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Modification of human monocytes and macrophages by magnetic nanoparticles in vitro for cell-based delivery

Perekucha N.A.[1], Smolina P.A.[1], Demin A.M.[2], Krasnov V.P.[2], Pershina A.G.[1, 3]

[1] *Siberian State Medical University*
2, Moscow Trakt, Tomsk, 634050, Russian Federation

[2] *Postovsky Institute of Organic Synthesis, Ural Branch of Russian Academy of Sciences*
22, S. Kovalevskoy Str., Yekaterinburg, 620990, Russian Federation

[3] *National Research Tomsk Polytechnic University*
30, Lenina Av., Tomsk, 634050, Russian Federation

ABSTRACT

The aim of the study was to develop a method for the modification of human monocytes/macrophages by iron oxide magnetic nanoparticles *in vitro*.

Materials and methods. Iron oxide magnetic nanoparticles were obtained by a co-precipitation method and coated with a thin SiO_2 layer and polyethylene glycol 3000. Murine macrophage-like cell line RAW 264.7, primary human monocytes and macrophages were incubated with magnetic nanoparticles for 1–24 hours. The efficiency of cellular uptake of nanoparticles was measured using a ferrozine-based method and microcopy with Perls' Prussian blue staining. The cell viability was tested by fluorescent flow cytometry using SYTOX Green.

Results. Incubation of RAW264.7 cell, human monocytes and macrophages with magnetic nanoparticles at a concentration > 5 μg/mL on a rotator for 1 hour at 37 °C provides the loading of nanoparticles into > 99% of cells. The magnetic nanoparticles have no adverse effect on the cell viability. The RAW264.7 cells modified with nanoparticles showed no change in migration activity. The efficiency of the nanoparticle uptake by macrophages was >50 pkg (Fe)/cell.

Conclusion. According to the proposed method, macrophages loaded with magnetic nanoparticles have proved viable, they retain the ability to migrate, and therefore can be used as cell-based delivery systems for tumor diagnostic and therapy.

Key words: magnetic nanoparticles, monocytes, macrophages, cell-based delivery system.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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✉ *Pershina Alexandra G.,* e-mail: allysyz@mail.ru.

Модификация макрофагов и моноцитов человека магнитными наночастицами *in vitro* для доставки, опосредованной клетками

Перекуча Н.А.[1], Смолина П.А.[1], Дёмин А.М.[2], Краснов В.П.[2], Першина А.Г.[1, 3]

[1] *Сибирский государственный медицинский университет (СибГМУ)*
Россия, 634050, г. Томск, Московский тракт, 2

[2] *Институт органического синтеза (ИОС) им. И.Я. Постовского Уральского отделения Российской академии наук (УрО РАН)*
Россия, 620990, г. Екатеринбург, ул. С. Ковалевской, 22

[3] *Национальный исследовательский Томский политехнический университет (НИ ТПУ)*
Россия, 634050, г. Томск, пр. Ленина, 30

РЕЗЮМЕ

Цель исследования – разработать протокол модификации макрофагов и моноцитов человека магнитными наночастицами оксида железа (Fe_3O_4) *in vitro.*

Материалы и методы. Магнитные наночастицы оксида железа получены методом со-осаждения, покрыты силоксановой оболочкой и полиэтиленгликолем 3000. Макрофаги мыши линии RAW 264.7, моноциты периферической крови и макрофаги человека инкубировали с магнитными наночастицами в течение 1–24 ч. Эффективность захвата наночастиц клетками оценивали феррозиновым методом и методом микроскопии с окрашиванием на железо по Перлсу. Исследование жизнеспособности клеток выполняли методом проточной цитофлуориметрии с использованием красителя SYTOX Green.

Результаты. Инкубация макрофагов с магнитными наночастицами в концентрации >5 мкг/мл в течение 1 ч на ротаторе при 37 °С обеспечивает загрузку наночастиц в >99% клеток. Исследуемые магнитные наночастицы не оказывают негативных эффектов на жизнеспособность клеток. Клетки линии RAW 264.7, поглотившие наночастицы, сохраняют миграционную активность. Эффективность загрузки макрофагов магнитными наночастицами составляет >50 пкг (Fe)/клетку.

