CLINICAL-DIAGNOSTIC STUDIES

COMPARATIVE ANALYSIS OF THE EFFECT OF HYBRID VATERITE MICROPARTICLES WITH VARIOUS POLYSACCHARIDES ON NEUTROPHILS ACTIVITY

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Carriers based on natural biominerals attract much attention in the context of the development of new drug delivery systems. In this study, the effects of native (CC) and hybrid vaterite microparticles with the inclusion of dextran sulfate (CCDS), chondroitin sulfate (CCCS), heparin (CCHE), fucoidan (CCFU), and pectin (CCPE) have been investigated on the viability and functional activity of neutrophils. Among the tested preparations, only CCFU exhibited a slight cytotoxic effect. Native CC stimulated actin cytoskeleton rearrangements and cell production of reactive oxygen species (ROS), which decreased in the presence of diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase assembly. The CC-induced NADPH oxidase activation was reduced in the presence of inhibitors of non-receptor tyrosine kinases of the Src family, phosphatidylinositol 3-kinase (PI3K), and phospholipase C (PLC). Similar to native CC, hybrid vaterite microparticles also initiated ROS production by neutrophils. After addition of CC and hybrid vaterite microparticles (except CCDS), an increase in the number of neutrophils characterized by higher values of the side scattering value was detected thus indicating a change in the morphological characteristics of the cells. Given the ability of hybrid vaterite microparticles with polysaccharides to activate neutrophil NADPH oxidase, they could be promising systems for the delivery of antibacterial and antiviral drugs.

Keywords: vaterite microparticles; polysaccharides; neutrophils; cytotoxicity; NADPH oxidase

DOI: 10.18097/PBMCR1561

INTRODUCTION

Although results of numerous work have been published in recent decades in the field of biomedical technologies aimed at the development of new drug delivery strategies, translation of fundamental research into clinicasl practice is quite slow; this is associated with problems of biocompatibility and safety, complex procedures for approval and government regulation of drugs. In this regard, researchers pay attention to delivery systems based on materials that have already been approved for biomedical use. One of them is calcium carbonate ($CaCO_3$) [1]. This non-toxic natural biomineral is used as a safe buffering agent for food products, hygiene products and cosmetics. $CaCO_3$ can exist in the form of several polymorphs, among which vaterite is particularly interested researchers.

In the context of biomedical use vaterite has a number of advantages over other $CaCO_3$ forms. It is characterized by ease of production, particle size controlled by synthesis conditions, porous structure allowing vaterite to include various biologically active molecules, mucoadhesive properties [2], and the ability to easily dissolve in body fluids (for example, at low pH). Due to these properties, vaterite is considered as a promising container for drug delivery. However, vaterite has some disadvantages that limit its use. These include metastability, fairly rapid recrystallization in aqueous media into more stable calcite, as well as rapid release of positively charged molecules [1]. These disadvantages can be eliminated by additionally introducing polymers during the formation of vaterite microparticles (CC) and preparation of so-called “hybrid” CC [3–5]. CC modification with various biopolymers not only changes their physicochemical properties (surface area, pore size, zeta potential) [3, 5], but can also have an additional therapeutic effect. For example, it has been shown that in the presence of vaterite microparticles with pectin (CCPE), growth of the *E. coli* laboratory strain Mg1655 increased, while the bacterial growth of the clinical isolate SharL from a patient with Crohn's disease, on the contrary, decreased; at the same time, the bacterial cell wall permeability was impaired due to its damage,

as evidenced by an increase in the extracellular ATP concentration [6]. Inclusion of dextran and its derivatives in the composition of CC increased their adhesion to endothelial cells [7, 8].

Significant progress has been recently achieved in the development of methods for immobilization of various biologically active substances in vaterite, such as proteins [9], including enzymes [10], and also RNA [11], synthetic drugs [1], including antifungal and inorganic nanoparticles [8, 12].

However, for the effective use of these carriers as drug delivery vehicles, a comprehensive analysis of their effect on cells that are primarily involved in the body's inflammatory responses is clearly needed. This is due to the fact that introduction of micro/nanosized structures into the body can lead to the activation of immune cells.

Neutrophils, also known as polymorphonuclear granulocytes, are the most numerous population of immune cells circulating in blood. They are the first to reach the sites of inflammation, where they perform antimicrobial effector functions, including: phagocytosis, generation of reactive oxygen (ROS) and halogen (RHS) species, degranulation with secretion of cytotoxic granule enzymes (myeloperoxidase (MPO), elastase, lysozyme, etc.) and release of neutrophil extracellular traps (NETs), which capture microorganisms to isolate them and prevent subsequent spread. At present, the effect of hybrid CC on neutrophils has not been sufficiently studied. It has been shown that hybrid CC with mucin activate ROS generation by neutrophils, aggregation of these cells, and the formation of NETs-like structures [13]. The inclusion of pectin in CC abolishes the effect of adsorbed mucin on neutrophil activation [14].

It is important to note that hybrid CC are not only more stable in biological environments and more effectively retain the delivered compounds (e.g., cationic molecules), but they are also capable of exhibiting additional biological activity associated with the polymers.

Recently, a number of hybrid CC with various polysaccharides have been synthesized and characterized [3]. Their properties depend on the nature of the polysaccharide included in their composition. In this work, we performed for the first time, a comparative analysis of the effect of hybrid CC with dextran sulfate (CCDS), chondroitin sulfate (CCCS), heparin (CCHE), pectin (CCPE), fucoidan (CCFU) on the functional activity of neutrophils.

