} (14)	-	14b	-	-	-	-	-

Ionic fluorosurfactants were obtained by reaction of wide range of fluorinated carboxylic acid **1-14** with an appropriately selected amine **a-g** (Table 1). Briefly, acid (1 eq.) and amine (1 eq.) were dissolved in MeOH (C = 2 M; or mixture MeOH/H_2O for amine insoluble in MeOH) (Fig. 1; d). The mixture was then slowly heated to dissolve. The salt was evaporated under reduced pressure and dried.

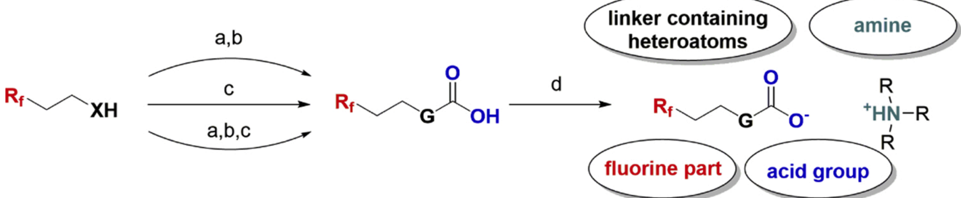


Fig. 1. General synthesis of new fluorosurfactants. X = O, S; a) TMG, alkylation agent acetone, 50 °C; b) H+, c) anhydride, DMAP, THF, 100 °C; d) amine, MeOH, ΔT.

conductivity of the surfactant solution before and after the formation of micelles. The conductivity increases linearly with increasing surfactant concentration until a certain point is reached, beyond which the conductivity changes its slope. The CMC point is visible as a refraction in the diagram of the dependence of conductivity on the concentration of the tested surfactant.The tested surfactant concentration range was 0.002–40 mM. Ultrapure water (Milli-Q) was used as solvent. The CMC value was given as an average of 3 measurements.

2.5. Preparation of the emulsion

Ternary systems consisting of surfactant, water and perfluorocarbon (perfluorodecalin) were studied. 180 mg of the surfactant was dissolved in 9 mL of ultrapure water (Milli-Q), then 1 mL of perfluorodecalin was added to the solution. The mixture was sonicated using an UP400 St ultrasonic homogenizer (Hielscher, Germany). Operation parameters of the device: A = 90 % (amplitude), continuous operation mode, sonotrode type - H14. Ultrasonic homogenization was carried out for about 2 min, while the reaction vessel was cooled in an ice bath. 10 mL of the O/W emulsions containing 10 % (*v/v*) of perfluorodecalin and 1.8 % (*w/v*) of investigated surfactant were prepared.

2.5.1. Particle size distribution and zeta potential

The emulsion droplets size and zeta potential were evaluated by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments. Ltd. Worcestershire. UK). Size of droplets is presented as an averaged hydrodynamic diameter (d_z). The samples were diluted in water to a final concentration range of 4 mg/mL for particle size measurement and 20 mg/mL for zeta potential (in triplicate).

2.6. The cellular toxicity

The cellular toxicity of surfactants was studied using murine fibroblasts (L929, ECACC, 85011425, UK) and human epithelial cell lines (HMEC-1, ATCC® CRL-3243, US). Cells were cultivated in a humidified atmosphere at 37 °C in 5% CO_2 incubator (standard condition).

Surfactant *in vitro* cytotoxicity evaluation was conducted in accordance with XTT procedure similar to procedure described in ISO norm 10993-5:2009(E) „Biological evaluation of medical devices — Part 5: Tests for *in vitro* cytotoxicity". Each experiment was repeated at least 3 times ($n \geq 3$).The percentage of the viable cells in each well comparing to the controls was determined. Whereas viability decreased below 70 % indicated cytotoxic influence on cells. The highest non-cytotoxic concentration of tested compounds were determined and summarized in Table S2, Supporting Information. [25] Initially, each synthesized surfactant (Table S1, supporting information) was tested at four concentrations: 0.25; 0.50; 1.00; 2.00 % (*w/v*). When toxicity was revealed, the compounds were further examined at the lower ones: 0.01; 0.02; 0.05; 0.10; 0.20 % (*w/v*).

24 h before the experiment, surfactant was weighted in two glass vessels, dissolved in supplemented culture media and sterilized by filtration (through a sterile syringe filter with 0.22 μm pores). In case of insoluble compound, extraction at 37 °C for 24 ± 2 h was conducted as described in ISO norm 10993-12:2004(E) "Biological evaluation of medical devices — Part 12: Sample preparation and reference materials". Obtained suspension was centrifuged, supernatant aspirated, filtered and used. Varied concentrations of the obtained solutions were prepared in sterile condition shortly before applying to cells.

Emulsion for the cytotoxicity tests was prepared as described in section '*2.5. Determination of emulsion parameters*', except that perfluorodecalin was replaced with a mixture of two perfluorocarbons: perfluorooctyl bromide (PFOB) and perfluorodecyl bromide (PFDB) (97: 3 w/w). The O/W emulsion contained 10 % (v/v) of the PFC phase and 1.8 % (w/v) of the tested surfactant. 96-well culture plates with inserts made of permeable membrane support were used for the assay. Cells were seeded in 100 μL of growth medium per well on the lower compartment of a plate and 100 μL of emulsion was added onto the membrane of the upper compartment of each well. After 24 -hs, inserts and growth medium were removed and replaced with fresh 150 μL. The XTT assay was then performed as described in the supporting material. Three concentrations were tested: 50 %, 25 %, 12.5 % (v/v).

2.7. In vitro hemolytic properties

Surfactants hemolytic properties was analysed adapting ASTM International Standard E2524 – 08: Standard Test Method for Analysis of Hemolytic Properties of Nanoparticles (Reapproved 2013). Aforementioned test is based on the quantitative determination of hemoglobin released into supernatant after blood exposure to tested material. Each surfactant was tested at least in 4 different concentrations (0.25 %. 0.5 %. 1.0 %. 2.0 %) and was repeated at least 3 times ($n \geq 3$).

2.8. In vivo studies

2.8.1. Sample preparation

All tested substances: **4b, 4c, 6c,** Soyabean Lecithin and Plurinic F-68 were administrated as a 0.5 % (*w/v*) solutions in Voluven. 40 mL of filtered solutions (pore size 0.22 μm, Merck Milipore) were closed in 100 mL sterile glass vials with septum under aseptic contitions and stored in 2−8 °C. Solutions were mixed and preheated up to 36 °C before administration.

2.8.2. Animals

The study was conducted according to the standards of Annex III of the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The study protocol was approved by the Local Ethical Committee (No. WAW2/158/2019), Warsaw University of Life Sciences, Warsaw, Poland.

Male Sprague Dawley rats (n = 30; weight, 369 ± 52 g; age, 8–12 weeks) were obtained from the Mossakowski Medical Research Centre, Polish Academy of Sciences (Warsaw, Poland). Animals were kept in the air flow cabinet (Bioscape Fa. UniProtectTHF 3378-V02) in individual cages (temp. 23 ± 1 °C; humidity 55 % ± 5 %), and 12:12 h light:dark cycles (100−250 lx) with free access to food and water. Air flow cabinet kept 20 air conversions cycles per hour. Animal were under 24 h veterinary care and clinical examination were done every morning.

2.8.3. Anesthesia, analgesia, blood pressure monitoring and catheter placement

12 h before the experiment, feedstuff were removed from animals' cages without restrictions to the water. Animals were weighted for circulating blood volume calculation according to Lee and Blaufox [26] and anesthetized by intramuscular injection of the mixture of Ceptor - 0.1 mL (Scanvet), Butomidor - 0.1 mL (Richter Pharma AG) and Bioketan - 0.1 mL (Vetoquinol). Within 2 min after injection, animals were placed in the dorsal position, on Thermo-Controlled Surgery Platform (Braintree Scientific, USA).

Silicone catheter (Scientific Commodities INC, USA) was placed within the right jugular vein and fixed with the surgical suture (4−0) to prevent any displacement. In between blood collection, catheter was fulfilled with Ringer's solution, patency of the catheter was kept without the anti-clotting agent. Rats were placed on the thermo-controlled platform GN 1/1 (temp. 37 ± 1 °C, Bartscher, Poland) and the cuff (MLT125R, ADI, Australia) for NIBP (non-invasive blood pressure) measurements were placed on tail and connected to the eight-channel PowerLab system (ADI, Australia) and PC. The tail cuff pressure was continuously recorded with a solid-state pressure sensor.

