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Encapsulation of clozapine in polymeric nanocapsules and its biological effects

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ABSTRACT

Clozapine is an effective atypical antipsychotic drug that unfortunately exhibits poor oral bioavailability. Moreover, the clinical use of the compound is limited because of its numerous unfavorable and unsafe side effects. Therefore, the aim of the present study was the development of a new nanocarrier for a more effective clozapine delivery. Here, clozapine was encapsulated into polymeric nanocapsules (NCs). Polyelectrolyte multilayer shells were constructed by the technique of sequential adsorption of polyelectrolytes (LbL) using biocompatible polyanion PGA (Poly-L-glutamic acid, sodium salt) and polycation PLL (poly-L-lysine) on clozapine-loaded nanoemulsion cores. Pegylated external layers were prepared using PGA-g-PEG (PGA grafted by PEG (polyethylene glycol)). Clozapine was successfully loaded into the PLL-PGA nanocarriers (CLO-NCs) with an average size of 100 nm. In vitro analysis of the interactions of the CLO-NCs with the cells of the mononuclear phagocytic system (MPS) was conducted. Cell biocompatibility, phagocytosis potential, and cellular uptake were studied. Additionally, the biodistribution and behavioral effects of the encapsulated clozapine were also studied. The results indicate that surface modified (by PEG grafting) polymeric PLL-PGA CLO-NCs are very promising nanovehicles for improving clozapine delivery.

1. Introduction

Encapsulation of active compounds is a promising strategy in pharmacology. It has been reported that nanoparticulate systems (NP) used as drugs carriers improved therapeutic efficiency by enhancement of tissue specific delivery [1]. Increased drug permeability and absorption have been shown for various drug molecules [2,3]. Maintaining a drug concentration within acceptable therapeutic levels by controlled release to desired sites over a prolonged period of time leads to reduced dosing frequency, as well as minimizing side effects [3–6]. Many active compounds exhibit poor systemic bioavailability after oral administration, which is connected with enzymatic and non-enzymatic degradation in the gastrointestinal tract and first-pass metabolism in the liver [1,7]. Additionally, intravenously administered drugs undergo rapid clearance, which decreases their circulation half-life. Drug encapsulation enhances protection of active molecules from the destructive influence of an external environment, and thus increases drug exposure [1,8,9].

This strategy might be particularly useful for antipsychotic drugs. Although these drugs have a well-established role in the therapy of schizophrenia, the numerous side effects associated with their long-term administration diminish their clinical efficacy. Clozapine, a lipophilic atypical antipsychotic agent, has been found to be effective in the treatment of both the positive and the negative symptoms of schizophrenia, and has few extrapyramidal side effects [10,11]. However, the clinical use of the compound is limited because of the induction of unfavorable actions

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leading to arrhythmias, weight gain, metabolic dysfunction linked to diabetes, and, above all, agranulocytosis [11]. Although clozapine is well absorbed after oral administration, it undergoes extensive first-pass hepatic metabolism, resulting in poor oral bioavailability [1,12]. In addition, its low plasma half-life points toward extensive metabolic clearance [1]. In view of the above aspects, the encapsulation of clozapine, allowing its controlled release, appears to be very important and promising because controlled release may improve its therapeutic potential.

Several different strategies have been described to incorporate antipsychotic drugs into nanoparticles (NPs). Studies have indicated that olanzapine loaded micelles [13], risperidon [14,15] or haloperidol loaded PLGA NPs [16], clozapine loaded chitosan microparticles [17], and clozapine [12] or risperidone loaded solid lipid NPs [18,19] exhibit better properties than simple drug suspension. Currently, biodegradable polymeric NCs have attracted much attention as delivery vehicles due to their safety and sustained-release capabilities [20,21]. Recently, it has been shown that polymeric NCs formed by the layer by layer (LbL) method using polycation PLL (poly-L-lysine) and polyanion PGA (poly-L-glutamic acid, sodium salt) are promising nanocarrier candidates in pharmacology due to their relatively low toxicity and biocompatibility [22–25]. Additionally, polyelectrolyte multilayer NCs are flexible and multifunctional. Combination of various polyelectrolytes and possibility of modification of the polyelectrolyte shell by organic molecules, polymers, inorganic NPs, carbon nanotubes, antibodies, lipids [26–29]. Zhu and McShane [27] enables functionalization of the capsules' surface and allows optimization of their action. The encapsulation of a liquid core eliminates the primary drawbacks of the sacrificial core such as incomplete dissolution of the sacrificial core and low efficiency in the loading of active substances [30]. Therefore, in the present work, we focused on designing novel polymeric PLL-PGA clozapine-containing NCs, which, in our opinion, may lead to an improved therapeutic activity and may make the profile of clozapine more secure.

2. Materials and methods

2.1. Materials

2.1.1. CLO-NCs preparation

Clozapine was obtained from Tocris. The polycations poly-L-lysine hydrobromide (PLL, MW ~15000–30000) and poly(fluorescein isothiocyanate allylamine hydrochloride) (FITC-PAH, MW ~70000); the polyanions poly-L-glutamic acid sodium salt (PGA, MW ~15000–50000) and methoxypolyethylene glycol amine (Me-PEG-NH2, MW ~5000); *N*-hydroxysulfo succinimide sodium salt (NHS, ≥98.5%), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC, ≥97%), docusate sodium salt (AOT), chloroform, and sodium chloride were obtained from Sigma–Aldrich. All of the materials were used as received without further purification. PGA-g-PEG, with various grafting ratios, was synthesized according to the procedure described by Szczepanowicz et al. [31]. The approximate PEG grafting ratios were 14%, 39% and 61%. These copolymers are further on referred to as PGA-g(14)-PEG, PGA-g(39)-PEG, and PGA-g(61)-PEG, respectively. The distilled water used in all of the experiments was obtained with the three-stage Millipore Direct-Q 5UV purification system.

2.1.2. Materials for cell culture assays

The mouse murine macrophages cell line RAW 264.7 was obtained from Sigma–Aldrich. All of the cell culture materials, including DMEM/F12 media, heat-inactivated fetal bovine serum (FBS), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), endocytosis inhibitors (chlorpromazine, amiloride, filipin III), and sulforhodamine B, were purchased from Sigma–Aldrich. The lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was obtained from Clontech Laboratories. The Vybrant Phagocytosis Kit was obtained from Molecular Probes (Life Technologies). Collagenase type II and dispase were obtained from Gibco.

