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Carbon nanoparticles as transporters of melittin to glioma grade IV U87 cells in *in vitro* model

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Abstract: *Carbon nanoparticles as transporters of melittin to glioma cells in* in vitro *model.* Substances derived from nature have natural cytotoxic properties, melittin, the main component of bee venom is one of them. It has the ability to destroy any lipid bilayer, therefore to be used in a cancer treatment it needs to be targeted. The aim is to create the drug delivery system, which would efficiently deliver the active substance to glioma cells. Carbon nanoparticles are considered to be a good agent in biomedical applications, due to their biocompatibility and small sizes. In this study five types of nanoparticles were used: pristine graphene (GN), nanographene oxide (nGO), graphite (G), nanodiamond (UDD) and hierarchical nanoporous carbons (HNCs) to target the melittin to cancer cells. The visualization of the drug delivery complexes of melittin and nanoparticles was done with transmission electron microscopy, the influence of the complexes on cell morphology and structure was pictured with scanning electron microscope. Moreover, in order to check the viability of the cells treated with melittin and the complexes of melittin and nanoparticles the PrestoBlue™ assay was done, also to specify the way of the cell death the annexin V/PI assay was carried out. The results indicate that various nanoparticles behave differently in a complex with melittin. The UDD, GN and nGO nanoparticles resulted in higher mortality than the melittin itself. Creating and applying such complexes of melittin with nanoparticles in glioma cancer treatment may be a promising solution in the therapy.

Key words: brain tumor, nanoparticles, melittin, drug delivery system

INTRODUCTION

The use of nanoparticles in cancer treatment has been already widely investigated (Yezhelyev et al. 2006, Peer et al. 2007). There are several different approaches of applying them in *in vitro* experiments. Carbon nanoparticles, such as graphene, nanotubes or nanodiamond, can be applied as a drug itself. Diamond nanoparticles are highly biocompatible and already tested to be effective in inhibition the brain tumor angiogenesis (Grodzik et al. 2011). Nanotubes can be used as both, in detection of cancerous cells and as a drug delivery of small therapeutic molecules to these cells (Ji et al. 2010). Recently, single-walled carbon nanotubes were used in three--dimensional (3D) localization of cancer (Lin et al. 2016). Although the toxicity of graphene nanoparticles is not entirely established, there are some tests already carried out showing that the genotoxicity and cytotoxicity of graphene sheets depends on their concentration and size. The smaller size and the higher

concentration of graphene sheets there is, the higher mortality of the cells occur (Akhavan et al. 2012). Moreover, the influence of reduced graphene oxide was tested on glioma giving the increase in apoptotic cell death (Jaworski et al. 2015). The current research focuses on the most effective treatment, which is targeted drug delivery. Nanoparticles and nanomaterials with the size smaller than 100 nm have high reactivity and can react with other substances practically without complementary energy. A share of surface atoms in nanoparticles is considerably greater than in bulk material and increases with reduction of particle size. Chemical bonds of nanoparticles surface atoms are not compensated and it results in appearance of new electrical, chemical, mechanical, toxic and other properties. That is why, nanoparticles can be easily functionalized with other substances and they seem to be perfect for carrying drugs into the cancer cells (Haley 2008, Blanco et al. 2015). Furthermore, many different substances derived from nature has been investigated due to their toxicity towards cancer cells, e.g. curcumin inhibits pancreatic cancer cells growth (Su et al. 2016). Melittin, the major component of bee venom, containing 25 amino acids, is considered to have lytic properties after spontaneous integration into lipid bilayers (Terwilligert et al. 1982). This small protein has already been tested as anticancer drug, on ovarian cancer cells and in cancer immunotherapy (Jo et al. 2012, Liu et al. 2016), giving the promising results. The aim of the work was to determine the effect of five different carbon nanoparticles as nanocarriers of melittin to glioma cells. The TEM was used to visualize the melittin-nanoparticle complexes and SEM to see the morphology and structure of the exposed cells, as well as the *in vitro* experiments were designed to test the viability and the way of cell death. The results of this study may advance the future application of carbon nanoparticles combined with active substance in cancer treatment.