MATERIALS AND METHODS

Reagents

The following reagents have been used in the study: fucoidan (FU) from *Fucus vesiculosus* (20–200 kDa, sulfur content 7–11%), pectin (PE) from apples (30–100 kDa, esterification degree of 50–75%, 6.7% methoxy groups, 74% galacturonic acid), chondroitin sulfate A (CS) from bovine trachea (20–30 kDa), scopoletin, horseradish peroxidase, phorbol 12-myristate 13-acetate (PMA), NaOCl, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), histopaque-1077, dextran T70, trypan blue, methoxyverapamil, $NiCl_2$, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2), wortmannin, cytochalasin b (cyt b), 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122), Triton X-100, Fluoromount aqueous mounting medium, and ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA; Sigma-Aldrich, USA); dextran sulfate (DS) from *Leuconostoc* ssp. (500 kDa, 2.3 sulfo groups per unit; Fluka, Germany); heparin (HE) (10–20 kDa; Spofa, Czech Republic); paraformaldehyde (Panreac, Spain); poly-L-lysine (Santa Cruz Biotechnology, USA); fura-2AM, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), propidium iodide (PI), Alexa Fluor 488-conjugated phalloidin and diphenyleneiodonium chloride (DPI; Molecular Probes, Netherlands); LDH-2-OLVEX and CALCIUM-OLVEX kits (OLVEX DIAGNOSTICUM, Russia). Other reagents were produced by Reakhim (Russia) and Belmedpreparaty (Belarus).

Preparation of Native and Hybrid Vaterite Microparticles

Native and hybrid CC were prepared by spontaneous crystallization by mixing $CaCl_2$ and Na_2CO_3 solutions in 0.05 M Tris buffer (pH 7.0), as described previously [3, 5]. For synthesis of hybrid CC with polysaccharides, the $CaCl_2$ solution was pre-mixed with polysaccharides. The precipitates of native and hybrid CC were washed with bidistilled water and lyophilized. The average diameter of spherical native and hybrid CC, determined by scanning electron microscopy (Zeiss Merlin microscope, Zeiss, Germany), was: 3.6±0.7 μm for CC, 3.8±0.8 μm for CCDS, 1.9±0.5 μm for CCCS, 2.0±0.3 μm for CCHE, 2.1±0.5 μm for CCFU, 2.3±0.7 μm for CCPE [3]. The zeta potential of native and hybrid CC, which was studied by the dynamic light scattering method on the Malvern Zetasizer Nano ZS setup (Malvern Panalytical, UK), was: 2±1 mV for CC, -13±2 mV for CCDS, -12±2 mV for CCCS, -9±2 mV for CCHE, -12±2 mV for CCFU, -12±2 mV for CCPE [3].

Neutrophil Isolation

Neutrophils were isolated from venous blood of healthy donors obtained at the Republican Scientific and Practical Center for Transfusiology and Medical Biotechnology (Minsk, Belarus) as described in [15]. Blood stabilized with sodium citrate (109 mM) in a ratio of 9:1 (v/v) was mixed with 6% dextran T70

in a ratio of 5:1 (v/v) and kept for 40 min for erythrocyte sedimentation. Residual erythrocytes in leukocyte-rich plasma were removed by osmotic lysis by sequential addition of 3 ml of 0.2% NaCl and 3 ml of 1.6% NaCl containing 20 mg/ml D-glucose to the cell suspension. Leukocyte-rich plasma was layered on histopaque-1077 and centrifuged at 450 *g* for 15 min. The neutrophil pellet was washed with phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4/KH_2PO_4, 2.7 mM KCl, pH 7.4) and resuspended in 1 ml PBS containing 1 mg/ml D-glucose. The isolated cell suspension contained at least 95% neutrophils; their viability was at least 95% according to the trypan blue test.

Neutrophil Viability Assessment

To assess the effect of native and hybrid CC on neutrophil viability, cells (1×10^6 cells/ml) in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were incubated for 1 h at 37°C with test samples or polysaccharides (0.25 mg/ml) and then PI (1 μg/ml) was added to the samples, which were incubated for 5 min at room temperature in a light protected place. Hypochlorous acid solution (HOCl, 250 μM), prepared by diluting a commercial NaOCl solution in PBS, was used as a positive control. The HOCl concentration in the diluted commercial NaOCl solution was determined spectrophotometrically at 290 nm using a molar absorption coefficient of 350 M^{-1} cm^{-1} at pH 12.0 [16]. Since at physiological pH values HOCl (pK_a = 7.5) exists both in a molecular form and as an anion, in this work HOCl will imply a mixture of OCl^- and HOCl. After incubation, the samples were diluted 3-fold with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and analyzed on a CytoFocus 820 flow cytometer (Healicom, China). For each sample, at least 20,000 single cells were analyzed. The neutrophil population was determined by a ratio of forward (FSC) and side (SSC) light scatter. A laser with a wavelength of 488 nm was used for excitation of PI fluorescence, and a 620±20 nm filter (ECD channel) was used for registration. The percentage of PI-positive cells in the neutrophil population was used as a quantitative parameter characterizing the viability of neutrophils, which was determined using the software supplied with the flow cytometer.

The viability of neutrophils incubated with native or hybrid CC was also determined by the activity of lactate dehydrogenase (LDH) assayed in the neutrophil supernatant using a commercial LDH-2-OLVEX kit. For this purpose, 10 μl of the neutrophil supernatant was added to 1 ml of the working reagent and the optical density of the solution was recorded at 340 nm at 1-min intervals for 3 min using a PB 2201 spectrophotometer (SOLAR, Belarus). Next, the average value of the optical density change per 1 min (ΔD_{340}/min) was calculated.

Registration of ROS Production by Neutrophils

Extracellular H_2O_2 production by neutrophils was assessed by a fluorescence method using the scopoletin-horseradish peroxidase system [16]. Scopoletin (fluorescent substrate of horseradish peroxidase, 1 μM), horseradish peroxidase (20 μg/ml), NaN_3 (catalase and MPO inhibitor, 1 mM), and native or hybrid CC or polysaccharides (0.25 mg/ml) were added to a neutrophil suspension (1×10^6 cells/ml) in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. In a series of experiments, before adding CC, neutrophils were preincubated for 5 min at 37°C with inhibitors 20 μM DPI, 100 μM PP2, 100 nM wortmannin, 5 μM cyt b, 1 mM $NiCl_2$, 100 μM methoxyverapamil, 620 nM U73122. The decrease in scopoletin fluorescence intensity was recorded at 460 nm (with fluorescence excitation at 350 nm) using a computerized spectrofluorimeter CM 2203 (SOLAR). For quantitative characterization of the extracellular H_2O_2 production by neutrophils, the maximum slope of the recorded kinetic curves of the decrease in scopoletin fluorescence intensity was calculated.