2.2. Methods

2.2.1. NCs formation

NCs containing clozapine were prepared according to the method described previously [31–33]. Emulsion droplets containing clozapine were formed by the addition of AOT, which is a negatively charged, oil-soluble, FDA approved surfactant, and clozapine dissolved in chloroform to an aqueous solution of polycation PLL, which was slowly mix with a magnetic stirrer. The optimal ratio of surfactant (AOT) to polycation was determined by measuring the zeta potential of the emulsion drops and examining their stability. After formation of stable emulsion droplets, sequential adsorption of polyelectrolytes was performed using the LbL technique [34] with the saturation method [35–38]. The shell formation was followed by the zeta potential measurements [22,23,36]. To create a pegylated shell, positively charged nanocapsules were coated with a layer of PGA-g-PEG. The pegylated PGA was synthesized according to the method described by Boulmedais et al. [39] and Szczepanowicz et al. [31]. Briefly, 0.1 g of PGA, 0.46 g (1.3 and 2.1 g) of Me-PEG-NH_2 (MW 5000) and 0.014 g of NHS were dissolved in 10 mL of water. Then, 0.16 g of EDC was added to the mixture while stirring. The reaction was allowed to proceed for 6 h at room temperature. After filtration, the reaction mixture was dialyzed 3 times (cut off at MW 12400) for 24 h with deionized water (2 L). Using 1H NMR, the area that represents the glutamic acid side chain, one monomer of glutamic acid (4.16 ppm I~1) in this case, was compared with the PEG area (3.55 ppm I~64, 177 and 278) to determine the graft ratio of the copolymer. It was assumed that one molecule of PEG (MW 5000) possesses 456H hydrogen nuclei. The grafting percentage was equal to a ratio of 177/456. The achieved coupling rate was approximately 14%, 39% and 61%, and these polymers are referred to as PGA-g(14)-PEG, PGA-g(39)-PEG, and PGA-g(61)-PEG, respectively. For the preparation of fluorescently labeled NCs, FITC-PAH was used instead of one PLL layer.

Because pharmaceutical products require elimination of toxic solvents from the final product, chloroform was removed from the suspension by rotary evaporator. The final concentration of chloroform determined by GC–ECD analysis was 0.0397 mg/dm^3 [22]. This meets the requirements of the U.S. Department of Health and Human Services Food and Drug Administration, the Center for Drug Evaluation and Research (CDER), and the Center for Biologics Evaluation and Research (CBER), as published in guidance for industry [40].

2.2.2. Particle size, visualization, concentration and zeta potential measurements

The size distribution (hydrodynamic diameter) and the zeta potential (electrophoretic mobility) of the capsules were determined by the dynamic light scattering (DLS) and microelectrophoretic methods, respectively, both with the Zetasizer Nano Series from Malvern Instruments. The obtained size values were an average of at least three runs with 10 measurements. For the zeta potential measurements, an average was calculated from the three subsequent runs with 20 measurements. Additionally, the size and concentration of the nanocapsules was measured by the Nanoparticle Tracking Analysis (NTA) technique with a NS500 instrument (NanoSight). All of the measurements were performed at 25 °C in 0.015 M NaCl. For cryo-SEM imaging, the capsule suspension was placed on the sample holder, which was immediately

immersed (frozen) in liquid nitrogen using the Quorum PPT2000 cryo-preparation stage (Polaron). The holder with the frozen sample was cryo-transferred to the chamber of the cryo-unit where the sample was subjected to sublimation at −70 °C for 15–30 min. (until all visible ice crystals disappeared). The sample was then sputter coated with platinum (5 nm thickness). Following coating, the specimen was transferred to the cooled stage of the Jeol JSM 7600F field emission scanning electron microscope FESEM (Jeol Ltd.).

2.2.3. UV–vis spectrophotometry

UV–vis spectroscopy was used to confirm the encapsulation and measure the kinetics of releasing clozapine from the nanocapsules. The UV absorbance measurements were performed using a UV-1800 spectrophotometer (Shimadzu).

2.2.4. Stability studies

To evaluate the colloidal and biological stability of the CLO-NCs, the size distribution (hydrodynamic diameter) and the zeta potential of the CLO-NCs were monitored in time. Freshly prepared suspensions of the CLO-NCs were stored in 0.015 M NaCl cell culture medium and FBS solutions. Aggregation was also monitored during cell culturing.

2.2.5. In vitro drug release study

The release profile of clozapine from NC was determined by the direct dialysis method [38]. 5 mL of drug-loaded capsules, with one layer for the core and 2, 5, and 6 layers of polyelectrolyte, were placed in a dialysis cellulose tubing (Sigma–Aldrich, 12 400 Da). This was then immersed in 200 mL of phosphate buffer (PBS), containing 2% (w/v) Tween 20 for improving the solubility and the stability of the released drug, and was stirred at 100 rpm. The analysis of the drug content in the medium was performed using UV–vis spectroscopy at various time intervals. 2 mL of the medium was collected and was replaced with the same volume of fresh PBS containing Tween 20. All of the experiments were performed with magnetic stirring at room temperature. In the release kinetics for all the studied NCs, the absorbance of the released drug was plotted against time and was normalized in the range of 0–1. The obtained data were expressed in mg/mL.

2.2.6. Cell culture

RAW 264.7 cells were grown in DMEM medium supplemented with 1% L-glutamine, high glucose and 10% heat-inactivated fetal bovine serum (FBS). Cells were cultured at 37 °C inside a humidified incubator in an atmosphere of 5% CO_2.

2.2.7. Cell viability and cytotoxicity assays

2.2.7.1. MTT reduction test. An evaluation of cell viability was performed using the MTT reduction test. Two days before the experiments, RAW 264.7 were seeded into appropriate 96-well plates at a density of 3×10^4 cells per well. Various types of CLO-NPs (with various numbers of polyelectrolyte layers and surface modifications) resuspended in 0.015 M NaCl were added in various doses to the fresh medium in each well. Doses of 100 μL, 50 μL, and 20 μL per well were used, which was equivalent to approximately 0.85×10^6, 0.4×10^6, and 0.15×10^6 NCs per cell, respectively. After 24 h, the cell culture media with CLO-NCs was removed, and cells were incubated with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and were resuspended in the serum-free media for 4 h at 37 °C, in a 5% CO_2 atmosphere. After incubation, the medium was removed, and 100 μL of DMSO was added to each well. Yellow tetrazolium salt is converted by viable cells to purple formazan, which was dissolved in dimethyl sulfoxide. The absorbance of the product was detected at 570 nm using a microplate reader (TECAN Infinitive200Pro). Untreated cells served as a control. Six replicates for each type of CLO-NCs were measured. The results represent the average cell viability from different experiments repeated 3 times.