MATERIAL AND METHODS

Preparation and characterization of nanoparticles-melittin complex

Pure melittin peptide was obtained from Sigma Aldrich (Munich, Germany) in a powder form. Then it was dissolved in 1 ml of Milli-Q water. There were prepared five different complexes from the melittin stock solution of 20 μg/ml and the solution of five different nanoparticles: pristine graphene – GN, nanographene oxide – nGO, graphite – G, nanodiamond – UDD (SkySpring Nanomaterials, Hudson, USA) and hierarchical nanoporous carbons- HNCs (Faculty of Advanced Technologies and Chemistry, Military University of Technologies). Melittin was added to each type of nanoparticle in order to obtain five different complexes in concentration of 20 μg/ml. Then, the complexes were incubated in 37°C and vortexed for 15 min. Previous studies have showed that the concentration of 20 μg/ml of nanoparticles was not toxic to glioma cells, therefore to check the effect of melittin itself the same concentration was used (Jaworski et al. 2015). Melittin, five different nanoparticles and complexes of nanoparticles and melittin were investigated by the transmission electron microscope (TEM) JEM-1220 (JEOL, Tokyo, Japan) at 80 KeV, with

a Morada eleven-megapixel camera (Olympus Soft Imaging Solutions, Münster, Germany). Samples for the TEM were prepared by placing droplets of hydrocolloids on to Formvarcoated copper grids (Agar Scientific, Stansted, UK). Immediately after drying the droplets in the room temperature, the grids were inserted into the TEM.

Cell culture

Human glioma U87 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cell line was cultured in Dulbecco's Modified Eagle's culture Medium containing 10% fetal bovine serum (Life Technologies, Houston, TX, USA) and 1% penicillin and streptomycin (Life Technologies) at 37°C in a humidified air atmosphere containing 5% carbon dioxide in a DH AutoFlow CO_2 air-jacketed incubator (NuAire, Plymouth, MN, USA).

Cell morphology

In order to check the morphology of cells after treatment with tested complexes the SEM examination was done. The U87 cells were seeded in six-well plates ($5 \times \times 10^5$ cells per well) and incubated for 24 h. Then the medium was removed, and the complexes of melittin with different nanoparticles as well as the melittin itself (20 μg/ml) were introduced to the medium. The next day, cells were washed in PBS (0.01 M, pH 7.2, Sigma), fixed in 2.5% glutaraldehyde (Sigma) for 1 h, washed twice in PBS, and placed on aluminum SEM stubs. The SEM stubs were kept in a moist atmosphere for 1 h, washed in PBS, post fixed in 1% osmium tetroxide (Sigma) for 1 h, rinsed in distilled water, and dehydrated with progressive alcohol solutions (30–50–70–90–95–99%). The preparations were further dehydrated with a critical point-dried (Polaron CPD 7501, Quorum Technologies, Newhaven, East Sussex, UK) and covered by a thin layer of gold (JEE-4C, JEOL Ltd., Tokyo, Japan). The samples were inspected by SEM at 1 KeV (FEI Quanta 200, FEI Co., Hillsboro, OR, USA).

Cell viability

Human glioma U87 cells were cultured in 96-well plates (5×10^3 cells per well) and incubated for 24 h. Then the medium was removed, and the complexes of melittin with five different nanoparticles as well as the melittin itself in concentration of 20 μg/ml were introduced to the medium. The blank tests, medium with nanoparticles, were also prepared. After 24 h, 10 μl of PrestoBlue™ reagent was added to each well and incubated for an additional 2 h at 37°C. The optical density of each well was recorded on ELISA reader (Infinite M200, Tecan, Durham, NC, USA). Cell viability was expressed as the percentage (ODtest-ODblank)/ /(ODcontrol-ODblank), where "ODtest" is the optical density of cells exposed to melittin complexes, "ODcontrol" is the optical density of the control sample, and "ODblank" is the optical density of wells without glioma cells. Test was performed in triplicates.