Intracellular ROS production by neutrophils was studied by flow cytometry using H_2DCFDA, applicable for evaluation of the total level of oxidants inside the cell [17]. Neutrophils (1×10^6 cells/ml) in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were incubated with H_2DCFDA (2.5 μM) for 10 min at room temperature, and then native or hybrid CC (0.25 mg/ml), polysaccharides (0.25 mg/ml) or PMA (50 nM) (used as a positive control) were added and samples were incubated for 30 min at 37°C. In a series of experiments, before adding CC, neutrophils were preincubated for 5 min at 37°C with inhibitors 20 μM DPI, 100 μM PP2, 100 nM wortmannin, 5 μM cyt b, 1 mM $NiCl_2$, 100 μM methoxyverapamil, 620 nM U73122. Then, the cell suspension was diluted 3-fold with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and analyzed on a CytoFocus 820 flow cytometer. For each sample, at least 20,000 single cells were analyzed. The neutrophil population was determined by the FSC and SSC ratio. A laser with a wavelength of 488 nm was used to excite 2′,7′-dichlorodihydrofluorescein (DCF) fluorescence, and a 525±40 nm filter (FITC channel) was used for its registration. The median DCF fluorescence intensity in the population of DCF-positive neutrophils was used as a quantitative parameter characterizing the intracellular ROS production by neutrophils; it was determined using the software supplied with the flow cytometer.

Determination of the Intracellular Concentration of Free Calcium Ions in Neutrophils

Intracellular concentrations of free calcium ions ($[Ca^{2+}]_i$) in neutrophils were determined using the fura-2AM fluorescent probe, as described previously [18]. After addition of 2 μl of fura-2AM (0.5 mM) to 1 ml of washed neutrophils (10^7 cells/ml)

in 20 mM HEPES buffer (pH 7.4) containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 4.4 mM $MgSO_4$, 1.7 mM $CaCl_2$, and 11 mM D-glucose, the mixture was incubated for 40 min at 37°C under constant stirring. The probe-loaded cells were washed twice from the incubation medium with $CaCl_2$-free HEPES buffer and sedimintation at 400 *g* for 5 min. The washed neutrophils were stored as the initial suspension (10^7 cells/ml). For $[Ca^{2+}]_i$ measurement 0.9 ml of HEPES buffer and 100 μl of the original cell suspension were added to the spectrofluorimeter cuvette followed by addition of different concentrations of CC. Fluorescence intensity was measured at 510 nm (fluorescence excitation at 340 nm and 380 nm) at 37°C in kinetic mode using a computerized spectrofluorimeter CM 2203. Neutrophil $[Ca^{2+}]_i$ was calculated as described previously [19], using the ratiometric calibration method.

Study of Morphological Characteristics of Neutrophils

Analysis of morphological characteristics of neutrophils was performed by flow cytometry [20]. Neutrophils were incubated with native or hybrid CC (0.25 mg/ml) in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, 1 h at 37°C. Samples incubated with the tested preparations were analyzed by the percentage of cells with a higher SSC value (pulse area) than the threshold value determined in the sample without CC (percentage of positive events <10%).

Changes in the organization of the actin cytoskeleton of neutrophils incubated with CC were investigated using confocal microscopy as described previously [16]. Neutrophils (3×10^6 cells/ml) in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were incubated with or without CC (0.25 mg/ml) for 15 min at 37°C; cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and then the samples were washed from paraformaldehyde twice by centrifugation at 450 *g* for 5 min and resuspension of the pellet in PBS. Washed neutrophils were applied to coverslips pre-coated with poly-L-lysine for 1 h. After cell attachment to the slides, the samples were washed 3 times with PBS. Next, the coverslips with neutrophils were incubated for 5 min with 0.1% Triton X-100 for membrane permeabilization and then the samples were washed 3 times with PBS. The coverslips with neutrophils were then incubated in the dark for 40 min at room temperature with phalloidin (0.165 μM) conjugated with Alexa Fluor 488; after the incubation slides were washed from excess of fluorescently labeled probe with PBS (2 times for 10 min) and distilled water (1 time for 10 min). The coverslips with neutrophils were fixed on microscope slides, pre-cleaned with 96% alcohol, using Fluoromount aqueous mounting medium. The resulting samples were analyzed using a spectral analytical complex based on a Nanofinder scanning confocal microscope (Tokio Instruments, Japan). Fluorescence excitation of Alexa Fluor 488 dye was performed by a laser with a wavelength of 488 nm, fluorescence detection was performed by using a filter of 505–550 nm. The experimental results were processed using the NanoFinder Data Viewer software.

Statistical Data Processing

The results are presented as the mean value ± standard error of the mean (M ± SEM). The minimum number of independent experiments carried out for each experimental point was 5–7, unless otherwise indicated. The significance of differences in mean values was calculated using the Student's *t*-test, differences were considered as statistically reliable at $p<0.05$. The kinetic curves are presented as typical for a series of 5–7 independent experiments. Statistical data processing was performed using the Origin 7.0 software package (OriginLab Corporation, USA).

RESULTS

Cytotoxicity Evaluation of Vaterite Microparticles and Polysaccharides

The viability of neutrophils in the presence of polysaccharides, native or hybrid CC has been evaluated by flow cytometry using nucleic acid-binding PI; the latter does not penetrate living cells and is usually used to detect dead cells in the population. Figure 1a shows that none of the used polysaccharides (0.25 mg/ml, 1 h at 37°C) disrupted the intactness of the neutrophil plasma membrane. The study of the effect of native and hybrid CC (0.25 mg/ml, 1 h at 37°C) on neutrophil viability has shown that only after incubation of neutrophils with CCFU the number of nonviable cells increased approximately 2-fold compared to the control. It should be noted that the cytometric method with PI is inapplicable for evaluation of the effect of CCDS on the viability of neutrophils due to PI intercalation of into them; this resulted in simultaneous staining of both neutrophils and CCDS (data not shown). Therefore, a spectrophotometric method for LDH activity assay in the extracellular medium was additionally used to assess neutrophil viability. After incubation of neutrophils with CCFU, LDH activity in the extracellular medium significantly increased, thus indicating impaired neutrophil viability and consistent with results of the PI test. After incubation of neutrophils with CCDS, a tendency toward an increase in LDH activity in the extracellular medium was noted, but no significant differences were found (Fig. 1b).