Заключение. Макрофаги, загруженные магнитными наночастицами согласно предложенному протоколу, являются жизнеспособными, сохраняют способность к миграции и перспективны в качестве систем доставки, опосредованной клетками, для диагностики и терапии опухоли.

Ключевые слова: магнитные наночастицы, моноциты, макрофаги, системы доставки на основе клеток.

Конфликт интересов. Авторы гарантируют отсутствие потенциальных и явных конфликтов интересов, связанных с публикацией данной статьи.

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INTRODUCTION

Magnetic nanoparticles (MNPs) are being actively studied to develop alternative approaches to therapy [1] and diagnostics of malignant neoplasms [2, 3], including controlled drug delivery [4, 5] and monitoring the effectiveness of tumor chemotherapy [6].

However, the problem of efficient delivery of MNPs to the tumor still remains unresolved [7]. One of the promising approaches to solving this problem is using autologous leukocytes as biocontainers for the delivery of nanoparticles [8, 9]. Leukocytes are capable of active migration, as they can pass through the

endothelium of capillaries and penetrate into the intercellular space, which makes them ideal "couriers" [10], allowing them to increase the delivery efficiency by more than 100 times [11].

Among leukocytes, monocytes and macrophages are the most attractive populations for the development of a nanoparticle delivery system within cells. An important feature of monocytes and macrophages, in contrast to neutrophils, is the ability to maintain their viability for a long time after phagocytosis of particles. The pronounced phagocytic capacity of monocytes and macrophages favorably distinguishes them from lymphocytes; to efficiently load the latter with nanoparticles, special methods are needed [12].

The ability of monocytes and macrophages due to chemotaxis [13] to penetrate into hypoxic zones of tumor promises to solve the problem of impact on cancer cells in these areas, which are practically inaccessible for penetration of conventional pharmacological drugs [14].

The first step in implementing cell-based delivery systems is creating complexes of immune cells with nanoparticles. There exist several approaches [15, 16]; the main method is *in vitro* assembly, when nanoparticles either attach to the surface of the cell membrane or are internalized in cells [17, 18]. An alternative approach is using nanoparticles coated with isolated cell membranes [19].

Optimization of the parameters of cell modification with nanoparticles is important in the development of delivery systems. The modification should not lead to significant changes in the cellular immune response, the rate of differentiation, the migration ability of cells, or a decrease in viability [20].

The aim of this work was to develop a method for loading iron oxide (Fe_3O_4) MNPs into human macrophages/monocytes *in vitro*.

MATERIALS AND METHODS

Iron oxide (Fe_3O_4) MNPs with an average diameter of 10 nm were obtained by a method of co-precipitation from aqueous solutions of Fe^{2+} and Fe^{3+} salts, coated with SiO_2 and covalently modified with O-[N-(6-Maleimidohexanoyl)aminoethyl]-O′-[3-(N-succinimidyloxy)-3-oxopropyl]polyethylene glycol 3,000 (PEG). To obtain fluorescent labeled particles, Cyanine5 NHS ester (Lumiprobe, Cy5, Russia) was used. The received nanoparticles (MNP-PEG) formed stable colloidal suspensions in aqueous buffers with an average hydrodynamic diameter of 190 nm (PdI < 0.06).

RAW264.7 cells were cultured in DMEM/F12 complete medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), L-glutamine (Glutamax, Gibco) and gentamicin at 5% CO_2, 37 °C. A scraper was used to remove adhered cells.

To isolate monocytes, peripheral blood from healthy donors was collected from the cubital vein into a vacutainer with heparin (Green Vac-Tube with Li-heparin, Green Cross) in a volume of 30 ml. Isolation of mononuclear cells from the peripheral blood of the healthy donors was carried out by centrifugation on a Ficoll 1.077 density gradient (PanEco, Russia). Isolation of monocytes from the mononuclear fraction was carried out on magnetic columns (Miltenyi Biotec) using magnetic particles with antibodies to CD14 (Miltenyi Biotec Inc., Germany). Monocytes were cultured in RPMI 1640 complete medium (PanEco) mixed with 1% L-glutamine (Glutamax, Gibco), 10% FBS, and 1% penicillin-streptomycin (PanEco). The purity of the human monocyte population was assessed using PE Mouse Anti-Human CD14 (Clone M5E2) antibodies (BD Biosciences, USA). The purity of the monocyte population was at least 97%.