Mode of cell death

Type of cell death was evaluated with an annexin V/PI assay (Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit-Invitrogen, Carlsbad, CA, USA). After 24-hour incubation of U87 glioma cells in 75 ml flasks (1×10^6 cells per flask),

the medium was removed, and the complexes of melittin in concentration of 20 μg/ml and melittin in complexes with different nanoparticles were added at 20 μg/ml. After a further 24-hour incubation, the medium was removed and the cells were washed in ice-cold PBS and trypsinized. Harvested cells were suspended in 100 μl annexin-binding buffer (Invitrogen) and afterwards 5 μl of annexin V linked with Alexa Fluor 488 and 1 μl of PI were added (Invitrogen). Cells were analysed using FACStrak (Becton-Dickinson, Germany; software – SimulSet), measuring the fluorescence emission at 530 and 575 nm using excitation at 488 nm.

Statistical analysis

One-way variance analysis of viability was performed by Tukey's multiple range test. Differences were considered significant at $P \leq 0.05$. For the analysis the Statgraphics Centurion software (StatPoint Technologies, Warrenton, VA, USA) was used.

RESULTS AND DISCUSSION

Drug delivery systems have been lately widely investigated (Torchilin et al. 2010). The observation of connections in tested complexes and cytotoxicity of them is crucial in order to use those complexes in cancer treatment. Moreover, the components have to be easily functionalized. The nanoparticles are such multifunctional agents, which can interact with different type of cells (Singh et al. 2009). When it comes to carbon nanoparticles, they are more biocompatible compared with different materials (Liu et al. 2008, Zhu et al. 2012), accordingly they are broadly used in cancer therapies of cervical cancer cells and glioma cells (Kim et al. 2008, 2011, Grodzik et al. 2011). The nanocarriers with graphene oxide are used to deliver cancer drug, doxorubicin, directly to the nucleus of the cell (Zhou et al. 2014), as well as nanodiamonds-mediated doxorubicin is used to inhibit the lung metastasis of breast cancer (Xiao et al. 2013). In this work the carbon nanoparticles were tested as components of drug delivery system for melittin, the lytic agent, on glioma cells in *in vitro* cell culture. The self-organization of complexes (melittin with different nanoparticles) was checked with TEM. The morphology of cells after treatment was investigated with SEM. Moreover, the viability was tested with PrestoBlue™ reagent and the way of cell death with flow cytometry using an annexin V/PI assay.

The TEM pictures visualize the complexes, which were done through self-organization, connecting the melittin with different nanoparticles: UDD, nGO, GN, G and HNCs (Fig. 1). Self-organization of the melittin in complexes was different with different nanoparticle. In order to compare how nanoparticles look in the complexes and by themselves, the TEM pictures of only nanoparticles are shown on Figure 2. For UDD and nGO this small peptide behaved as a linker, but it also stayed on the outside of the complex what allows it to interact well with cancer cells (Fig. 1B, D). Whereas the HNCs and graphite cover the melittin from every side, what may result in inefficient treatment (Fig. 1E, F). Graphene, which is the one atom-thick layer of bonded carbon (Stankovich et al. 2006), in a complex with melittin resulted in very low viability of glioma cells, the peptide was evenly dispersed on graphene sheet (Fig. 1C).