The Effect of Vaterite Microparticles and Polysaccharides on ROS Production by Neutrophils

The effect of native and hybrid CC, as well as polysaccharides on ROS production by neutrophils was studied in two ways: by the fluorescence

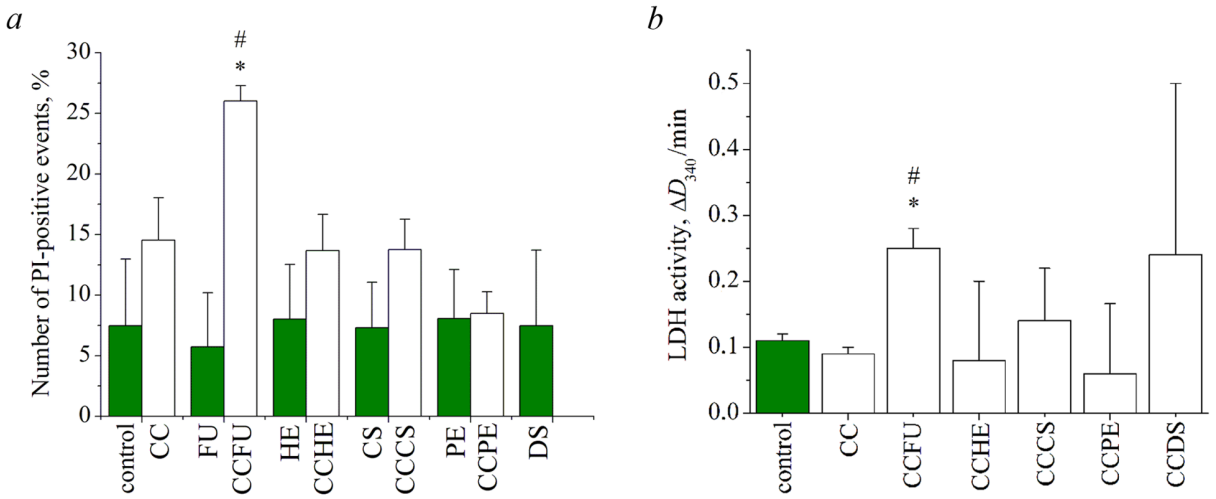

Figure 1. The effect of native and hybrid CC (0.25 mg/ml) (**a** (PI test) and **b** (LDH test)) and polysaccharides (0.25 mg/ml) (**a** (PI test)) on neutrophil viability. Neutrophils were incubated with the tested preparations for 1 h at 37°C. *p<0.05 compared to the control. #p<0.05 compared to the effect of CC.

method using scopoletin, registering extracellular production of H_2O_2, and by the flow cytometry method using H_2DCFDA, detecting mainly intracellular production of various ROS. Figure 2a shows typical kinetic curves of scopoletin oxidation in a suspension of neutrophils activated by native CC used in different concentrations. It is evident that CC initiate a dose-dependent (Fig. 2a,b) H_2O_2 production by neutrophils, which is consistent with the literature data [21, 22]. As shown in Figure 2c, addition of hybrid CC to neutrophils was also accompanied by cell activation. It should be noted that the effect of CCHE on the production of H_2O_2 (scopoletin test) was higher compared to the effect of native CC; in the presence of CCPE the activation of the respiratory burst of neutrophils was lower (Fig. 2c) compared to the effect of CC.

Figure 2c shows that in the presence of HE, as well as in the presence of FU, DS, or CS, H_2O_2 production by neutrophils was also registered, but the effects of these polysaccharides were less pronounced compared to the effects of native and hybrid CC. A comparative analysis of the effect of native and hybrid CC on neutrophil activity showed that the inclusion of polysaccharides in the CC led to a change in the properties of CC in such a way that the activation of neutrophils increased (in the case of the inclusion of HE in the CC) or decreased (in the case of the inclusion of PE in the CC), thus indicating that the effect of hybrid CC was not additive (the sum of the effects of CC and polysaccharides).

Activation of neutrophil NADPH oxidase by polysaccharides, as well as native and hybrid CC, was studied by flow cytometry using the fluorescent probe H_2DCFDA, applicable for evaluation of the intracellular production of ROS in cells [16]. Figure 2d shows that after incubation with native CC, the DCF fluorescence intensity in neutrophils increased. However, it should be noted that the effect of the CC was one order of magnitude lower than the effect of the standard neutrophil activator, phorbol ester PMA (data not shown). A comparative analysis of the effect of hybrid CC on intracellular ROS production by neutrophils has shown that the effects of hybrid CC were comparable to the effects of the CC, with the exception of CCPE, which had a significantly higher effect on the DCF fluorescence intensity as compared to the effect of CC (Fig. 2d). Although all the studied polysaccharides caused a small intracellular production of ROS by neutrophils (Fig. 2d) their effects much weaker than the effects of CC. Activation of H_2O_2 production by neutrophils treated with CC decreased in the presence in the incubation medium of DPI, an inhibitor of NADPH oxidase assembly. This indicates activation of the respiratory burst of neutrophils (Fig. 3a). In addition, inhibition of CC-induced H_2O_2 production by neutrophils was observed in the presence of PP2, an inhibitor of non-receptor cytosolic tyrosine kinases of the Src family (Lck, Fyn, and Hck), wortmannin, a selective and irreversible PI3K inhibitor, and U73122, a PLC inhibitor (Fig. 3a). This suggests involvement of these signaling molecules in the respiratory burst of neutrophils in response to CC. Cyt b, which inhibits actin polymerization, did not affect the extracellular H_2O_2 production by neutrophils in response to CC.

In response to CC in a calcium-free medium, a slight decrease in the rate of scopoletin oxidation was noted (Fig. 3a). In the presence of $NiCl_2$, an inorganic blocker of T-type Ca^{2+} channels, or methoxyverapamil, an inhibitor of L-type voltage-gated Ca^{2+} channels, CC-induced H_2O_2 production by neutrophils decreased by 20–60% (Fig. 3a).