2.2.7.2. LDH cytotoxicity detection kit. Two days before the experiments were carried out, the cells were seeded into 96-well plates at a density of 1×10^4 cells per well. A cytotoxicity assay was performed in RAW 264.7 cells after 4 h incubation with various types and doses of CLO-NCs (analogous to the MTT assay). The LDH (lactate dehydrogenase) release into the cell culture medium that correlates with the damage of the cell membrane was determined using a Cytotoxicity Detection Kit (LDH) according to the manufacturer's protocol. The plates were centrifuged at $250 \times g$ for 10 min. Then, 100 μL of the supernatant was taken and incubated for 30 min in the dark at room temperature with 100 μL of the reaction mixture. LDH level was determined by a colorimetric assay. The absorbance was measured at 490 nm (with a reference wavelength at 610 nm) (TECAN Infinitive200). The following two controls were performed. Low control measures the activity of spontaneous LDH release from the untreated cells as well as the cells incubated with 0.015 M NaCl. High control indicates the maximum LDH level released in response to Triton X-100, which causes cell death. Six replicates for each types of CLO-NCs were measured. The results represented the cytotoxic effect from different experiments repeated 3 times.

2.2.8. Vybrant phagocytosis assay

A phagocytosis assay was performed using Vybrant Phagocytosis Assay Kit according to the manufacturer's instruction. Briefly, cells cultured in 96-well microplates (1×10^5 RAW 264.7 cells per well) were incubated for 2 h in standard culture conditions in DMEM medium containing 0% and 10% FBS in the presence of various types of CLO-NCs resuspended in 0.015 M NaCl in a dose of 15 μL per well (ca. 0.15×10^6 NCs/cell). After incubation, the medium containing CLO-NCs was removed, and the cells were incubated for 2 h with fluorescein-labeled *Escherichia coli* particles resuspended in HBSS buffer at 37 °C. The negative control was free medium, and the positive control consisted of non-NCs treated cells. The fluorescence resulting from non-internalized *E. coli* was quenched by adding Trypan Blue. The results were acquired using fluorescence plate reader (TECAN Infinitive200Pro; 488 nm excitation, and 520 nm emission wavelengths). Five replicates for each experimental condition were performed. The results represented the average from different experiments repeated 3 times.

2.2.9. Confocal microscopy imaging

Confocal microscopy was used to analyze internalization of the fluorescently labeled CLO-NCs in RAW 264.7 cells, which were seeded one day before the experiment on 40 mm plates with 15 mm diameter glass coverslips at a density of 1×10^5 cells per well. Images were acquired using a Leica LSC SP5 laser scanning confocal microscope (Leica) and a 63× HCX PL APO NA 1.4 oil immersion lens (Leica). Data were acquired in the sequential mode. FITC excitation was performed at 488 nm (Ar), and emission was measured at 500–550 nm; sulforhodamine B excitation was performed at 543 nm (HeNe), and emission was measured at 580–650 nm. Before the experiments, the medium was replaced with 1 mL of fresh F12 medium containing different types of CLO-NCs at a volume of 60 μL. Prior to microscopic examination, aqueous sulforhodamine B solution was added to the cell culture at a final concentration of 1.73 μM. Sulforhodamine B is not internalized by the cells, thus enabling visualization of the cell exterior [41]. Images of the living cells acquired at 37 °C were registered.

2.2.10. Flow cytometry experiments

The quantitative cellular uptake of different types of CLO-NCs in RAW 264.7 cells was measured by flow cytometry. Two days before the experiments were carried out, the cells were seeded into appropriate 6-well plates at a density of 3×10^5 cells per well. Before experiment, the cultured medium was replaced with fresh DMEM medium with 10% FBS containing fluorescently labeled (FITC-labelled) various types of CLO-NCs, at a volume of 30 μL per well. After 2 h incubation in standard culture conditions, the cells were washed six times with cold PBS (phosphate buffered saline, pH 7.4) and eventually resuspended in 1 mL of cold PBS for analysis. To determine the degree of surface-bound NCs that may interfere with the analysis of cellular uptake, corresponding experiments were performed under cold conditions (4 °C). To estimate the influence of the different endocytosis inhibitors on the cellular uptake of CLO-NCs RAW 264.7, the cells were pre-incubated for 1 h in standard culture conditions with the following inhibitors: chlorpromazine (CPZ 8 μg/mL), filipin III (1 μg/mL), and amiloride (50 μM). The final inhibitors concentrations that were not toxic to the cells were evaluated using an MTT assay (data not shown). Following the pre-incubation step, CLO-NCs were added to the culture medium containing inhibitors. The uptake was analyzed using a BD FACSCalibur flow cytometer and CellQuestPro. Ten thousand events were acquired for each sample. The results for cells incubated with 0.015 M NaCl as well as non-fluorescent CLO-NCs indicated background fluorescence (autofluorescence of the cells).

2.2.11. CLO-NCs biodistribution

150 μL of CLO-NCs, VI-PGA and CLO-NCs PGA-g(39)-PEG were injected intravenously into a mouse (C57Bl6xFVB) tail vein. 4 h after the injection, the mice were sacrificed and the spleens, lungs, hearts, kidneys and livers were resected. Spleens were dissociated on 70 μm mesh (BD Biosciences). The lungs, kidneys, hearts and livers were cut into small pieces and digested with 1 mg/mL collagenase type II and 3 IU/mL dispase for 1 h at 37 °C. Then, the cells were washed, and the erythrocytes were lysed with red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM, $NaHCO_3$, and 1 mM EDTA). The cells were then analyzed on a BD LSR Fortessa cytometer (Becton Dickinson). All of the animal work was approved by the Local Ethical Committee for Animal Research at the Jagiellonian University (registration number 55/V/2009).

2.2.12. Behavioral studies

Experiments were performed in adult male C57BL/6J (Taconic, Denmark) mice weighing 25–30 g. Animals were housed 4–6 per standard plastic type III Macrolon® cage (41 × 25 × 15 cm) in a light-controlled room (12 h light/dark cycle, light on at 6.00 a.m.) at 21 °C and 60% humidity, with free access to standard lab chow (Ewos R36, Ewos AB, Sweden) and tap water up to the time of the experiments. All studies were approved by the Ethics Board of Animal Experimentation of Northern Stockholm. The mice were habituated to the animal facilities and were handled daily by the same experimenter for a period of five days before the experiments. Experiments were conducted in experimentally naïve mice that were used only once, to prevent possible carryover effects between tests. Experimental procedures involving animals and their care followed the provisions and recommendations of Sweden's animal protection legislation. The experimental procedures were approved by the local Animal Ethical Committees (Ethical Numbers N423/12) and conformed to the European Council Directive (86/609/EEC). Spontaneous locomotor activity was measured in several animals simultaneously by means of a multicage red and infrared-sensitive motion detection system (SOBI Kungsbacka Mät- & Reglerteknik AB). The system is fully computerized and uses beams of red and infrared lights in combination with vertical (infrared light sensitive photocells in the walls of the apparatus) and horizontal (red light sensitive photocells in the floor of the apparatus) arrays. The distance between photocells is 4 cm. Locomotor activity (horizontal activity, locomotion, and rearing activity) was measured as all movements of a distance of 4 cm or more, while rearing was detected by horizontal and vertical photocells, and represents a measurement of general activity. The animals were injected with 100 μl $100 \lceil 1$ suspension of clozapine, CLO-NCs PGA, and CLO-NCs-g(39)-PEG. The final concentrations of clozapine differed and were as follows: 25–30 μg, 0.16 μg, and 0.08 μg per animal. 10 min later each mouse was re-introduced to the locomotor cages for 60 min period. The data collection system was activated immediately after the animal was placed in the activity box.