To obtain macrophages, monocytes were incubated in RPMI complete medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma, USA) to a concentration of 100 ng/ml for 7 days, with a medium change every 3 days. Maturation was assessed using Pacific Blue anti-human CD11b Antibody (Clone ICRF44) (Biolegend, USA) and PE anti-human CD68 Antibody (Biolegend). The purity of the macrophage population was at least 91%.

To study the uptake of nanoparticles, 10^5 cells of the RAW264.7 line in 450 μl of DMEM/F12 complete medium were introduced into 2 ml tubes or seeded in the wells of a 24-well plate. Then, 1/10 of the MNP-PEG suspension (50 μl) in phosphate-buffered saline (PBS) was added to the cells. An equivalent volume of 1 × PBS was added to control cell samples. The cells in tubes were incubated at 37 °C for 1 or 2 hours on a rotator, cells in 24-well plates were incubated for 1 hour in a CO_2 incubator.

After the incubation with MNP-PEG on a rotator, the cells were centrifuged for 7 minutes at 180 × *g*, resuspended in 1 × PBS to wash away nanoparticles, centrifuged, and the pellet was resuspended in 200 μl of 1 × PBS. After the incubation with MNP-PEG, the adherent cells were washed twice with 1 × PBS, removed with a scraper, and transferred into

2 ml tubes. The cells were centrifuged for 7 minutes at 180 *g*, the pellet was resuspended in 200 µl of 1 × PBS.

Viability was assessed on a BD Accuri C6 flow cytometer (BD Biosciences) using SYTOX Green (Thermo Fisher Scientific). For confocal microscopy, cell nuclei were stained with Hoechst 33342 in PBS (5 µg/ml). The study was carried out on a Carl Zeiss LSM 710 confocal microscope. A Prussian Blue Iron Stain Kit (Polysciences, Inc., USA) was used to detect iron in the cells. The cells were analyzed using light microscopy (Leica DMi8). The iron content in cells was determined by the ferrozine method as described in [21]. The rate of cell migration was assessed according to the proposed method [22].

Statistical analysis of the data was performed using the GraphPad Prism 7 software package. The Shapiro – Wilk test was used to control the normal distribution of the data. Data with normal distribution were presented as mean and standard deviation ($M \pm SD$). The statistical significance of differences was determined using the Student's *t*-test. For multiple comparisons, one-way ANOVA with post-hoc Dunnett's test was used. The significance level was defined as $p < 0.05$.

RESULTS

To select optimal conditions for loading cells with nanoparticles, two incubation modes were tested on RAW 264.7 cells: with cells adhered to a culture plate and in a suspension with continuous mixing on the rotator. During the incubation of RAW 264.7 and MNP-PEG in a concentration range of 0.625–5 µg/ml, it was found that MNP-PEG were more efficiently absorbed by the cells in the suspension (when incubated on the rotator) than by adherent cells. Particularly, at a concentration of MNP-PEG of 5 µg/ml after 1 hour of incubation on the plate, the proportion of Cy5-positive cells was 38.0 ± 2.5%; while during the incubation on the rotator, the percentage of the cells that were loaded with nanoparticles was 99.7 ± 0.1%. With a 2-hour increase in the incubation period on the rotator, the number of the cells that were loaded with nanoparticles grew to 99.9 ± 0.04% (Fig. 1,*a*).