These data indicate involvement of Ca^{2+} in the signaling processes leading to the activation of the neutrophil respiratory burst in response to CC. Indeed, as shown in Figure 3b, addition of CC

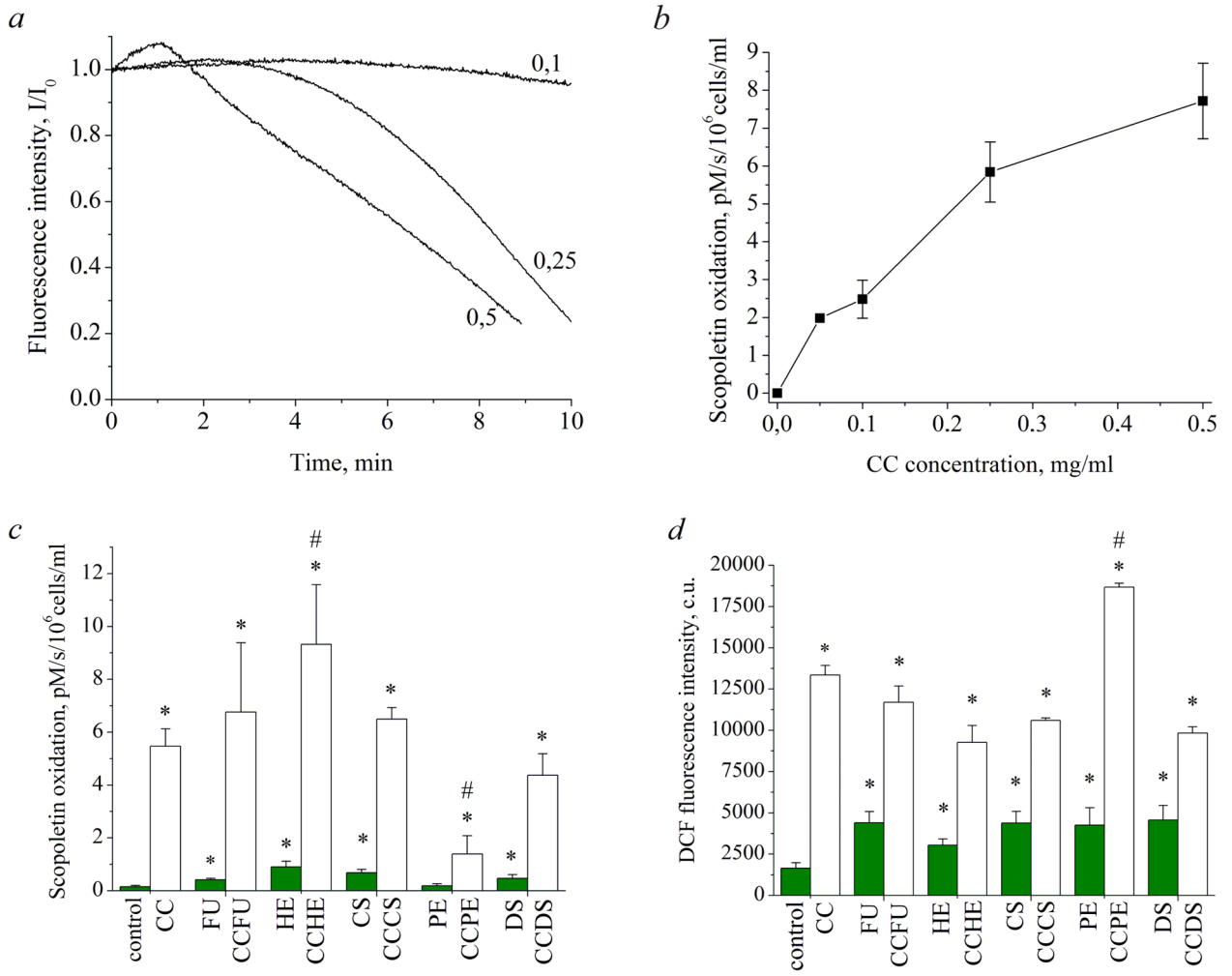

Figure 2. The effect of native and hybrid CC and polysaccharides on ROS production by neutrophils. **a** – Typical kinetic curves of scopoletin oxidation in the neutrophil suspension after addition of CC at concentrations of 0.1 mg/ml; 0.25 mg/ml; 0.5 mg/ml; **b** – Dependence of the scopoletin oxidation rate on CC concentration; **c** and **d** – The effect of native and hybrid CC (0.25 mg/ml), as well as polysaccharides (0.25 mg/ml) on extracellular (**c**) and intracellular (**d**) ROS production by neutrophils. The CC concentrations (in mg/ml) used in panel **a** are indicated by numbers next to the curves. **p*<0.05 compared to the control. #*p*<0.05 compared to the effect of native CC.

to the neutrophil suspension resulted in a dose-dependent increase in $[Ca^{2+}]_i$, which was reduced by 57% in the presence of $NiCl_2$ and by 48% in the presence of methoxyverapamil, thus indicating the role of plasma membrane Ca^{2+} channels in the CC-induced increase in $[Ca^{2+}]_i$ in neutrophils. It should be noted that the increase in Ca^{2+} entry into the neutrophil cytosol is not due to an increase in the Ca^{2+} concentration in the extracellular medium due to CC dissolution, because during CC incubation in buffer solutions (PBS or HEPES buffer) for 1 h, the amount of released Ca^{2+} was just 6–15 nM per 0.1–0.25 mg CC.

Next, we performed an inhibitory analysis of intracellular ROS production by neutrophils treated with CC. Figure 3c shows that intracellular ROS production by neutrophils in response to CC decreased in a calcium-free medium, in the presence of DPI, PP2, wortmannin, $NiCl_2$, U73122, and also in the presence of cyt b, impairing intracellular actin dynamics.

Analysis of Morphological Characteristics of Neutrophils in the Presence of Vaterite Microparticles

The interaction of CC with neutrophils can be accompanied by changes in cell morphology due to initiation of intracellular signaling processes and actin cytoskeleton reorganization [23]. The impact of CC on the actin cytoskeleton organization of neutrophils was studied using confocal microscopy. Figure 4a shows that control cells have a clearly expressed cortical cytoskeleton (at the cell margins) and a less intense distribution of F-actin (cytosolic cytoskeleton) throughout the cell; after incubation of neutrophils with CC, the formation of pseudopodia of various shapes was observed thus indicating actin cytoskeleton reorganization.