2.2.13. Statistical analysis

Data are presented as the means ± standard error (SEM). The statistical significance was evaluated using student *t*-test and Mann–Whitney *U*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results and discussion

Our previous studies have shown that polyelectrolyte shell NCs with emulsion cores can be successfully used as the nanocarriers for some model compounds, e.g., beta-carotene, vitamin A, and neuroprotective (MDL 28170) and anticancer drugs (Paclitaxel) [23,31,36]. Therefore, in this work, we decided to use those nanocapsules as antipsychotic drug carriers. NCs containing clozapine were synthesized according to the method described previously [31,33]. The oil phase for preparation of capsule cores was as follows: 0.1 g clozapine was dissolved in 1 mL of solution of AOT (FDA approved surfactant) in chloroform (340 g/dm^3). Then, 0.1 mL of the oil phase was added to the aqueous PLL solution (c = 0.1 g/dm^3) with continuous mixing using a magnetic stirrer at 300 rpm. The optimal ratio of surfactant (AOT) to polycation (PLL) was determined by measuring the zeta potential of the emulsion droplets and examining their stability. The optimal ratio for AOT/PLL was found when the zeta potential of the emulsion droplets reached a value close to that of the PLL in solution, just after overcharging. The optimal ratio was ca. 1. With this approach, the amount of unadsorbed polycation PLL was minimized as most of it was consumed to form the AOT/PLL interfacial complex [22,23,36]. The positive value of the zeta potential (+62 mV) of the emulsion containing clozapine demonstrated that they are electrostatically stabilized by high surface charges. The size of the CLO-NCs was measured by the DLS and NTA techniques and was approximately 80 nm with a polydispersity index (PDI) <0.2 (Fig. 1A, B).

In the following step, the cores of the CLO-NCs were encapsulated using consecutive polyelectrolyte layers by the saturation method of the LbL technique. The volume of the oppositely charged polyelectrolyte added to form a layer was considered to be optimal when the zeta potential of the capsule reached a value close to that of the polyelectrolyte used to form the outermost layer of the shell. This approach minimized the amount of unadsorbed PE in the nanocapsules suspension. The observed layer-to-layer variations in the zeta potential were from −40 mV for the polyanion to ca. 40 mV for the polycation layers, which indicated an electrostatic stabilization of the suspension (Fig. 1C).

To form pegylated CLO-NCs, the surface of the PLL-terminated nanocapsules with five polyelectrolytes layers (CLO-NCs V-PLL) were coated with PGA-g-PEG using the same procedure as for normal polyelectrolytes. Pegylation of the capsules with PGA-g-PEG (CLO-NCs VI-PGA-g-PEG) significantly decreased their zeta potential in comparison to the capsules with PGA layers. Therefore, the optimal volumes of PGA-g-PEGs used to form stable layers were achieved when the zeta potential of the NCs reached a value close to 0 (Fig. 1C); therefore, the measured zeta potential of the pegylated