2.8.4. Blood removal and administration of tested substances

After catheter placement rats were stabilized for 15 min. Shock was induced by removing 5 % of rat blood (calculated on the basis of the weight of individual rats [26]) and substitute in the same volume as removed blood with the tested substances according to the study design.

The initial values of systolic blood pressure in the studied rats under general anesthesia were in the range of 130−155 mmHg. Bleeding was continued until the blood pressure dropped to 45–55 mm Hg defined in the study as the humanitarian endpoint. Conducting of hemorrhagic shock below the defined level could led the physiological and histological alterations, [27] disorder study results interpretations, and not show possible toxic effect.

2.8.5. Blood analysis – gasometry and morphology

Venous blood samples collected during the experiment were analyzed in Siemes (EPOC Siemens) gasometer: pH, pO_2 (oxygen partial pressure [mmHg]), pCO_2 (carbon dioxide partial pressure [mmHg]), $cHCO_3^-$ (concentration of bicarbonate [mmol/L]), BE (b) (base excess in blood [mmol/L]), BE (ecf) (base excess in extra cellular fluid [mmol/L]), $cTCO_2$ (total amount of CO_2[mmol/L]).

Blood morphology analysis was done using Mindray analyzer: hematocrit (Hct, %); hemoglobin (g/L); red blood cell count (RBC, $\times 10^{12}$/L); white blood cell count (WBC, $\times 10^9$/L) and platelets number (PLT, $\times 10^9$/L). Middleton et al. (2006) demonstrated the consistency of gasometry measurements (pH, bicarbonate concentration, deficiency of buffering bases (BE), lactate concentration) from venous and arterial blood enabling assessment of acid-base balance in the body.

2.9. Histology

Tissue samples were fixed in 4 % buffered paraformaldehyde for 5 d and embedded in paraffin, manually sectioned with a microtome to obtain 5 μm-thick paraffin sections. Dewaxed sections were stained then stained with hematoxylin and eosin (H&E) for histopathological analysis under light microscope (Chapter S7; Supporting Information).

2.10. Statistical analysis

All statistical analyses were performed with Prism version 8.3 (GraphPad Software, San Diego, CA, USA). Results are shown as mean ± standard deviation (SD). Statistical analysis was done using a two – way ANOVA followed by Tukey`s multiple comparison test. Survival rate of Sprague Dawley rats was calculated with Kaplan–Meier estimator and the log-rank test was used to compare the survival curves. Differences were considered significant at a P value of <0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

3. Results and discussion

3.1. Characterisation of ionic fluorosurfactants

During our studies on the preparation of perfluorocarbon nanoemulsions, great attention was dedicated to new ionic fluorosurfactants. It is known that replacing hydrogen with fluorine atoms reduces their hemolytic activity. [28] In addition, several cytotoxicity tests reveal that nonionics have the least toxicity in the order as cationic > anionic > amphoteric > non-ionic. [29].

The length of the hydrophobic part is an important element affecting the properties of the compound (Fig. 2). The short fluorinated part gives the surfactants excellent solubility in water, but on the other hand, the emulsion is not stable enough and toxicity is higher. Elongation of the fluorinated chain increases the stability of the emulsion and decreases the toxicity of the surfactant but at the same time reduced solubility in aqueous solutions of the compound is observed. Therefore, compounds containing 6 or 8 fluorinated carbon atoms were selected, as the optimal length of the hydrophobic part.

The first studies showed that salts **4b**, **4c**, **6c** have unique properties, therefore, their description is presented in the main part of the article (Fig. 3). Information for others surfactants, can be found in Supporting Information.

3.2. CMC determination

The values of CMC for the surfactants **4b**, **4c** and **6c** were in the range of 0.45 to 0,63 mM (Fig. 4). In general, the values of individual CMCs depend on the construction of the compound, lower values were observed for surfactants with a longer fluorinated (hydrophobic) part. A negative correlation was also found between the alkylated chain length of the surfactant and the value of CMC. Critical concentration of micellization parameter was measured also for commercial surfactants (Fig. 4). The lowest CMC values were determined for Pluronics (0.0065 mM and 0.0218 mM) while SDS has the highest value: 7.06 mM.

3.3. Characterisation of nanoemulsion

The most representative surfactants **4b**, **4c**, **6c** were selected and their properties were compared to commercially available surfactants (Table 2).

Emulsions with surfactants **4b, 4c, 6c** immediately after receiving (0 h) were characterized by similar values of avereged diameter (d_z), which oscillate in the range 141–147 nm, as well as almost identical particle size distribution (Table 2). Also the polydispersity indexes (PDI) for the above-mentioned surfactants were small and equaled 0.06, which indicate highly homogeneous emulsions. The same properties were observed for strong ionic surfactants, such as SDS and CTAB (d_z value of 154 nm and 186 nm, respectively). PDI was equal to both groups of emulsions and had value 0.04. Emulsion with the addition of Soybean Lecithin, was characterized by the highest polydispersity index: 0.29, compared to the rest of the emulsions. Strong inhomogeneity of the Soybean Lecithin emulsion could be a result of the large amount of surfactant micelles (low peak at about 35 nm, in the particles size distribution plot (Fig. 5B). Moreover, the presence of micelles lowered the value of the averaged diameter, which was 153 nm, thus it was close to the value of d_z for emulsions with compounds **4b, 4c, 6c**. The emulsions with addition of Pluronics showed the worst properties: DLS analysis reported the highest values of d_z and broad particles size distribution (391 nm; 0.17 for Pluronic F-127 and 260 nm; 0.08 for Pluronic F-68).

The stability of nanoemulsion was examined after 48 h and 6 months of storage at 4 °C (the reduced temperature presents the possibility of long-term storage the final product, Fig. 5A). DLS measurements after 48 h of emulsion storage showed a slight increase in avereged diameter for

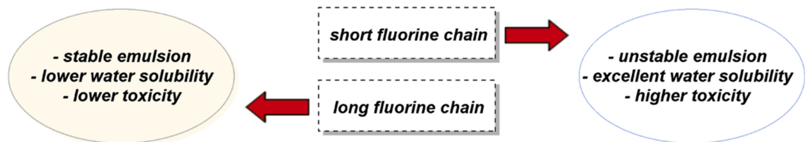


Fig. 2. Changes in surfactant properties depending on the length of the fluorinated chain.

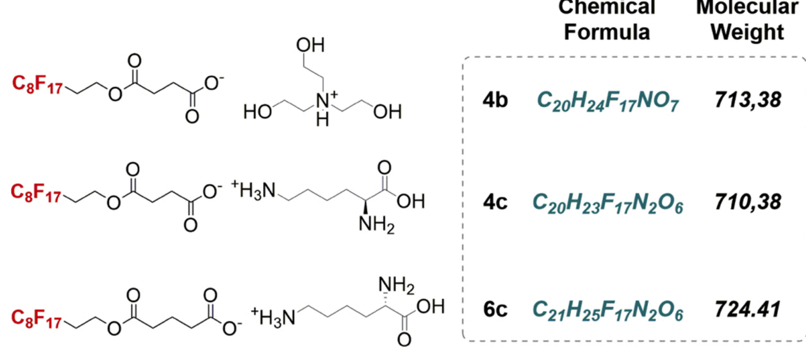
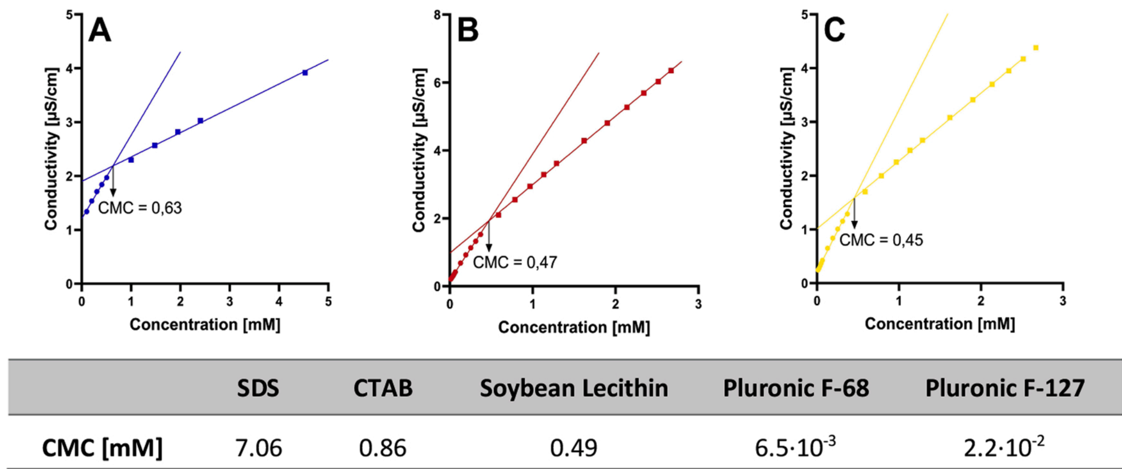


Fig. 3. Structure and numbering of surfactants with the unique properties.