Using the flow cytometry method it is possible to determine a wide range of parameters: the volume and morphological structure of cells,

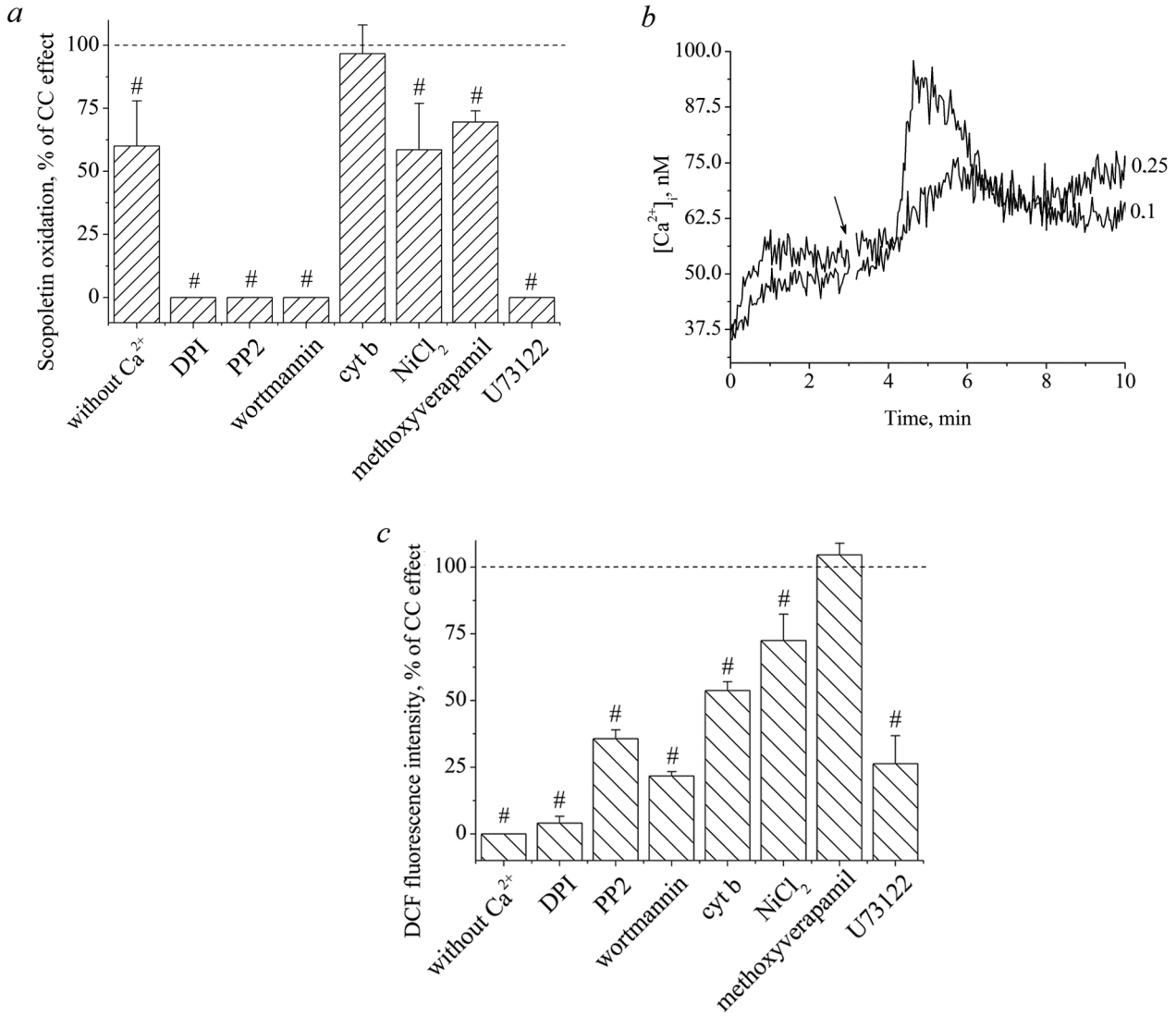

Figure 3. The effect of inhibitors on extracellular H_2O_2 production by neutrophils (**a**), change in $[Ca^{2+}]_i$ in neutrophils (**b**), intracellular ROS production by neutrophils (**c**) under the action of CC. The concentrations used: CC – 0.25 mg/ml, DPI – 20 μM, PP2 – 100 μM, wortmannin – 100 nM, cyt b – 5 μM, $NiCl_2$ – 1 mM, methoxyverapamil – 100 μM, U73122 – 620 nM. The concentrations of CC used in panel **b** are indicated by numbers next to the curves in mg/ml, the arrow shows the moment of CC addition. #p<0.05 compared to the effect of CC.

the state of the membrane, surface and intracellular cell markers, etc. The FSC value depends on the cell volume, while the SSC value, associated with such cellular parameters as the shape of the nucleus, the number and type of cytoplasmic granules and membrane roughness, depends on the morphology of the cell. It is known that during cell activation and changes in its morphology, the SSC value increases [22, 24]. Figure 4b shows the distribution of the control cell population and neutrophils incubated with CC (0.25 mg/ml, 1 h at 37°C) by SSC value. It is evident that after incubation of neutrophils with CC, the number of cells characterized by higher SSC values increased, possibly, due to adsorption and/or endocytosis of CC [25]. In the presence of all hybrid CC, except CCCS, the morphological characteristics of neutrophils changed (Fig. 4c). However, the highest changes in the SSC value were recorded under the action of CCPE, which also had the highest activating effect on intracellular ROS production (H_2DCFDA test).

DISCUSSION

In order to increase CC stability in biological systems and effective incorporation of target molecules into their composition for the development of drug delivery vehicles we have earlier prepared and characterized hybrid CC with polysaccharides (FU, HE, CS, PE, DS) [3, 4].