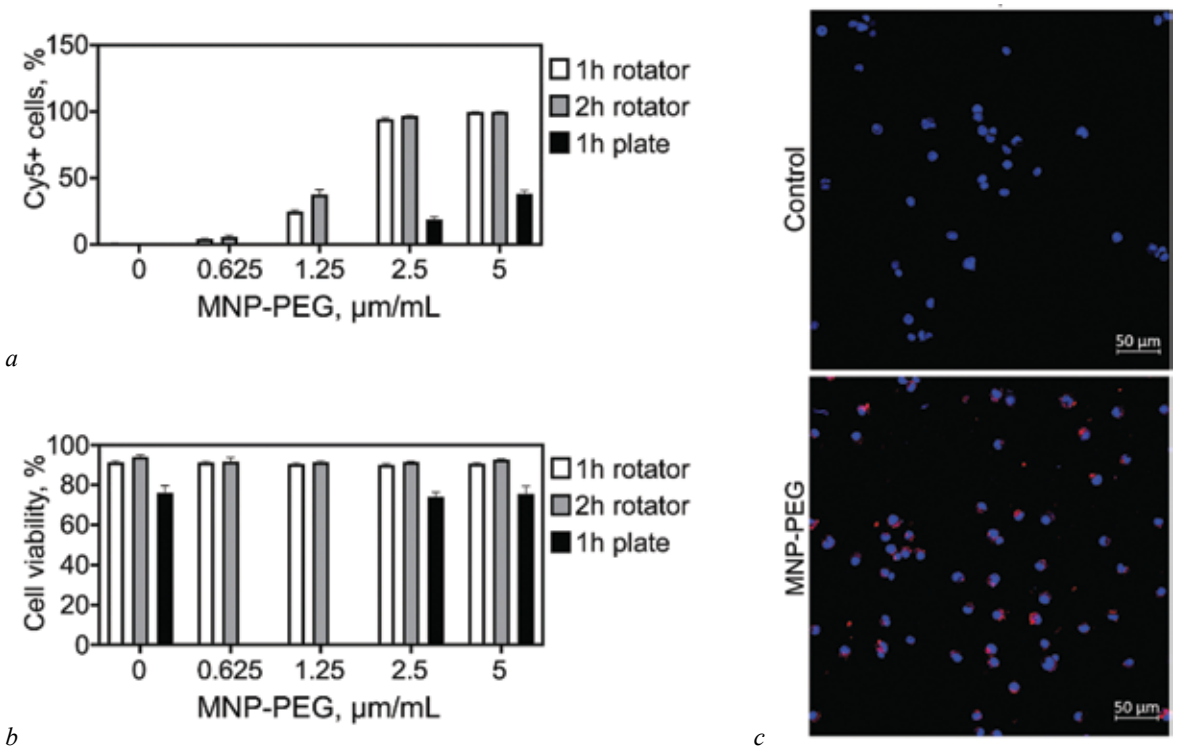


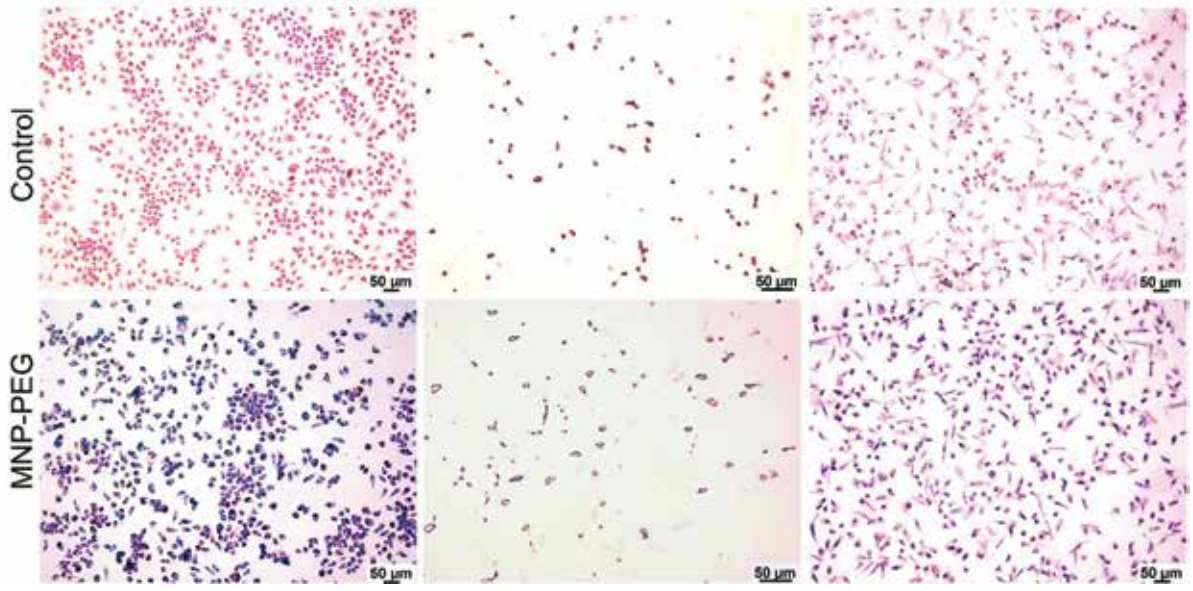
Fig. 1. Uptake of MNP-PEG by RAW264.7 cells: *a* – efficiency of MNP-PEG uptake by RAW264.7 cells under various incubation conditions; *b* – viability of RAW264.7 cells under various incubation conditions as determined by fluorescence flow cytometry with SYTOX Green; *c* – image of RAW264.7 cells incubated without nanoparticles (control) and after incubation with MNP-PEG for 1 hour on the rotator, obtained by confocal microscopy (Carl Zeiss LSM 710). Cell nuclei are colored blue (Hoechst 33342), nanoparticles are colored red (Cyanine5). Scale bar: 50 µm