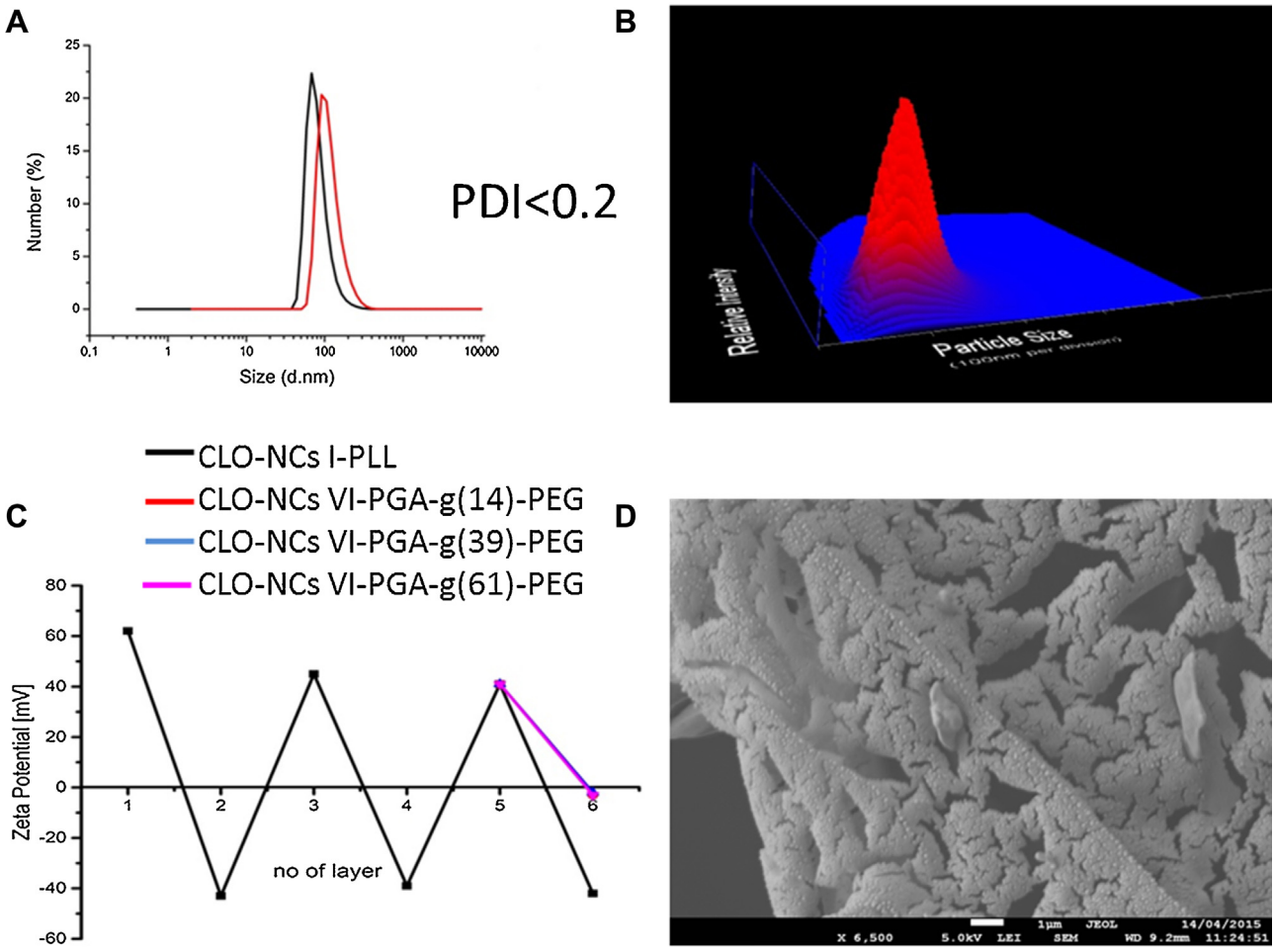

Fig. 1. (A) Size distribution of emulsion droplets containing clozapine (black) and pegylated multilayer NCs containing clozapine (red) measured by DLS; (B) Pegylated multilayer NCs containing clozapine measured by NTA; (C) Variation of zeta potential of NCs with numbers of polyelectrolyte layer; (D) The example of Cryo-SEM micrograph of CLO-NCs VI-PGA-g(39) PEG nanocapsules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

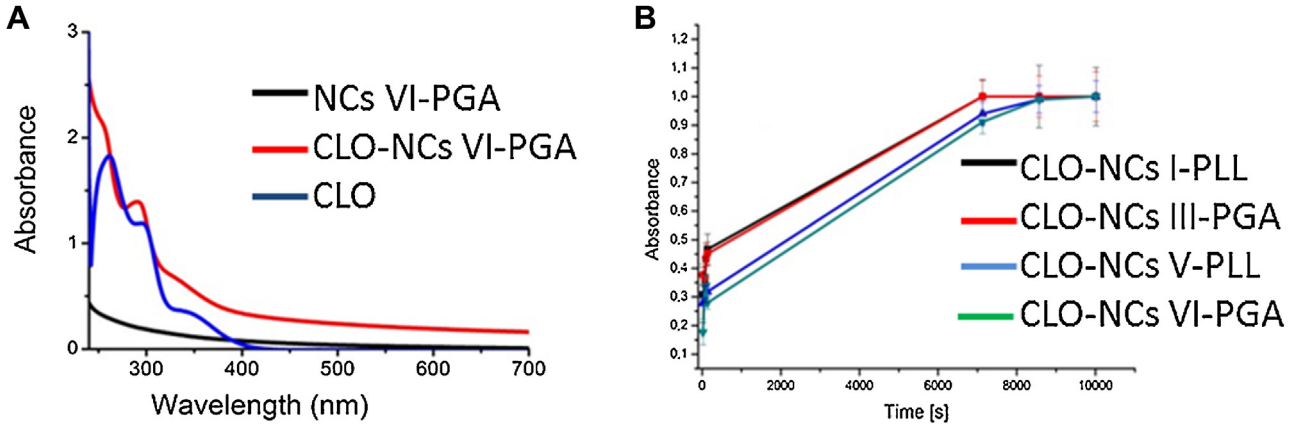

Fig. 2. (A) UV–vis spectra of empty pegylated NCs and clozapine containing NCs. (B) The kinetics of clozapine release from the NCs encapsulated with (PLL/PGA) polyelectrolyte shells with various number of polyelectrolyte layers.

nanocapsules was -4 ± 6 mV. The average size of the multilayer capsules was approximately 100 nm (measured by DLS and NTA), as shown in Fig. 1A, B. An example of the cryo-SEM micrograph of the pegylated nanocapsules (CLO-NCs VI-PGA-g(39)PEG) is shown in Fig. 1D. The size of most of the observed particles was ~100 nm, which is in agreement with the values obtained by DLS and NTA analysis. To establish whether the CLO-NCs would aggregate in a biosystem, the stability of the pegylated ones in the full culture medium (DMEM) with fetal foetal bovine serum (FBS) was tested [42]. We found that our pegylated clozapine containing nanocapsules retained their size without significant changes for at least 48 h irrespective of PEG grafting density, which means that the hydrophilic polymer tails at the surface of the particles provided sufficient steric stabilization. Encapsulation of clozapine

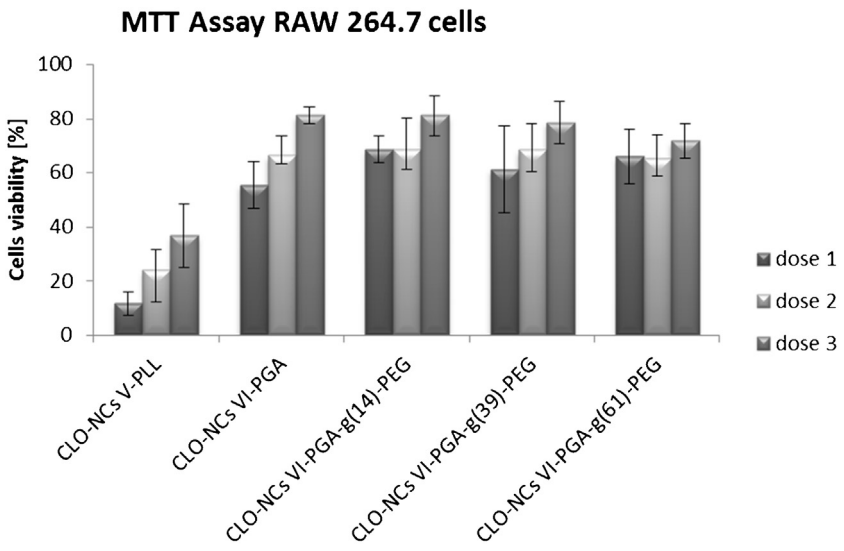

Fig. 3. Cell viability—MTT tests performed in RAW 264.7 cells after 24 h incubation with various types of CLO-NCs. Dose 1—0.85×10^6 NCs/cell, dose 2—0.45×10^6 NCs/cell, dose 3—0.15×10^6 NCs/cell.