	SDS	CTAB	Soybean Lecithin	Pluronic F-68	Pluronic F-127
CMC [mM]	7.06	0.86	0.49	$6.5 \cdot 10^{-3}$	$2.2 \cdot 10^{-2}$

Fig. 4. Determination of CMC by conductivity for: A) **4b** B) **4c** C) **6c** and comparison to commercial surfactants.

emulsions with surfactants: by 35 nm for **4b**, by 21 nm for **4c**, by 23 nm for **6c** and by 29 nm and 49 nm for SDS and CTAB, respectively. For Soybean Lecithin emulsions this value practically not changed. The worst results were obtained for Pluronics emulsions, where d_z value increased significantly: by 126 nm for Pluronic F-127 and by 75 nm for Pluronic F-68.

After 6 months of emulsion storage (4 °C), a huge difference in particle size between individual emulsions was observed (Fig. 5A). Emulsions with **4c** and **6c** surfactants exhibited outstanding stability compared to other surfactants. Their average diameter increased to 193 nm and 205 nm, respectively, while maintaining a very low PDI value (less than 0.05). The Soybean Lecithin emulsion also retained its average particle size (161 nm), however in the same time its PDI value increased significantly to 0.588. Also, very high PDI was measured for emulsions with **4b** (0.626), CTAB (0.487) and both Pluronic: F-127 (0.803) and F-68 (0.716). At such high values the average particle diameter are no longer reliable. This indicates low homogenity and polydispersity of emulsion. In the case of Pluronics emulsions their average diameter were larger than device's measuring range. For emulsion with SDS the average particle diameter and PDI value increased significantly: to 744 nm and 0.192. The increase in average particle diameter over time may be due to Oswald ripening, which is one of the most serious problem in maintaining emulsion stability.

The zeta potential values of emulsions with the addition of surfactants: **4b, 4c, 6c**, SDS and Soybean Lecithin were very similar to each other, ranging from -66.3 mV to -57.6 mV (Table 2). For Pluronics, which are non-ionic compounds, the value of ζ fluctuated from -6.7 mV to -3.7 mV and emulsions were stabilized only sterically. CTAB is a cationic surfactant, so the emulsion with its addition has a positive value of the zeta potential (67.3 mV).

3.4. Cellular toxicity

The highest noncytotoxic concentration was determined on L929 and HMEC-1 cell lines for 59 new surfactants and the results are summarized in Table S2 (Supporting Information). Of the all compounds, as many as 37 surfactants revealed good tolerability for at least one of the cell line at the high-range concentrations; 7 of them (**2 d; 4 b, c; 6 b, c, d, g**) exhibited great cells viability for both cultures at 1–2 % (*w/v*); 16 compounds were tolerable at lower than 0.25 % (*w/v*) and only 6 compounds (**1f** – **8f**) revealed high cytotoxicity even at 0.01 % (*w/v*) for both cell lines (Table S2; Supporting Information). Additionally, SDS,

Table 2
Summary of the most important parameters of emulsion.

No.	Surfactant		Stability 0 h	48 h	6 months	Zeta-potential [mV]
1	SDS	A	154 ± 2.3	184 ± 1.9	744 ± 15.4	−61.8 ± 1.1
		B	0.04 ± 0.02	0.04 ± 0.01	0.19 ± 0.04	
2	CTAB	A	186 ± 2.2	235 ± 2.3	1639 ± 177	67.3 ± 1.7
		B	0.04 ± 0.02	0.03 ± 0.02	0.49 ± 0.25	
3	Soybean Lecithin	A	153 ± 9.7	154 ± 2.3	161 ± 5.6	−65.9 ± 1.3
		B	0.29 ± 0.01	0.35 ± 0.04	0.59 ± 0.08	
4	Pluronic F-68	A	260 ± 3.1	335 ± 3.6	5770 ± 1362	−3.8 ± 0.1
		B	0.08 ± 0.03	0.14 ± 0.03	0.72 ± 0.32	
5	Pluronic F-127	A	391 ± 16	517 ± 7.5	5385 ± 595	−6.7 ± 1.0
		B	0.17 ± 0.02	0.29 ± 0.03	0.80 ± 0.34	
6	**4b**	A	147 ± 1.6	182 ± 2.9	654 ± 118	−66.3 ± 1.6[a]
		B	0.06 ± 0.02	0.05 ± 0.02	0.63 ± 0.14	
7	**4c**	A	146 ± 1.3	168 ± 1.7	193 ± 1.5	−62.3 ± 1.6[a]
		B	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.03	
8	**6c**	A	141 ± 1.4	164 ± 2.2	205 ± 1.8	−57.6 ± 2.4[a]
		B	0.06 ± 0.01	0.04 ± 0.02	0.04 ± 0.03	

A Mean droplet size [d. nm]; **B** Polydispersity index; [a] - zeta potential value for emulsions 10 times diluted.

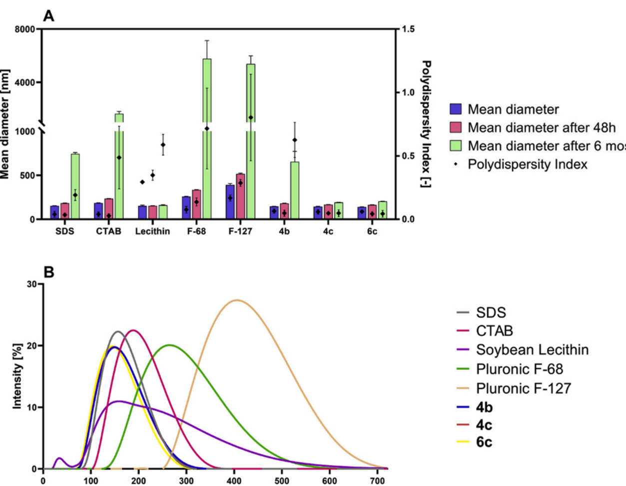


Fig. 5. A) The stability of nanoemulsion after 48 h and 6 months. Significant differences are shown in Supporting Information, Chapter 8 **B)** Size distribution by intensity.

CTAB, Soybean Lecithin, Pluronic F-127 and Pluronic F-68 were evaluated for their cytotoxic potential. Both Pluronics had no negative impact on cells. Surprisingly, Soybean Lecithin, which is widely used to produce nanoemulsions for medical uses [30–32] revealed cytotoxicity on HMEC-1 (36 % viability at 1% (*w/v*)) but not on L929 (100 % viability at 1% (*w/v*); data not shown). The ionic detergents: SDS and CTAB caused damages to both cultures as expected [33–37] even at 0,01 % (*w/v*) concentration.

Cells exposed for 24 h to medium containing 0.25 % *(v/w)* of each of the examined compound (**4b, 4c** and **6c**) independently exhibited great metabolic activity and showed no differences compared to Pluronics and Lecithin groups (Fig. 6A). Interestingly, mean viability of cells incubated with **4b** is higher than 100 % of control. The explanation of this phenomena could be either **4b** molecule disturbed normal metabolism and accelerated enzymatic activity due to stress or influenced proliferation ratio, therefore more XTT reagent was converted giving high amounts of formazan resulting higher than control group activity.

The medium containing emulsions (from the tested compounds **4b**, **4c**, **6c** and PFC) with a concentration below 25 % (v / v) did not show any cytotoxic properties (Fig. 6B). For the 50 % emulsion, a decrease in metabolic activity was observed only for the emulsion with the surfactant **4b** (60 % ± 4.2).