After the incubation of cells with MNP-PEG on the rotator, more than 90% of RAW264.7 cells retained their viability. In particular, after 1 hour of incubation with MNP-PEG at a concentration of 5 μg/ml on the rotator, the percentage of living cells was (90.7 ± 0.3)%, which did not differ from the values obtained for controls (91.4 ± 0.8)% (Fig. 1,*b*). After the incubation of adhered cells on a plate with the addition of MNP-PEG, the proportion of dead cells was 24.5 ± 4.0% and did not differ from the value in controls (24.0 ± 3.7)%. This outcome is probably due to the negative effect of using a scraper to remove adhered cells from the plastic surface.

The efficient uptake of MNP-PEG by RAW 264.7 cells was confirmed by confocal microscopy data. In particular, Cy5-positive inclusions were observed in the cytoplasm of RAW 264.7 cells after their incubation with MNP-PEG (Figure 1*b*).

The selected incubation mode, 1 hour on the rotator at 37 °C, was further used for quantitative assessment of the efficiency of the "loading" of RAW 264.7 cells. As many as 10^6 cells were incubated with MNP-PEG at a high concentration (50 μg/ml). Then the cells were washed and resuspended in 1 × MACS buffer. After sorting on the magnetic columns, the number of cells in the washing solutions and cell suspension eluted from the column after it was removed from the magnetic separator was counted. The efficiency of magnetic sorting for RAW 264.7 cells was 77%. "Magnetically positive" cells were transferred into the wells of a 6-well plate and incubated for 2 hours in a CO_2 incubator for cell adhesion; then they were additionally washed from nanoparticles twice with 1 × PBS. Then the cells were removed from the plastic surface to determine the iron content in them. According to the ferrozine test, the efficiency of the uptake of nanoparticles by RAW264.7 macrophages was (58 ± 14) pkg/cell. According to flow cytometry data with SYTOX Green, after the modification of the cells with MNP-PEG nanoparticles, the proportion of dead cells was (9.9 + 3.6)%, which did not differ from the values obtained in controls (11.8 + 4.6)%.

When examining the "magnetically positive" RAW264.7 cells with light microscopy, a large number of Prussian blue positive granules were observed in the cytoplasm (Fig. 2).



a *b* *c*

Fig. 2. Image of RAW264.7 cells (*a*), human peripheral blood monocytes (*b*) and (*c*) human monocyte-derived macrophages incubated without nanoparticles (control) and after loading with MNP-PEG and magnetic sorting. Prussian blue staining. Scale bar: 50 μm

The migration rate of RAW264.7 cells "loaded" with MNP-PEG was 12.6 μm/h (Fig. 3) and practically did not differ from the cell migration rate in controls (14.8 μm/h).

In the follow-up series of experiments, we studied the uptake of MNP-PEG by human peripheral blood monocytes and by macrophages differentiated from peripheral blood monocytes. Incubation was carried out under the conditions selected for RAW264.7 cells: non-adherent cells in a suspension were incubated with MNP-PEG at a concentration of 50 μg/ml for 1 hour on the rotator at 37 °C. MNP-PEG were absorbed by both

human monocytes and macrophages. Light microscopic images of "magnetically positive" human macrophages adhered to the plate are shown in Fig. 2,*b*.