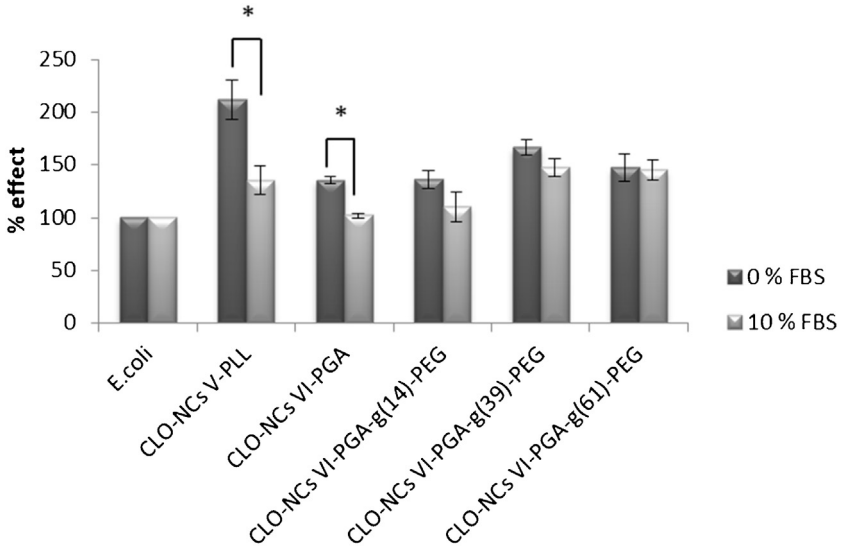

Fig. 4. The influence of CLO-NCs on phagocytic potential of RAW 264.7 cells. Experiment conducted in various conditions: in presence of 0% and 10% serum in the culture medium.

was confirmed by UV–vis spectroscopy. The characteristic peaks for clozapine could be observed in the UV–vis spectra, and a comparison of the spectra for empty and loaded capsules provided evidence on the successful encapsulation of clozapine (Fig. 2A). The final concentration of the CLO-NCs was $\sim 1 \times 10^{12}$ capsules per mL.

The release of clozapine from freshly prepared NCs was determined by dialysis performed in phosphate buffer (PBS) with the addition of 2% (w/v) Tween 20 to improve the solubility and the stability of the released clozapine. For the release kinetics studies, four types of drug loaded NCs were selected: two covered with positively charged PLL (core and the 5 layers) and two covered with negatively charged PGA (2 layers and 6 layers). Fig. 2B presents the experimental results for the kinetics of clozapine release from the nanocarriers encapsulated with (PLL/PGA) polyelectrolyte shells with various number of polyelectrolyte layers. One can see that the second layer of PGA on the AOT/PLL stabilized emulsion drop does not exert any significant resistance to clozapine release, but five or six layers of polyelectrolytes delay the release of clozapine. The permeability of the shell decreases with the number of polyelectrolyte layers, first due to an increase in the thickness of the PE membrane, and, second, due to reduced porosity [38,43]. Similar dependence of release on the number of polyelectrolyte layers has been described by others [44]. Our results indicate that by the optimization of the number of polyelectrolyte layers in a multilayer shell, the release properties can be controlled for in the proper application of NCs.

Exploitation of nanomaterials in pharmacology requires extensive studies of the cellular interactions of nanocarriers, especially with the cells of the mononuclear phagocytic system (MPS). It has been reported that significant quantities of systemically administered NCs are eliminated by the cells of MPS before they reached their desired sites of action [45]. It leads to a reduction in the circulating half-life of a drug and restricts usefulness of NCs as effective nanocarriers. Rapid NCs recognition by the immune system, and their clearance, depends on the sizes, shapes, and surface properties of the capsules [46–48]. Therefore, in the present study, the interactions of the various types of CLO-NCs with phagocytic cells were estimated. For that reason, PLL terminated nanocapsules (positively

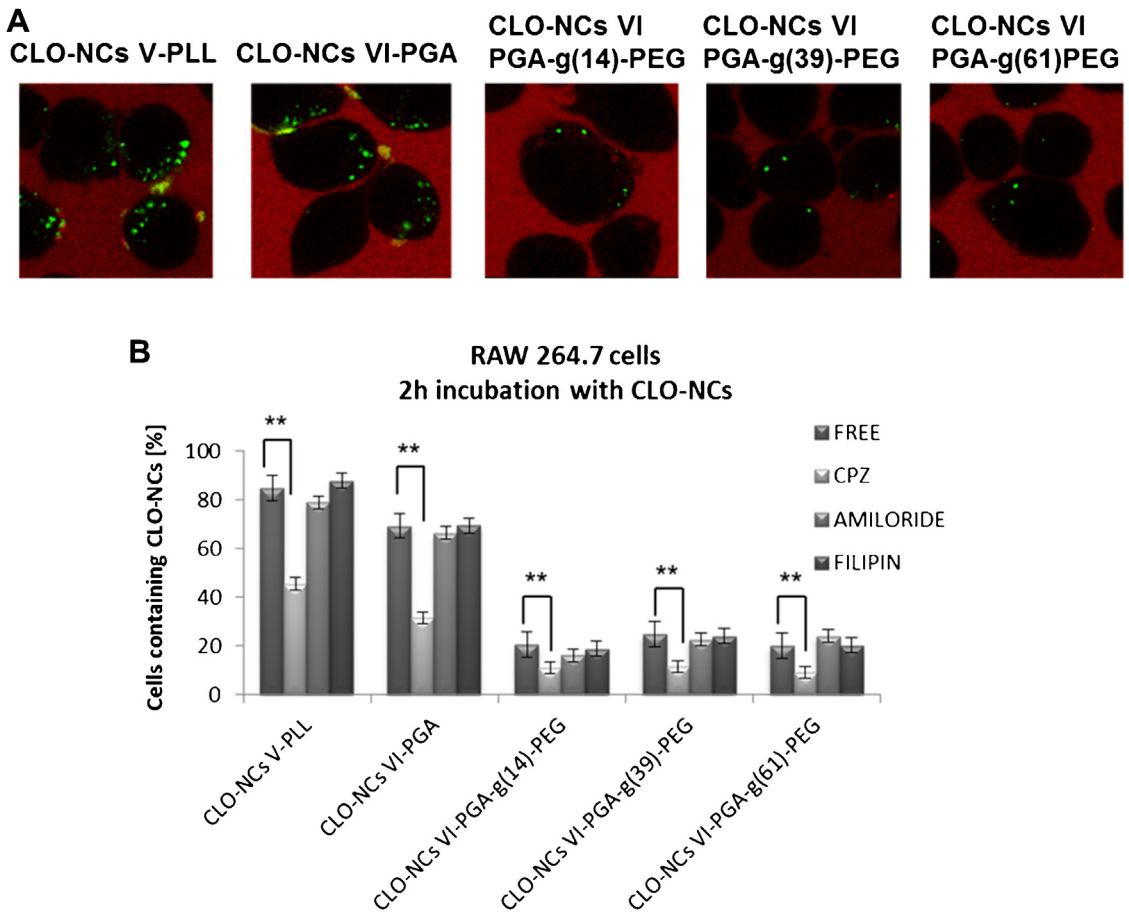

Fig. 5. (A) Confocal microscopy analysis of CLO-NCs internalization by RAW 264.7 cells after 2 h incubation of the cells with NCs; (B) Flow cytometry experiments of CLO-NCs uptake by RAW 264.7 cells after 2 h incubation of the cells with NCs.

charged), PGA terminated nanocapsules (negatively charged), and pegylated nanocapsules were chosen for the biotests.