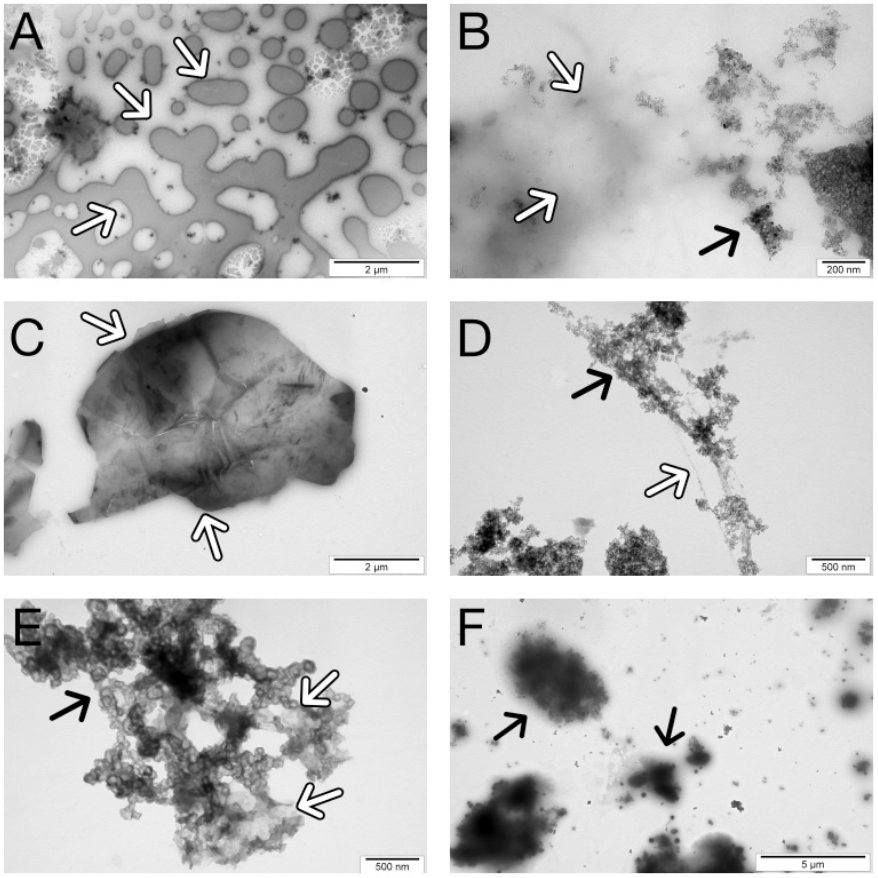

FIGURE 1. Complexes of melittin and nanoparticles. A – melittin, B – melittin with UDD, C – melittin with GN, D – melittin with nGO, E – melittin with HNCs, F – melittin with G (white arrow points the melittin and black arrow points the nanoparticles agglomerates)

In the pictures of glioma cells after treatment with melittin (Fig. 3B) and complexes of melittin with different nanoparticles – UDD, nGO and GN (Fig. 3C, D, E) there were seen the changes, especially in the protrusions of the cells, treated cells have them less and they are thinner. Whereas, cells in a control group are thick and have long protrusions (Fig. 3A). In reference to HNCs and graphite, the morphology of the cells remained unchanged, they looked as in the control group.

Different nanoparticles have diverse effects on *in vitro* cultured cells. What is more, their cytotoxicity depends on a given dose, their size, surface chemistry and also the type of the cells and excretion (Jia et al. 2005, Firme et al. 2010). There was a significant difference between control group and cells treated with melittin in a complex with nanodiamond, where almost the half of the cells died. It was already settled that nanodiamond is the most biocompatible among