According to the data on the iron content in cells after their incubation with MNP-PEG, it was found that the efficiency of the uptake of nanoparticles by monocytes was (46 ± 6) pkg/cell, and by macrophages, (369 ± 96) pkg/cell. The efficiency of magnetic sorting of macrophages after their "loading" with MNP-PEG was 93%. Despite the 100% modification of the cell population with nanoparticles (according to flow cytometry data), the loading density of each individual cell might vary. As a result, some cells lack enough particles to effectively retain them on the magnetic column. It is important to note that the viability of the cells loaded with MNP-PEG did not differ from the values obtained in controls.

The research into the influence of MNP-PEG on the viability of human peripheral blood mononuclear cells and monocytes after 24-hour co-incubation showed that MNP-PEG had no negative effects on cell viability (Fig. 4).

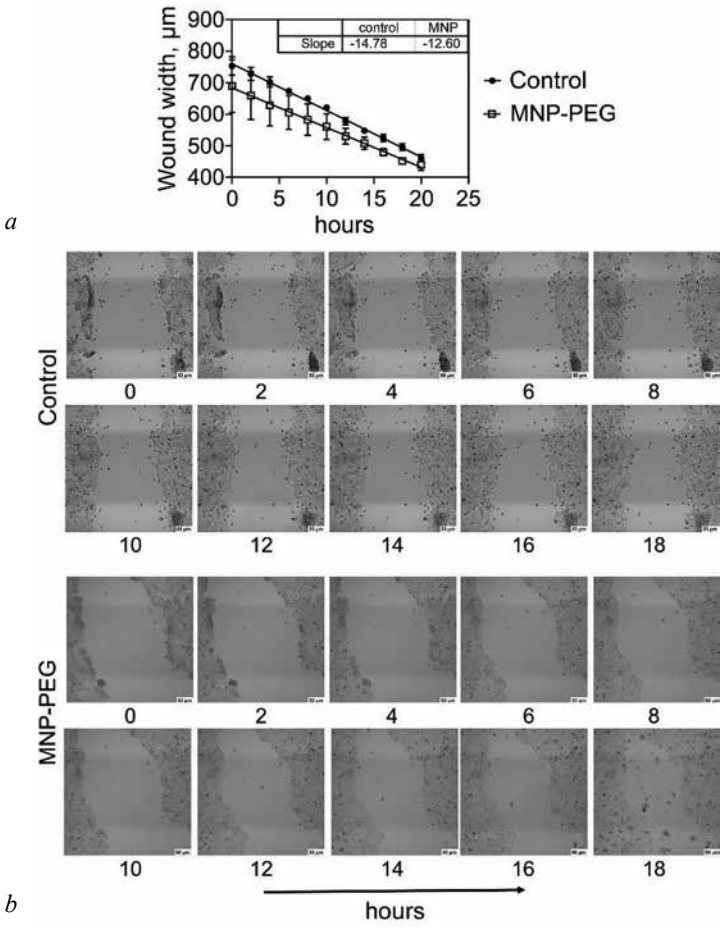


Fig. 3. Study of the migration activity of RAW264.7 cells: *a* – calculation of the migration rate of RAW264.7 cells in controls and cells loaded with MNP-PEG: *b* – images of RAW264.7 cells incubated without nanoparticles (control) and after loading with MNP-PEG and magnetic sorting, obtained within 18 hours after the formation of a "wound" on the adhesion layer