Our results indicate that the surfactants **4b**, **4c**, **6c** are noncytotoxic at less than 1 % (*w/v*) and noncytotoxic in emulsion at concentrations below 25 %. Thereafter, these surfactants were chosen to be tested in animal studies at safe 0.5 % (*w/v*) concentration intravenously. No acute toxicity has occurred and rats survived as long as the control group, which confirmed lack of toxicity observed during *in vitro* tests. Nevertheless, different type of cytotoxicity assay (based on ATP amount or fluorometric staining with cytometric measurements) should be further performed as XTT assay was limited in several experiments.

3.5. Hemolysis assay

Hemolysis was another parameter of biocompatibility that was tested. All of the synthesized salts **1b-14c** were examined and summarised in Table S2 (Supporting Information).

Hemolytic activity is strongly reduced and often suppressed when fluorinated chains are in the structure of surfactant. For example, the maximum non-hemolytic concentration for compound **3b** and **3c** ($R_f = C_6F_{13}$), was 0.02 and 0.05 %, respectively. Whereas salts **4b** and **4c** with elongated fluorinated chain ($R_f = C_8F_{17}$) had this parameter much higher (> 2.00 % for both compounds). This effect is almost invisible in the case of acid salts **7** and **8**, where the carbon part was considerably extended (*e.g.* **7c** *vs.* **8c**, respectively 0.01 %, 0.02 %). The introduction of the aromatic ring has a very adverse effect on hemolytic activity. Even the elongation of the fluorinated chain does not reduce the toxicity caused by this effect (*e.g.* **9b** *vs.* **10b**, respectively 0.02 %, 0.01 %).

As expected, the ionic surfactants SDS and CTAB exhibited strongly hemolytic properties, in all concentrations. In contrast, Soybean Lecithin, Pluronic F-68 and Pluronic F-127 were not hemolytic in the concentration range 0.25–1.00 %. Also results for surfactants **4b**, **4c** confirm no damage to red blood cells in the same concentration range. Interestingly, in the case of compound **6c**, the lengthening of the carbon chain by only one atom (compared to compound **4c**) caused a decrease in the maximum non-hemolytiction concentration to 0.50 % (Fig. 7). It should be emphasized that compounds **4b** and **4c** show a slightly smaller hemolyticity than the mentioned above Lecithin.

The obtained results clearly indicate that the longer fluorocarbon chain, the less hemolytic compound (opposite to hydrocarbon surfactants [17]). Fluorosurfactants can be considered as a potential group of compounds with non-hemolytic properties.

3.6. In vivo Trials

Acute toxicity study on rat model was carried out for all three developed surfactants (**4b**, **4c**, **6c** experimental group) as well as for Voluven, Soyabean Lecithin and Pluronic F-68 (control group). The model of hemorrhagic shock was chosen and the methodology is presented in Fig. 8A. Briefly, 5 % of rat blood was collected followed by administration of 5 % of tested fluids. The whole procedure was repeated until the blood pressure dropped to 45–55 mm Hg.

Survival data of rats after administration of individual preparations

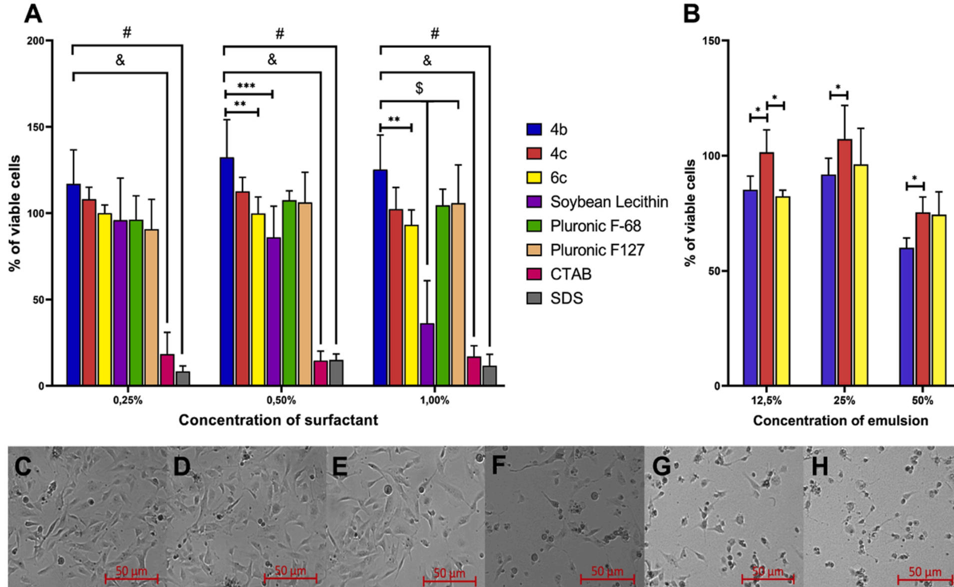


Fig. 6. Compared cytotoxicity results of representative: **A)** surfactants **4b**, **4c**, **6c** with SDS, CTAB, Pluronics and Soybean Lecithin and **B)** PFC nanoemulsion with surfactants **4b**, **4c**, **6c**; tested on HMEC-1 cell line. Exemplary HMEC-1 cells viability in 0.25 % (**C**), 0.5 % (**D**) and 1% (*E*) **4b** solution *versus* 0.25 % (**F**), 0.5 % (**G**), 1% (**H**) Soybean Lecithin solution are presented. & mark represents CTAB *vs* other surfactants (except SDS and 1% SL) – $p < 0.0001$. # mark represents SDS *vs* other surfactants (except CTAB and 1% SL) – $p < 0.0001$. $ mark represents Soybean lecithin *vs* other surfactants (except SDS and CTAB) – $p < 0.0001$. ***$p < 0.001$, **$p < 0.01$.

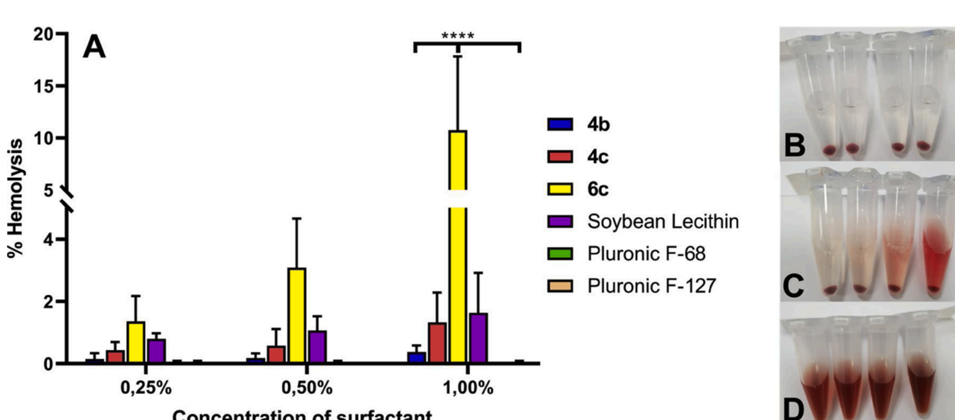


Fig. 7. **A)** Hemolysis results for tested surfactants; **B)** no hemolysis in the whole range of concentrations; **C)** hemolysis depending on concentration; **D)** hemolytic compound in the whole range of concentrations; samples are stacked with increasing concentrations of 0.25 %, 0.5 %, 1.0 %, 2.0 % (from left to right). **** $p < 0.0001$ (**6c** *vs* other surfactants).

are presented in the Fig. 8B. Survival in all groups decreased in a manner comparable without statistically significant differences. Pluronic F-68 and **4c** groups showed 100 % survival for the highest number of administrations (19 and 15, respectively). Finally, survival for groups **4b**, **4c** and **6c** was the longest from the tested preparations and amounted to 28, 29 and 31, respectively.

Data regarding the amount of compound administered and the volume percent of blood exchanged were shown in the Fig. 8C. The control group had the lowest and highest value of the compound weight per kg rat weight (for Soybean Lecithin – 250 mg/kg, for Pluronic F-68 – 340 mg/kg respectively). In the experimental group, intermediate values were observed (for **4b** – 310 mg/kg, for **4c** – 324 mg/kg and for **6c** – 315 mg/kg). There were no statistically significant differences between the groups. An analogous trend was noticed for the volume percent of exchanged blood. The lowest and highest values appeared in the control group: 68.5 % for Pluronic F-68, 62.6 % for Soybean Lecithin and 58.4 % for Voluven. In contrast, the values of the volume percent of exchanged blood in the experimental group were similar to each other and were in the middle (63.9 % for **4b**, 66.0 % for **4c**, 63.4 % for **6c**). There were no statistically significant differences between the groups.