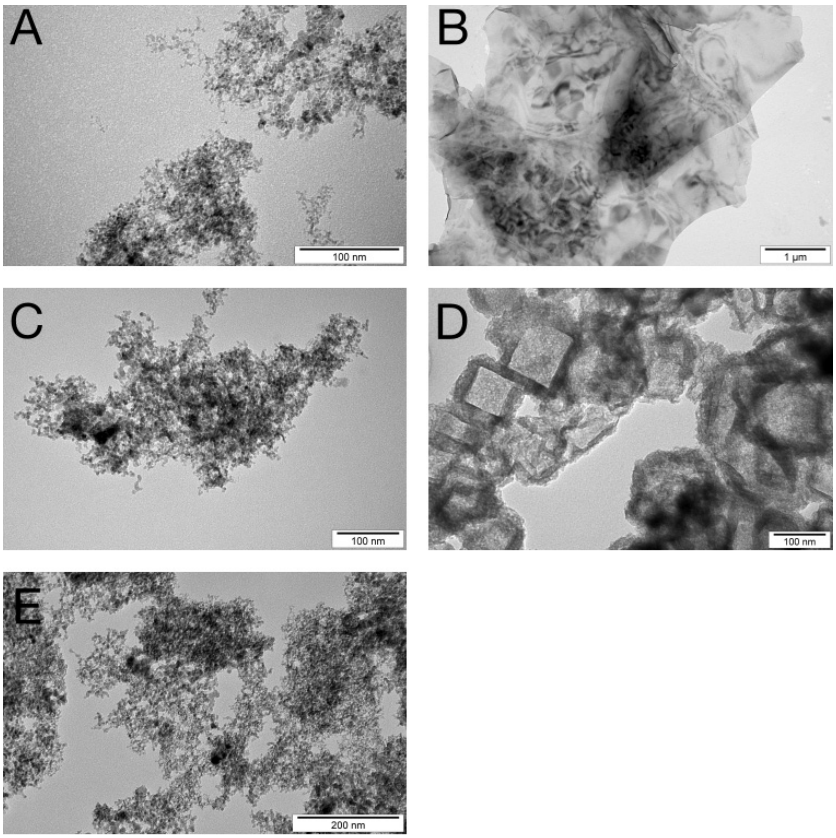

FIGURE 2. Carbon nanoparticles. A – UDD, B – GN, C – G, D – HNCs, E – nGO

other carbon nanoparticles (Zhu et al. 2012). Regarding graphene (GN) and nanographene oxide (nGO) the outcome was also promising, over 40% of dead cells occurred (Fig. 4). Melittin by itself had lower cytotoxicity than in a complex with mentioned above nanoparticles, which indicate their contribution to the more efficient uptake of the component by the cells. The influence of HNCs and graphite in complex with melittin resulted in high viability of glioma cells, what may be caused by their big tendency to agglomeration or their structure (Fig. 1E, F).

Apoptosis is a programmed cell death and it is a natural process, too little apoptosis may result in cancer, autoimmune or inflammatory diseases, the macrophages in apoptosis remove the cells' debris, so that the inflammation does not occur. Hence, the way the cells die is crucial in cancer treatment (Schwartzman et al. 1993). Thus the flow cytometry was used in order to analyze how many cells are apoptotic and how many are necrotic. For this assay only three nanoparticles were chosen (UDD, nGO and GN), which gave the best results in previous tests. The most cells,