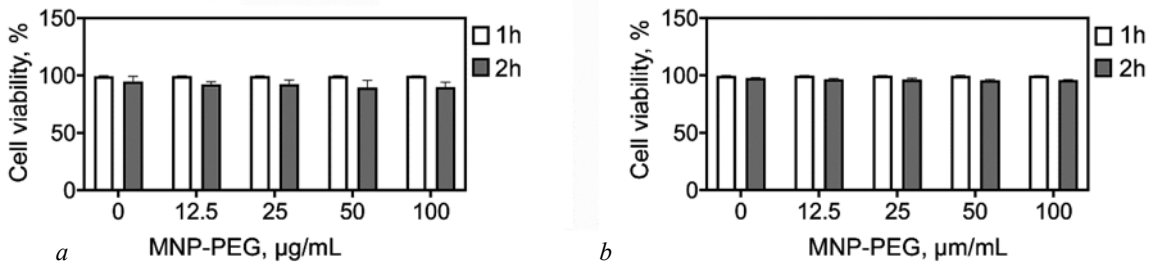


Fig. 4. Influence of MNP-PEG on the viability of human blood cells: viability of (*a*) mononuclear cells and (*b*) human peripheral blood monocytes after their incubation with MNP-PEG (12.5–100 mg/ml) for 1 and 24 hours according to fluorescence flow cytometry with SYTOX Green

DISCUSSION

Earlier experiments demonstrated successful creation of delivery systems based on monocyte-macrophage cell lines modified by nanoparticles [23], as well as on alveolar [24], peritoneal [25], and bone marrow macrophages [20]. However, research works on the uptake of nanoparticles by primary cultures of human monocyte/macrophage cells are very scarce.

The incubation period with MNPs required to achieve maximum accumulation varies considerably from 1 to 24 hours, according to the literature. It was shown that timing depends on the type of surface modification of nanoparticles [26]. According to our data, an increase in the incubation period up to 2 hours did not lead to a noticeable increase in the number of "loaded" cells; and therefore, to optimize the method, an incubation period of 1 hour was chosen.

This research work established that macrophages exhibited a more pronounced phagocytic activity towards nanoparticles, which is in line with the results of the comparative study on the uptake of nanoparticles by cells of the human monocyte-like cell line THP-1 and after their differentiation into macrophages dTHP-1 [27]: the efficiency was 6 and 50 pkg/cell for THP-1 and dTHP-1, respectively. Note that the uptake efficiency of MNP-PEG is much higher.

CONCLUSION

Iron oxide MNPs coated with SiO_2 and PEG are efficiently uptaken by RAW 264.7 mouse macrophage, human peripheral blood monocytes, and human monocyte-derived macrophages. The optimal loading conditions are incubation of non-adherent cells (in a suspension) with nanoparticles for 1 hour on the rotator at 37 °C. MNPs are absorbed by > 99% of cells in the suspension and have no cytotoxic effect on RAW 264.7 cells, human peripheral blood monocytes and macrophages. Therefore, macrophages loaded with MNPs, according to the proposed method, are viable, retain the ability to migrate, and can be used as systems for delivering magnetic nanoparticles to a tumor.

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Authors contribution

Perekucha N.A. – implementation of the experimental part of the study, interpretation and analysis of data, drafting of the manuscript. Smolina P.A. – implementation of the experimental part of the study. Demin A.M., Krasnov V.P. – synthesis of magnetic nanoparticles. Pershina A.G. – conception and design, interpretation and analysis of data, editing of the manuscript, critical revision of the manuscript for important intellectual content.

Authors information

Perekucha Natalya A., Junior Researcher, Center of Bioscience&Bioengineering, Central Research Laboratory, Siberian State Medical University, Tomsk, Russian Federation. ORCID 0000-0001-9310-3196.

Smolina Polina A., Student, Department of Biomedicine, Siberian State Medical University, Tomsk, Russian Federation. ORCID 0000-0001-9216-9572.

Demin Aleksandr M., Cand. Sci. (Chemistry), Senior Researcher, Laboratory of Asymmetric Synthesis, Postovsky Institute of Organic Synthesis, Yekaterinburg, Russian Federation. ORCID 0000-0002-4132-232.7

Krasnov Viktor P., Dr. Sci. (Chemistry), Professor, Head of the Laboratory of Asymmetric Synthesis, Postovsky Institute of Organic Synthesis, Yekaterinburg, Russian Federation. ORCID 0000-0003-1411-6218.

Pershina Aleksandra G., Cand. Sci. (Biology), Head of the Center of Bioscience&Bioengineering, Central Research Laboratory, Siberian State Medical University; Associate Professor, Research School of Chemical and Biomedical Engineering, National Research Tomsk Polytechnic University, Tomsk, Russian Federation. ORCID 0000-0001-7604-9722.

(✉) **Pershina Alexandra G.,** e-mail: allysyz@mail.ru.

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