For the intended use of CC as a specific transport system for biologically active molecules, biocompatibility plays a decisive role. Good evidence exists that CCDS, CCCS, CCHE, and CCPE do not exhibit cytotoxicity towards neutrophils,

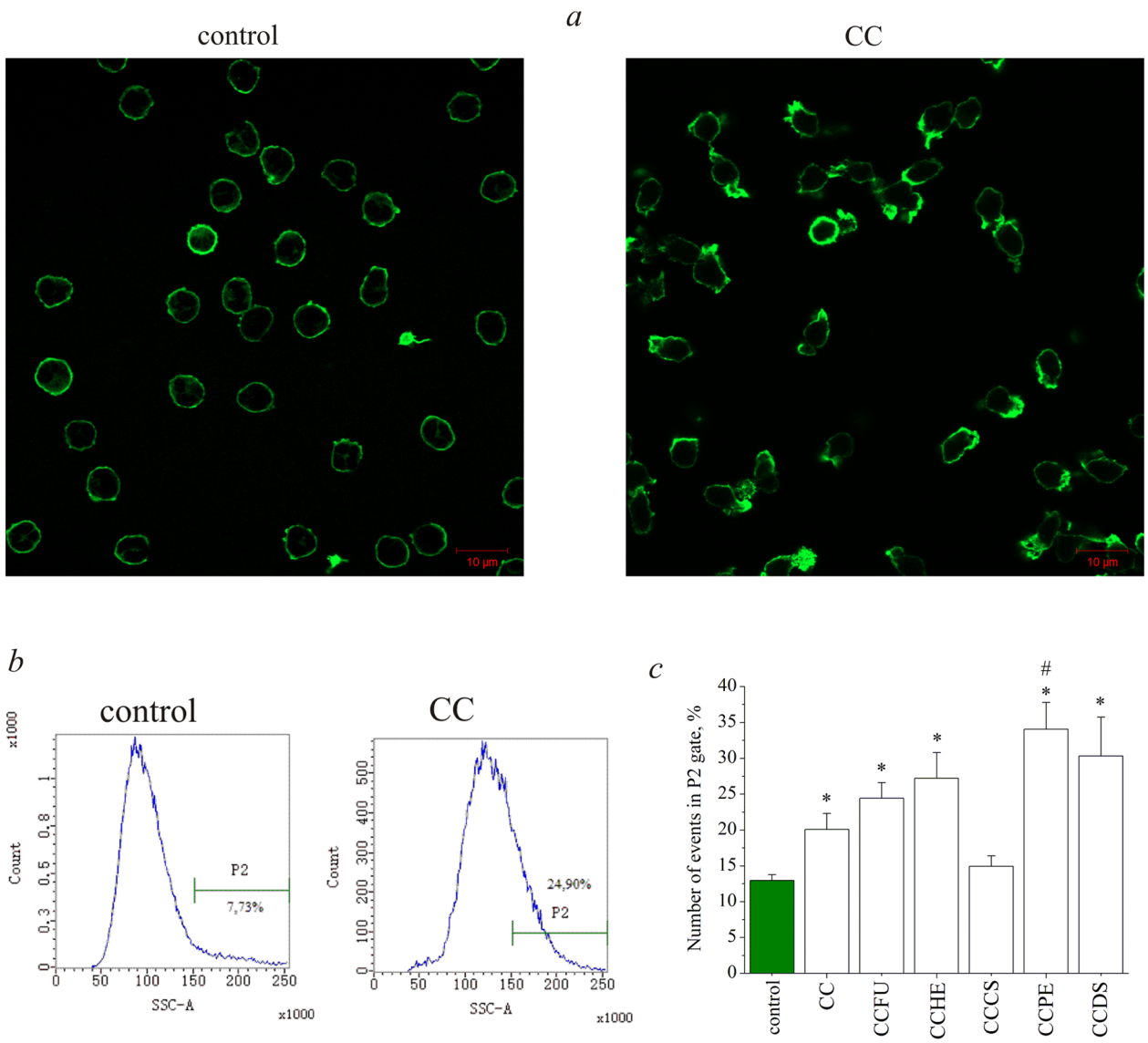

Figure 4. The effect of native and hybrid CC on neutrophil morphological characteristics: **a** – actin cytoskeleton organization in control cells and in neutrophils incubated with CC (0.25 mg/ml); **b** – distribution of control cells and neutrophils incubated with CC (0.25 mg/ml) by SSC value; **c** – number of neutrophils in gate P2 in the control and after incubation with native or hybrid CC (0.25 mg/ml). *$p<0.05$ compared to control. #$p<0.05$ compared to the effect of native CC. The scale bar in panel **a** is 10 μm.

which are a key link in innate immunity (Fig. 1a,b). Among the tested hybrid CC, only CCFU had a slight cytotoxic effect (Fig. 1). FU itself did not disrupt the intactness of the neutrophil membrane (Fig. 1a). This is consistent with the data of [26], which showed that FU did not affect the viability of tumor and non-tumor cell lines.

The study of the effect of CC on the respiratory burst of neutrophils has shown that CC could activate neutrophil NADPH oxidase (H_2DCFDA and scopoletin tests). In the presence of DPI, an inhibitor of NADPH oxidase assembly, as well as in the presence of inhibitors of non-receptor tyrosine kinases of the Src family, PI3K and PLC inhibitors, ROS production by neutrophils decreased (Fig. 3a,c). ROS production by neutrophils treated by CC decreased in the presence of the T-type Ca^{2+} channel blocker $NiCl_2$. In addition, the scopoletin test revealed a decrease in ROS production by CC-treated neutrophils in the presence of methoxyverapamil, a blocker of L-type Ca^{2+} channels. An increase in $[Ca^{2+}]_i$ in neutrophils was detected using the fluorescence method employing fura-2AM. Previously it was shown, that another $CaCO_3$ isomorph (aragonite) increased $[Ca^{2+}]_i$ in cells [27].

The H_2DCFDA test showed that activation of NADPH oxidase in the membrane of endo/phagosomes in response to CC was also reduced in the presence of cyt b, an inhibitor of actin cytoskeleton reorganization (Fig. 3c). It is possible that CC larger than 1 μm can be endocytosed by neutrophils via macropinocytosis [23]. So, activation

of neutrophils treated with CC was also accompanied by actin polymerization with the formation of filopodia at one margin of the cell (Fig. 4a). This is considered as one of the stages of plasma membrane invagination and vesicle formation [28]. In addition, after incubation of neutrophils with CC, the number of cells with a higher SSC value (Fig. 4b,c) (i.e., with higher scattering properties) increased, thus indicating a change in neutrophil morphology due to activation of cytoskeleton reorganization processes and possible CC uptake by the cells [25]. However, additional studies are needed to confirm the CC penetration into neutrophils.