One major issue in evaluating the biocompatibility of the NCs is assessing their cytotoxicity potential. Therefore, at first, the influence of the positively charged CLO-NCs (formed by five PE layers—CLO-NCs V-PLL), the negatively charged CLO-NCs (formed by six PE layers—CLO-NCs VI-PGA), and the pegylated CLO-NCs (CLO-NCs VI-PGA-g-PEG) on the viability of the RAW 264.7 cells was analyzed. Using the colorimetric MTT test, the metabolic activity of the cells was determined, which reflected the number of viable cells.

As shown in Fig. 3, the observed effects were concentration-dependent. Moreover, the lesser unfavorable influence on the cells was recorded in the case of the negatively charged CLO-NCs. Numerous studies have suggested that plasma membrane disruption is probably a common property of cationic nanomaterials [49,50]. The presence of high levels of lactate dehydrogenase (LDH) in the media of the cells treated with positively charged CLO-NCs points toward a harmful effect of the NCs on cell membrane integrity, resulting in cell death (Table 1). The pegylation process (irrespective of the PEG grafting ratio) did not cause any change in cell viability (Table 1). We also did not observe any LDH release.

As it was mentioned above, characterization of the interaction between the CLO-NCs and the phagocytic cells enables estimation of more effective and efficient nanocarrier action. Therefore, first of all, the ability of the CLO-NCs to modulate the phagocytic potential of the macrophages was determined. The results obtained depended on the experimental conditions. Both types of CLO-NCs (positively and negatively charged) up-regulated the phagocytosis when there was no serum in the culture medium. The observed effect was partially abated in the presence of serum in the sample. Lunov et al. [51] have shown that in serum-containing medium, human macrophages and THP-1 cells internalized much less polystyrene NPs than in buffer. In the present work, negatively charged NCs consisting of six polyelectrolyte layers seemed to exhibit the best properties as clozapine nanocarriers. As shown in Fig. 4, this type of CLO-NCs did not cause any change in the phagocytic potential of the RAW 264.7 cells. Although the pegylation process slightly increased the phagocytosis, the observed effect was not statistically significant.

It is well known that the mechanism of NC uptake is specific of a cell type [52]. Several reports have suggested that the NCs are taken up by a phagocytic or an endocytic process, but some studies have pointed to a passive mode of uptake. To study cellular uptake, all types of synthesized fluorescent CLO-NCs were incubated with macrophages (RAW 264.7 cells). Two different methodologies were adopted: flow cytometry and confocal microscopy imaging. As

Table 1
Cytotoxicity assay: LDH release from RAW 264.7 cells after 4 h incubation with different types CLO-NCs.

	Cytotoxicity [%] 0.85×10^6 NCs/cell	Cytotoxicity [%] 0.45×10^6 NCs/cell
CLO-NCs V-PLL	68 ± 12	14 ± 5
CLO-NCs VI-PGA	–	–
CLO-NCs VI-PGA-g(14)PEG	0.5 ± 0.07	–
CLO-NCs VI-PGA-g(39)PEG	0.9 ± 0.2	–
CLO-NCs VI-PGA-g(61)PEG	–	–

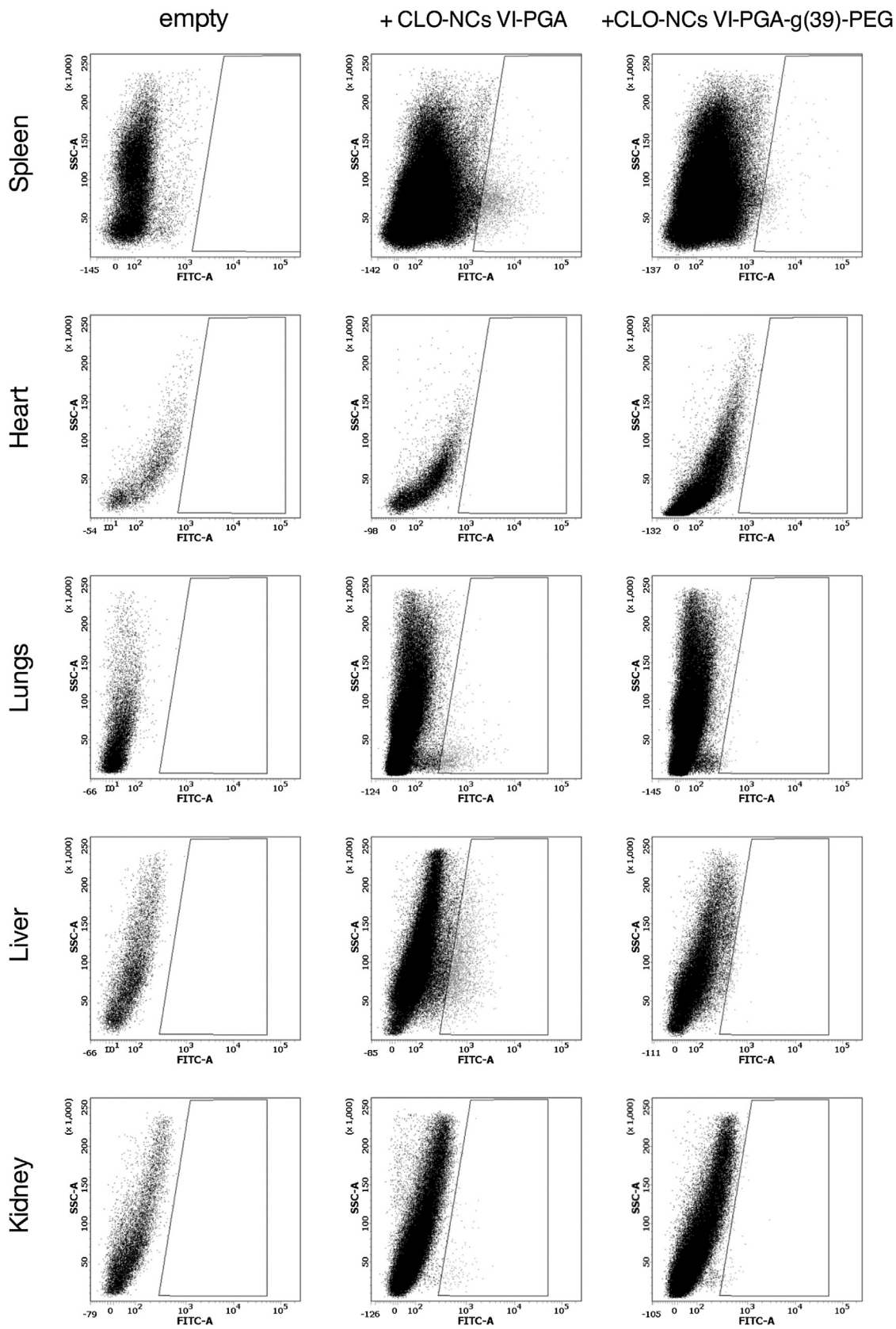

Fig. 6. CLO-NCs biodistribution studies: The animals were injected with 150 μL suspension of CLO-NCs VI-PGA as well as CLO-NCs VI-PGA-g(39)-PEG. 4 h after the injection, the cells of various organs were analyzed using flow cytometry.