3.6.1. *Blood morphology*

Blood morphology is the basic diagnostic test of blood that determines quantitatively and qualitatively the content of red blood cells (RBC, HGB - hemoglobin, HCT - hematocrit), as well as white blood cells (WBC) and platelets (PLT) [38,39]. The successive decrease of individual parameters in all treated groups of animals was associated with a reduction in the content of blood components (5 % of blood was collected at each interval; Fig. 9). During the experiment, no differences were found in the effect of individual test fluids on changes in blood counts in rats (for the details see Supporting Information; Chapter 4; Fig. S1).

3.6.2. *Gasometry*

The best expression of organism's acid-base homeostasis is gasometry [40,41]. The lungs and kidneys are the two main organs that regulate the acid-base levels. In the case of poor lung ventilation, carbon dioxide level in the blood increases which leads to a pH drop (respiratory acidosis). Blood pH and pCO_2 was in line with reference values during the whole experimental period for all tested group, which proves the proper functions of the lungs and kidneys are maintained.

The pO_2, $cHCO_3$, BE (b), BE (ecf) and $cTCO_2$, levels constantly decreased with the increase of the number of applications, however

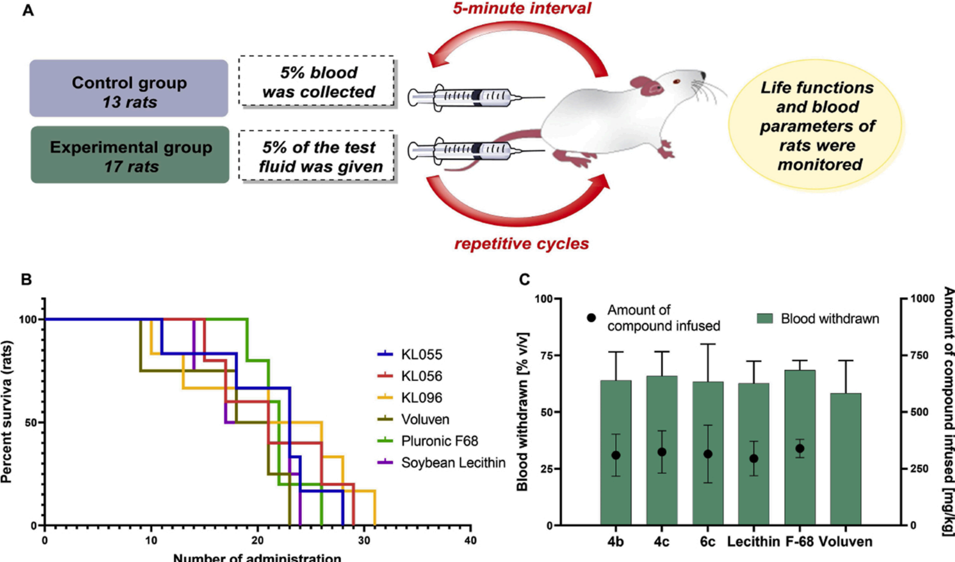
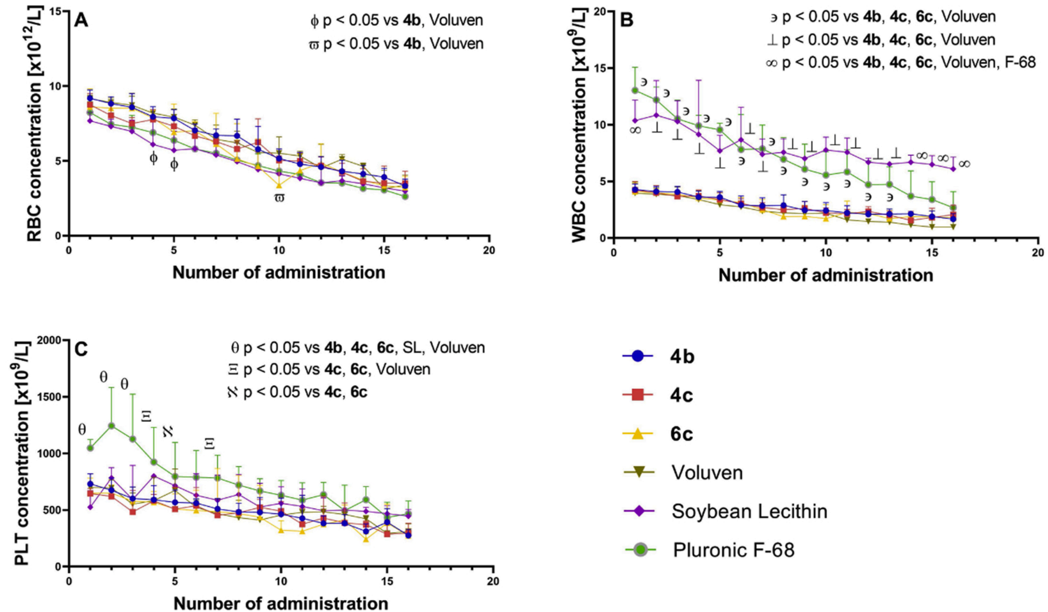


Fig. 8. A) Research methodology on a small animal model; **B)** Kaplan-Meyer survival chart for Sprague Dawley rats after administration of test fluids; Gasometry data depending on the number of administrations: **C)** Average computed amount of surfactant infused [mg] per kilogram of rat and mean computed blood withdrawn [% *v/v*].



Blood morphology		Reference values	Initial values	Final value
	RBC (x 10^{12}/L)	6.39 – 8.01	7.7 ± 0.6 - 9.2 ± 0.5	2.6 ± 0.7 – 3.5 ± 0.8
	WBC (x 10^{9}/L)	3.00 – 9.22	4.0 ± 1.0 - 13.0 ± 2.0	1.0 ± 0.6 – 6.1 ± 1.0
	PLT (x 10^{9}/L)	735 – 1418	526 ± 145 - 1048 ± 76	274 ± 63 – 466 ± 115
	HGB [g/L]	139 – 173	145 ± 23 – 158 ± 14	46 ± 11 – 55 ± 12
	HCT [%]	38.9 – 55.2	44 ± 2.7 – 49 ± 3.8	14 ± 3.8 – 18 ± 4.2

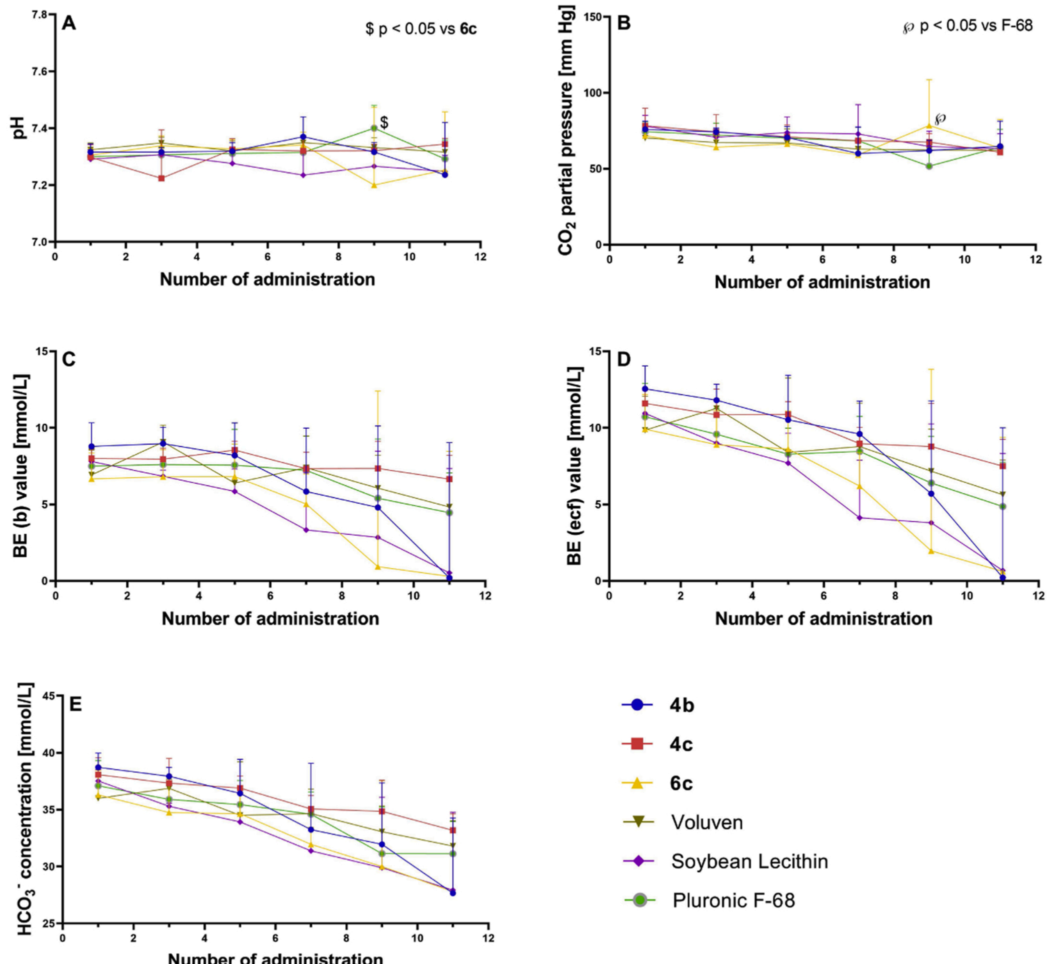
Fig. 9. Blood morphology data depending on the number of administrations and comparison with literature values: **A)** RBC concentration [x 10^{12}/L] **B)** WBC concentration [x 10^{9}/L] **C)** PLT concentration [x 10^{9}/L]; φ, ∞ and ⊥ marks represent Soybean Lecithin; ϖ marks represent **6c**; ∍, θ, Ξ and ℵ marks represent Pluronic F-68.