Similarly to native CC, hybrid CC also initiated ROS production by neutrophils. However, it should be noted that, in contrast to native CC, the effect of CCHE on H_2O_2 generation in the scopoletin test was higher, while the effect of CCPE was lower. The study of the effect of hybrid CC on ROS production inside cells (H_2DCFDA test), revealed that CCPE had the highest stimulating effect. As in the case of native CC, after addition of all the studied hybrid CC, with the exception of CCDS, an increase in the number of neutrophils characterized by higher SSC values was noted thus indicating a change in the morphological characteristics of the cells. The number of neutrophils characterized by higher SSC values was detected in the sample incubated with CCPE, which caused the most pronounced intracellular ROS production.

Differences in the effect of hybrid CC on neutrophil activation may be due to the contribution of different polysaccharides included in their composition. For example, all polysaccharides except PE caused a small but statistically significant increase in the oxidation rate of scopoletin. The production of ROS inside the cells was recorded using the H_2DCFDA test, its significant increase was noted for all polysaccharides. According to the literature data [29, 30], polysaccharides can bind to the so-called pattern-recognition receptors and trigger intracellular signaling cascades that mediate functional cellular responses. For example, FU possesses the immunomodulatory activity: delay in neutrophil apoptosis and induction of their production of proinflammatory cytokines [31, 32], enhancement of TNF-α-induced formation of NETs [33], priming of the respiratory burst of phagocytes [34], enhancement of the phagocytic activity of macrophages [35]. HE causes stimulation of ROS production by neutrophils [36] and monocytes [37]. Binding of HE to isolated neutrophils causes stimulation of MPO release [38]. Neutrophils isolated from blood stabilized with HE were characterized by increased $[Ca^{2+}]_i$ and enhanced activation in response to PMA [39]. PE also increased neutrophil migration and their phagocytic activity and also increased the formation of ROS in these cells [40, 41].

CONCLUSIONS

The inclusion of biopolymers in CC not only changes the physicochemical properties of CC [2–6], but also modulates neutrophil functions. Hybrid CC capable of enhancing ROS production in the cytosol of neutrophils (e.g., CCHE) can be used to develop delivery systems for antibacterial and antiviral drugs, which can increase their action due to additional activation of neutrophils. On the other hand, CCPE are of interest in the context of drug delivery to inflamed tissues without releasing ROS into the extracellular space.

FUNDING

The work was supported by a joint grant from the Russian Science Foundation (project No. 23-45-10026) and the Belarusian Republican Foundation for Basic Research (project No. B23RNF-093). Obtaining vaterite microparticles was carried out under partial support from theme No. 121041500039-8 (Lomonosov Moscow State University). The study of the effect of vaterite microparticles on neutrophils viability was supported by a grant from the Ministry of Education of the Republic of Belarus (agreement No. 002/2024-BSU).

COMPLIANCE WITH ETHICAL STANDARDS

All procedures performed in studies involving people comply with the ethical standards of the National Research Ethics Committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed voluntary consent was obtained from each of the participants included in the study. This study was approved by the decision of the Ethics Committee of the State Institution "Republican Scientific and Practical Center for Transfusiology and Medical Biotechnology" (decision No. 1 dated 10.04.2022, Minsk, Belarus).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Received: 25. 02. 2025.
Revised: 10. 04. 2025.
Accepted: 14. 04. 2025.

СРАВНИТЕЛЬНЫЙ АНАЛИЗ ВЛИЯНИЯ НА АКТИВНОСТЬ НЕЙТРОФИЛОВ ГИБРИДНЫХ МИКРОЧАСТИЦ ВАТЕРИТА С РАЗЛИЧНЫМИ ПОЛИСАХАРИДАМИ

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В последнее время для разработки новых систем доставки лекарств всё чаще используют носители на основе природных биоминералов. В данной работе были исследованы эффекты нативных (CC) и гибридных микрочастиц ватерита с включением декстрансульфата (CCDS), хондроитинсульфата (CCCS), гепарина (CCHE), фукоидана (CCFU) и пектина (CCPE) на жизнеспособность и функциональную активность нейтрофилов. Среди протестированных препаратов только CCFU оказывали небольшое цитотоксическое действие. Сами по себе CC стимулируют реорганизацию актинового цитоскелета, а также продукцию активных форм кислорода (АФК) клетками, которая снижалась в присутствии хлорида дифенилениодония (DPI), ингибитора сборки NADPH-оксидазы. CC-индуцированная активация NADPH-оксидазы снижалась в присутствии ингибиторов нерецепторных тирозинкиназ семейства Src, фосфатидилинозитол-3-киназы (PI3K) и фосфолипазы C (PLC). Подобно нативным CC, гибридные микрочастицы ватерита также инициировали продукцию АФК нейтрофилами. После добавления CC и гибридных микрочастиц ватерита (за исключением CCDS) было зарегистрировано увеличение количества нейтрофилов, характеризующихся бóльшими значениями величины бокового светорассеяния, что свидетельствует об изменении морфологических характеристик клеток. Учитывая способность гибридных микрочастиц ватерита с полисахаридами активировать NADPH-оксидазу нейтрофилов, они представляются перспективными системами для доставки антибактериальных и противовирусных препаратов.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: микрочастицы ватерита; полисахариды; нейтрофилы; цитотоксичность; NADPH-оксидаза

Финансирование. Работа выполнена при поддержке совместного гранта Российского научного фонда (проект № 23-45-10026) и Белорусского республиканского фонда фундаментальных исследований (проект № Б23РНФ-093). Получение микрочастиц ватерита проведено при частичной поддержке темы 121041500039-8 МГУ имени М.В. Ломоносова. Исследование влияния микрочастиц ватерита на жизнеспособность нейтрофилов было поддержано грантом Министерства образования Республики Беларусь (договор № 002/2024-БГУ).

Поступила в редакцию: 25.02.2025; после доработки: 10.04.2025; принята к печати: 14.04.2025.