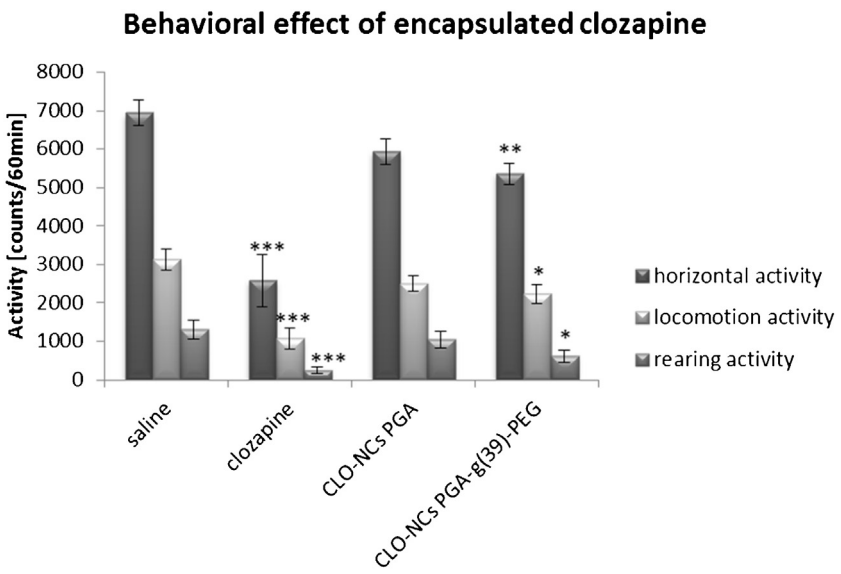

Fig. 7. Behavioral studies of CLO-NCs: Locomotor activity was measured in C57Bl/6J male mice. The animals were injected with 100 μL suspension of various forms of clozapine containing different amount of the drug: clozapine—25–30 μg, CLO-NCs PGA—0.16 μg, CLO-NCs PGA-g(39)-PEG—0.08 μg.

shown in Fig. 5A, all types of CLO-NCs were able to penetrate into the interior of the cells.

Using flow cytometry, the quantitative results for the internalization of the CLO-NCs were determined. A rigorous washing protocol and experiments performed at 4 °C ensured that all non-internalized CLO-NCs had been discarded from the surface of the cells. At low temperatures, NCs uptake was inhibited (data not shown). A summary of the flow cytometry experiments is presented in Fig. 5B. The non-pegylated (naked) CLO-NCs, especially those that were positively charged, were internalized to the highest extent. The uptake of the surface modified CLO-NCs, irrespective of the PEG grafting ratio, was notably reduced. It confirmed our hypothesis that the pegylation process made the nanocarriers more invisible to the phagocytic cells.

In our previous studies, it has been postulated that clathrin-mediated endocytosis is the major contribution in the internalization of polymeric PLL-PGA NCs. Therefore, in the present study, we assessed whether clozapine encapsulation altered the endocytic pathway. As shown in Fig. 5B, for clozapine loaded capsules, endocytosis through clathrin vesicles also played a key role during their internalization, which was independent of the surface charge of the capsules.

The results indicated that clozapine encapsulation within polymeric PLL-PGA shell nanocarriers seems to be very promising. Therefore, it was also important to estimate the biodistribution of the CLO-NCs in vivo. Although this type of experiment was only qualitative, as shown in Fig. 6, the biodistribution of the CLO-NCs depended on the surface modification of the capsules. The CLO-NCs, regardless of their surface coating, were found to be present in mouse kidneys, livers, spleens, and lungs, but not in hearts 4 h after intravenous injection. Non-pegylated CLO-NCs were found mainly in the spleen and the liver. Pegylation significantly decreased the accumulation of the CLO-NCs in the investigated organs.

Additionally, to confirm that the proposed nanocapsules are promising candidates for clozapine delivery, in vivo functional studies were performed. These preliminary behavioral results indicated that encapsulated clozapine reduced mice locomotor activity in a manner characteristic of clozapine, with the strongest effect on rearing (Fig. 7). The effect was induced only by the pegylated, negatively charged nanocarriers. The non-pegylated nanocarriers were not effective, probably due to their elimination by the circulating macrophages. This is indicated by the in vitro results obtained in the present study. The overall weaker activity of the encapsulated CLO in comparison to the drug administered by classical route was, most probably, due to lower doses of the encapsulated drug. One limitation during the synthesis of the capsules was variations in the concentration of clozapine, and the dose could not be increased further. However, the main goal of the study was to obtain a CLO-NCs formulation that showed an effect of clozapine in vivo. This aspect needs to be studied further; the present work focused primarily on the in vitro effects of the synthesized nanocarriers, which were then subjected to preliminary in vivo experiments.

4. Conclusion

In the present work, we proposed a new method for the encapsulation of clozapine using the LbL technique. To function effectively and efficiently in the defined and desired site of action, the nanocarriers should be invisible to the phagocytic cells. This feature is very important because it prolongs the circulation half-life of the drug enclosed within the NCs. The synthesized CLO-NCs consisted of a hydrophobic core, which enabled the dissolution of a lipophilic drug, and a hydrophilic surface formed by the PEG corona, which prevented an interaction between the core and the blood components. This ensured steric stabilization, thereby allowing longer drug circulation.

Fluctuating levels of clozapine, and especially its peripheral action, give rise to serious side effects. Hence, the formation of appropriate carriers for clozapine, as has been achieved in the present study, is very important as that may enable a steady level of the drug during long-term administration, as used in clinical practice. However, further studies are necessary to determine a strategy for targeted delivery of the encapsulated drug to the brain. The preliminary in vivo results obtained in this study indicate that the synthesized pegylated CLO-NCs are a promising alternative for the delivery of clozapine.

Conflict of interest

The authors report no conflicts of interest in this work. The authors also declare no competing financial interest.

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