there were no significant changes between groups (P > 0.05). Data of blood gas levels are shown in Fig. 10. Interestingly, for the base excess: BE (b) and BE (ecf) at the end point, two groups can be distinguished: **4c**, Voluven and Pluronic F-68 (with values closer to initial ones) and **4b**, **6c**, Soybean Lecithin (with values significantly lower than initial ones). It is worth mentioning that values of $cHCO_3$, BE (b) and BE (ecf) parameters for surfactant **4c**, showed the smallest decrease and remain close to initial values despite continues blood replacement.

3.6.3. Blood biochemistry

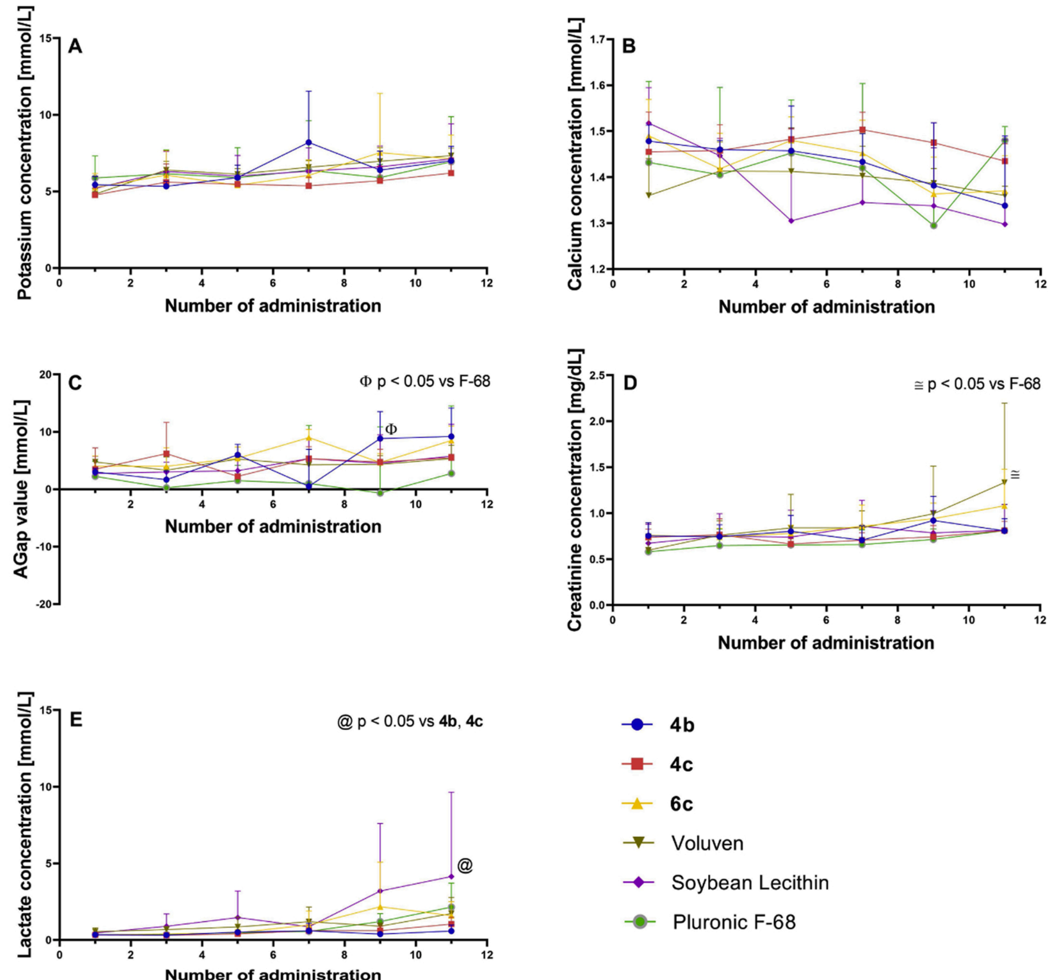
During the biochemical examination of the blood, the composition of the plasma is analyzed, determining the level of enzymes, hormones, proteins, electrolytes and trace elements in the body (Fig. 11).

The concentrations of individual electrolytes slightly changed, but they were within the literature norms [39,40]. For all the groups, potassium and chloride concentration, parameters had slightly increasing trend, while in the case of calcium and sodium some groups have shown a decreasing trend (for calcium: **4b**, **4c**, **6c** and Soybean Lecithin, for



		Reference values	Initial values	Final value
Gasometry	pH	7.25 - 7.38	7.29 ± 0.03 - 7.32 ± 0.02	7.23 ± 0.18 - 7.34 ± 0.02
	pCO_2 (mm Hg)	26 - 54	70 ± 6.5 – 78 ± 11.5	61 ± 1.5 – 65 ± 16.4
	$cTCO_2$ (mmol/L)	13 – 27.1	38 ± 2.4 – 41 ± 1.3	30 ± 6.2 – 35 ± 1.5
	pO_2 (mm Hg)	12 - 58	32 ± 1.6 - 37 ± 6.6	17 ± 5.0 - 26 ± 4.6
	HCO_3^- (mmol/L)	12.2 – 25.4	36 ± 2.2 – 39 ± 1.3	28 ± 6.6 - 33 ± 1.5
	BE (ecf) (mmol/L)	3.8 – 7.6	9.9 ± 2.2 - 12.6 ± 1.5	0.2 ± 9.8 - 7.5 ± 1.7
	BE (b) (mmol/L)	2.8 – 10.3	6.7 ± 1.7 - 8.8 ± 1.5	0.2 ± 8.8 - 6.7 ± 1.6

Fig. 10. Gasometry data depending on the number of administrations and comparison with literature values **A)** pH; **B)** CO_2 partial pressure [mm Hg]; **C)** Base excess [mmol/L]; **D)** Base excess in the extracellular fluid [mmol/L]; **E)** HCO_3^- concentration [mmol/L]; $ and ∝ marks represent Pluronic F-68; ℘ and ⊗ marks represent 6c; •, ∅ and @ represent Soybean lecithin; Φ mark represents **4b**; ≅ mark represents Voluven.



		Reference values	Initial values	Final value
Blood biochemistry	Glu (mg/dL)	111 - 359	344 ± 99 - 542 ± 71	307 ± 146 - 590 ± 205
	Crea (mg/dL)	0.1 - 5.6	0.6 ± 0.2 - 0.8 ± 0.1	0.8 ± 0.1 - 1.3 ± 0.9
	Lac (mmol/L)	5.0 – 8.5	0.3 ± 0.03 - 0.5 ± 0.2	0.6 ± 0.4 - 4.1 ± 5.5
	K^+ (mmol/L)	3.96 – 7.87	4.8 ± 0.5 – 5.9 ± 1.4	6.2 ± 0.9 – 7.3 ± 0.5
	Cl^- (mmol/L)	97 – 116	96.5 ± 2.4 – 102.7 ± 3.3	101 ± 4.8 – 109 ± 6.8
	AGap (mmol/L)	No data	2.3 ± 2.6 – 4.8 ± 0.5	2.8 ± 11.8 – 9.2 ± 4.9
	AGapK (mmol/L)	No data	7.8 ± 2.5 – 9.8 ± 1.0	11.5 ± 1.3 – 16.4 ± 5.6
	Ca^{2+} (mmol/L)	0.27 - 1.26	1.4 ± 0.1 – 1.5 ± 0.1	1.30 ± 0.18 – 1.48 ± 0.03
	Na^+ (mmol/L)	129 – 155	136.8 ± 3.8 – 143.0 ± 1.4	134.4 ± 12 - 144.8 ± 3

Fig. 11. Blood biochemistry data depending on the number of administrations and comparison with literature values **A)** Potassium concentration [mmol/L]; **B)** Calcium concentration [mmol/L]; **C)** AGap value [mmol/L]; **D)** Creatinine concentration [mg/dL]; **E)** Lactate concentration [mmol/L]; @ represent Soybean Lecithin; Φ mark represents **4b**; ≅ mark represents Voluven.

sodium: Voluven. Soybean Lecithin and Pluronic F-68; for details see Supporting Information, Chapter 5). Moreover calcium level was stable for Voluven group. The smallest difference between the initial and final value for: sodium, chloride and calcium was observed for Voluven, for potassium and the AGap parameter for Pluronic F-68. However, in the case of the AGapK parameter, the smallest difference between the initial and final value was noted for **4c**.

Anion Gap (AGap) is defined as the difference between plasma/

serum sodium concentration and the sum of plasma/serum chloride and bicarbonate concentrations, while the definition of Anion Gap K (AGapK) additionally includes of plasma/serum potassium concentration [42].

Low initial values of serum Anion Gapes can be caused by frequently occurring measurement errors (for example: underestimation of sodium and potassium concentration, overestimation of chloride and bicarbonate concentration), while hypoalbuminemia (decrease of albumin concentration caused by blood loss) can induced low final values (from 7th blood removals) [43]. AGap values increased slightly, but did not exceed the reference intervals, so metabolic acidosis cannot be clearly determined on this basis, only high anion gap acidosis can be excluded.

Creatinine blood level [44] were stable during the experiment in **4b**, **4c**, Soybean Lecithin and Pluronic F-68 groups, this indicates the good efficiency of the glomeruli function, responsible for extracting harmful and unnecessary substances from the blood in order to remove them from the body along with urine. Creatinine blood level have tendency to increase in Voluven and **6c** groups (to 1.3 ± 0.9 and 1.1 ± 0.4, respectively), without statistically significant differences. If the creatinine concentration is elevated and exceeds the established norms, poor kidney function should be suspected.

In the Soybean Lecithin and Pluronic F-68 groups, after 7 administration the level of lactate concentration [41] increased greatly, means anaerobic respiration. It is a sensitive parameter indicating ischemia of peripheral tissues. Statistically significant differences were observed in the Soybean Lecithin group after administration of 9 and 11 (SL vs **4b** and SL vs **4b**, **4c**).

3.6.4. Histopathological examination

Macroscopic analysis after the end of the experiment did not reveal any pathological changes in the examined organs (liver, kidneys, lungs and heart tissue samples). Neither congenital changes nor changes that could be caused by the administration of the analyzed molecules were observed. In the overall picture, macroscopic pallor of the organs was observed in proportion to the duration of the experiment. The structure of the parenchyma of internal organs preserved without visible signs of inflammation, marked reduction or absence of red blood cells (Supporting Information; Chapter S7, Supporting Information).

4. Conclusions

In summary, a high-yielding synthesis of 59 new ionic fluorosurfactants consisting of perfluorohexyl- or perfluorooctyl-groups were reported. Most of obtained compounds have the appropriate physicochemical properties and biological criteria for medical applications. *In vitro* tests including hemolysis, proliferation of human HMEC-1 endothelial cells and mouse L929 fibroblasts have confirmed the biocompatibility of the surfactants. It is worth noticing that emulsions containing surfactants **4b**, **4c**, **6c** showed greater stability compared to emulsions with commercial surfactants: Soybean Lecithin, Pluronic F-68 and Pluronic F-127 after 48 h. Moreover, also the average droplet size was significantly lower in favor of formulations with synthesized surfactants. The stability of nanoemulsions prepared with addition of developed fluorosurfactants showed similar stability to formulations prepared with addition of strong ionic surfactants: CTAB and SDS after 48 h but definitely better stability (for **4c, 6c** surfactants) after 6 months and significantly lower toxicity. Additionally, the obtained nanoemulsions required lower concentration of surfactant (<2 % *w/v*) to remain stable than reported in the literature (5–10 % *w/v* for obtaining stable nanoemulsions [45]). Preclinical toxicity results on a rat model in gasometry and plasma analysis, indicate that developed surfactants are as good as registered substances without significant deviations. Despite the first overview and animal study limitation, results suggest that mentioned surfactants **4c**, **4b**, **6c** seem to be suitable solution for infusion fluids used in a therapy after hemorrhagic shock and trauma. The best liquid variant developed by our group as a leading product will be further tested on a large animal model. Finally, we proved that obtained surfactants display strong surface activity at the same time meet the requirements in hemocompatibility and biocompatibility.

CRediT authorship contribution statement

Agata Stefanek: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing. **Katarzyna Łęczycka-Wilk:** Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Sylwia Czarnocka-Śniadała:** Investigation, Methodology, Validation. **Wojciech Frąckowiak:** Formal analysis, Visualization. **Joanna Graffstein:** Investigation, Validation. **Agata Ryżko:** Investigation, Validation. **Aleksandra Nowak:** Investigation, Validation. **Tomasz Ciach:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors have declared that no competing interest exists.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2021.111603.

References

[1] L. Wu, X. Wen, X. Wang, et al., Local intratracheal delivery of perfluorocarbon nanoparticles to lung cancer demonstrated with magnetic resonance multimodal imaging, Theranostics 8 (2018) 563–574.

[2] M. Jaiswal, R. Dudhe, P.K. Sharma, Nanoemulsion: an advanced mode of drug delivery system, 3 Biotech 5 (2015) 123–127.

[3] A. Gupta, H.B. Eral, T.A. Hatton, P.S. Doyle, Nanoemulsions: formation, properties and applications, Soft Matter 12 (2016) 2826–2841.

[4] S.A. Hosgood, E. van Heurn, M.L. Nicholson, Normothermic machine perfusion of the kidney: better conditioning and repair? Transpl. Int. 28 (2015) 657–664.

[5] R.S. Schuh, F. Bruxel, H.F. Teixeira, Physicochemical properties of lecithin-based nanoemulsions obtained by spontaneous emulsification or high-pressure homogenization, Química Nova 37 (2014) 1193–1198.

[6] T. Delmas, H. Piraux, A. Couffin, I. Texier, F. Vinet, P. Poulin, M.E. Cates, J. Bibette, How to prepare and stabilize very small nanoemulsions, Langmuir 27 (2011) 1683–1692.

[7] E.P. Wesseler, R. Iltis, L.C. Clark, The solubility of oxygen in highly fluorinated liquids, J. Fluor. Chem. 9 (1977) 137–146.

[8] J.G. Riess, M.P. Krafft, Fluorinated materials for in vivo oxygen transport (blood substitutes), diagnosis and drug delivery, Biomaterials. 19 (1998) 1529–1539.

[9] K.C. Lowe, Perfluorochemical respiratory gas carriers: benefits to cell culture systems, J. Fluorine Chem. 118 (2002) 19–26.

[10] B.D. Spiess, Perfluorocarbon emulsions as a promising technology: a review of tissue and vascular gas dynamics, J. Appl. Physiol. 106 (2009) 1444–1452.

[11] Y. Que, Y. Liu, W. Tan, C. Feng, P. Shi, Y. Li, H. Xiaoyu, ACS Macro Lett. 5 (2016) 168–173.

[12] T. Zhang, Q. Zhang, J.-H. Tian, J.-F. Xing, W. Guo, X.-J. Liang, Perfluorocarbon-based nanomedicine: emerging strategy for diagnosis and treatment of diseases, MRS Commun. 8 (2018) 303–313.

[13] N. Rapoport, K. Nam, R. Gupta, Z. Gao, P. Mohan, A. Payne, N. Todd, X. Liu, T. Kim, J. Shea, C. Scaife, D.L. Parker, E. Jeong, A.M. Kennedy, Ultrasound-mediated tumor imaging and nanotherapy using drug loaded, block copolymer stabilized perfluorocarbon nanoemulsions, J. Control. Release 153 (2011) 4–15.

[14] M.P. Krafft, J.G. Riess, Chemistry, physical chemistry, and uses of molecular fluorocarbon– hydrocarbon diblocks, triblocks, and related compounds unique "apolar" components for self-assembled colloid and interface engineering, Chem. Rev. 109 (2009) 1714–1792.

[15] Y. De Smet, L. Deriemaekeri, R. Finsy, Ostwald ripening of alkane emulsions in the presence of surfactant micelles, Langmuir 15 (1999) 6745–6754.

[16] B. Arechabala, C. Coiffard, P. Rivalland, L. Coiffard, Y. J.; de Roeck-Holtzhauer, Comparison of cytotoxicity of various surfactants tested on normal human fibroblast cultures using the neutral red test, MTT assay and LDH release, J. Appl. Toxicol. 19 (1999) 163–165.

[17] A.M. Mahé, J. Manoux, A. Valla, R. Follana, L. Zarif, J. Greiner, P. Vierling, J. G. Riess, Perfluoroalkylated surfactants: relationships between structure and acute toxicity in mice, Biomater. Artif. Cells Immobilization Biotechnol. 20 (1992) 1025–1027.
[18] A. Diouf, E. Taffin De Givenchy, S.Y. Dieng, A. Dramé, S. Amigoni, T. Darmanin, F. Guittard, Surface properties of new catanionic semi-fluorinated hybrid surfactants, J. Fluorine Chemistry 161 (2014) 60–65.
[19] M. Kamei, Y. Matsumoto, Jph01193336a, 1987.
[20] S. Benita, M.Y. Levy, Submicron emulsions as colloidal drug carriers for intravenous administration: comprehensive physicochemical characterization, J. Pharm. Sci. 82 (1993) 1069–1079.
[21] M. Wulff-Pérez, A. Torcello-Gómez, M.J. Gálvez-Ruíz, A. Martín-Rodríguez, Stability of emulsions for parenteral feeding: preparation and characterization of o/w nanoemulsions with natural oils and Pluronic F-68 as surfactant, Food Hydrocoll. 23 (2009) 1096–1102.
[22] F.E. Antunes, L. Gentile, C.O. Rossi, L. Tavano, G.A. Ranieri, Gels of Pluronic F-127 and nonionic surfactants from rheological characterization to controlled drug permeation, Colloids Surf. B Biointerfaces 87 (2011) 42–48.
[23] Fuji Photo Film Co., Ltd.; Kato, Syunya; Yoshikawa, Masaru, KR101634475 B1, 2016.
[24] M. Włostowski, S. Czarnocka, P. Maciejewski, Efficient S-alkylation of cysteine in the presence of 1, 1, 3, 3-tetramethylguanidine, Tetrahedron Lett. 51 (2010) 5977–5979.
[25] N.W. Roehm, G.H. Rodgers, S.M. Hatfield, A.L. Glasebrook, An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT, J. Immunol. Methods 142 (1991) 257–265.
[26] H.B. Lee, M.D. Blaufox, Blood volume in the rat, J. Nucl. Med. 26 (1985) 72–76.
[27] R.A. Hoppen, C.O. Corso, T.J. Grezzana, A. Severino, F. Dal-Pizzol, C. Ritter, Hypertonic saline and hemorrhagic shock: hepatocellular function and integrity after six hours of treatment, Acta Cir. Bras. 20 (2005) 414–417.
[28] M.P. Krafft, Fluorocarbons and fluorinated amphiphiles in drug delivery and biomedical research, Adv. Drug Deliv. Rev. 47 (2001) 209–228.
[29] R.L. Grant, C. Yao, D. Gabaldon, D. Acosta, Evaluation of surfactant cytotoxicity potential by primary cultures of ocular tissues: I. Characterization of rabbit corneal epithelial cells and initial injury and delayed toxicity studies, Toxicology 76 (1992) 153–176.
[30] S. Hoeller, A. Sperger, C. Valenta, Lecithin based nanoemulsions: a comparative study of the influence of non-ionic surfactants and the cationic phytosphingosine on physicochemical behaviour and skin permeation, Int. J. Pharm. 370 (2009) 181–186.
[31] V. Klang, J.C. Schwarz, C. Valenta, Nanoemulsions in dermal drug delivery, Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement 18 (2015) 255–266.
[32] F. Karamustafa, N. Celebi, Development of an oral microemulsion formulation of alendronate: effects of oil and co-surfactant type on phase behavior, J. Microencapsul. 25 (2008) 315–323.
[33] M. Ishiyama, H. Tominaga, M. Shiga, K. Sasamoto, Y. Ohkura, K. Ueno, A combined assay of cell vability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet, Biol. Pharm. Bull. 19 (1996) 1518–1520.
[34] N. Vlachy, D. Touraud, J. Heilmann, W. Kunz, Determining the cytotoxicity of catanionic surfactant mixtures on HeLa cells, Colloids Surf. B Biointerfaces 70 (2009) 278–280.
[35] E.E. Connor, J. Mwamuka, A. Gole, C.J. Murphy, M.D. Wyatt, Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity, Small. 1 (2005) 325–327.
[36] T. Niidome, M. Yamagata, Y. Okamoto, Y. Akiyama, H. Takahashi, T. Kawano, Y. Katayama, Y. Niidome, PEG-modified gold nanorods with a stealth character for in vivo applications, J. Control. Release 114 (2006) 343–347.
[37] C. Aiello, P. Andreozzi, C. Mesa, G. Risuleo, Biological activity of SDS-CTAB cat-anionic vesicles in cultured cells and assessment of their cytotoxicity ending in apoptosis, Colloids Surf. B Biointerfaces 78 (2010) 149–154.
[38] Q. He, G. Su, K. Liu, F. Zhang, Y. Jiang, J. Gao, L. Liu, Z. Jiang, M. Jin, H. Xie, Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the nonparametric rank percentile method, PLoS One (2017) 12.
[39] Z.Z. Han, H.D. Xu, K.H. Kim, T.H. Ahn, J.S. Bae, J.Y. Lee, K.H. Gil, J.Y. Lee, S. J. Woo, H.J. Yoo, H.K. Lee, K.H. Kim, C.K. Park, H.S. Zhang, S.W. Song, Reference data of the main physiological parameters in control sprague-dawley rats from pre-clinical toxicity studies, Lab. Anim. Res. 26 (2010) 153–164.
[40] R. Uribe-Escamilla, P. Sánchez Aparicio, A. Córdova Izquierdo, A. Alfaro-Rodríguez, Reference values for electrolytes and blood gases in wistar rats with permanent cerebral ischemia: the effect of treatment with Glycine on gasometry and electrolytes, Multiciencias 11 (2011) 378–386.
[41] M.D. Baldissera, R.A. Vaucher, C.B. Oliveira, V.C. Rech, M.R. Sagrillo, D.R. Stainki, L.M. Stefani, Blood gas analyses and other components involved in the acid–base metabolism of rats infected by Trypanosoma evansi, J. Adv. Res. 6 (2015) 1079–1082.
[42] C.S. Deutschman, P.J. Neligan, Evidence-based Practice of Critical, second edition, Elsevier Health Sciences, 2016.
[43] J.A. Kraut, N.E. Madias, Serum anion gap: its uses and limitations in clinical medicine, Clin. J. Am. Soc. Nephrol. 2 (2007) 162–174.
[44] T. Matsuzawa, Y. Hayashi, M. Nomura, T. Unno, T. Igarashi, T. Furuya, K. Sekita, A. Ono, Y. Kurokawa, Y. Hayashi, A survey of the values of clinical chemistry parameters obtained for a common rat blood sample in ninety-eight Japanese laboratories, J. Toxicol. Sci. 22 (1997) 25–44.
[45] T. Tadros, P. Izquierdo, J. Esquena, C. Solans, Formation and stability of nano-emulsions, Adv. Colloid Interface Sci. 108–109 (2004) 303–318.