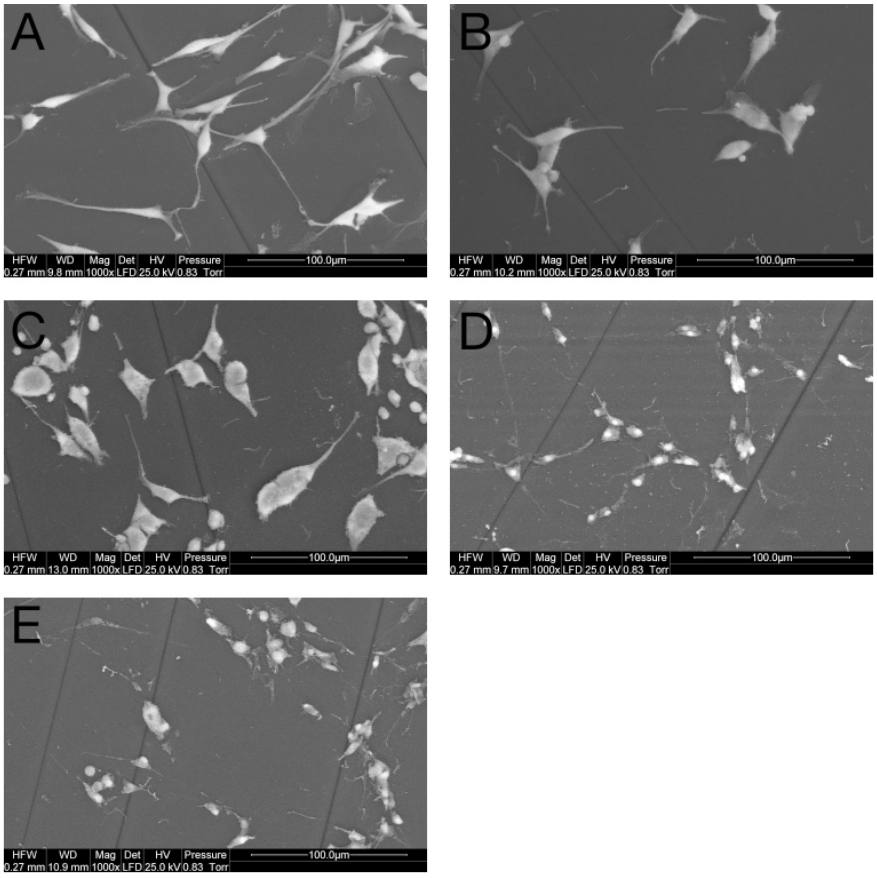

FIGURE 3. Cell morphology after treatment. A – control group, B – cells treated with melittin, C – cells treated with melittin with UDD, D – melittin with GN, E – melittin with nGO

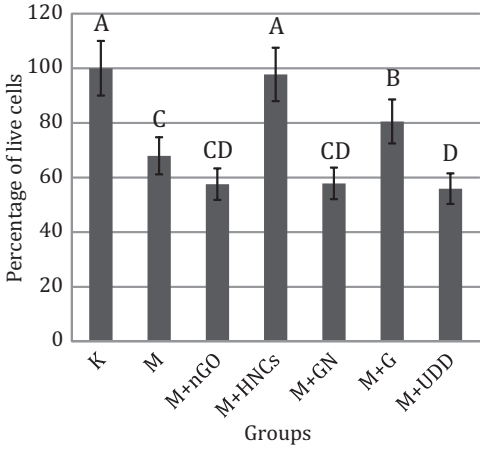

FIGURE 4. Effect of melittin and complexes of nanoparticles with melittin on viability of glioma cells. Columns marked with different letters (A–D) show significant differences between treated groups, they differ at $P = 0.0000$. C – control, M – melittin, M+nGO – melittin with nanographene oxide, M+HNCs – melittin with hierarchical nanoporous carbons, M+GN – melittin with pristine graphene, M+G – melittin with graphite, M+UDD – melittin with ultradispersed diamonds

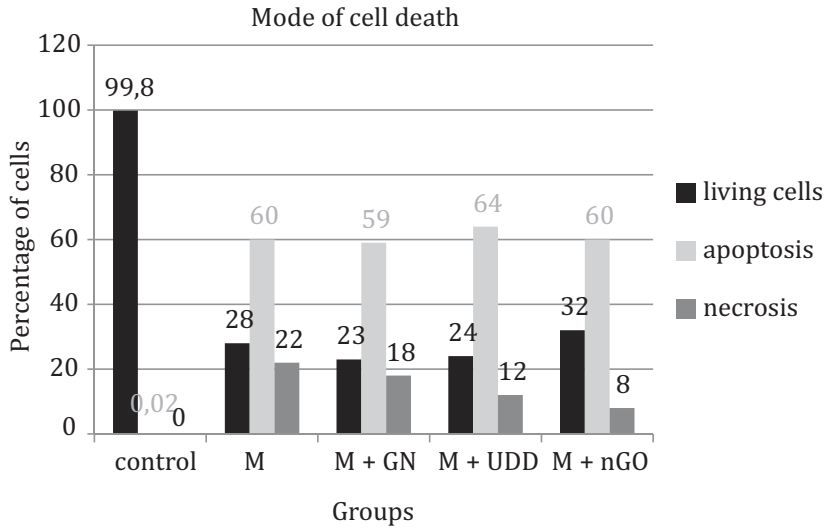

FIGURE 5. Comperation of cell deaths among different groups: C – control, M – melittin, M+GN – melittin with pristine graphene, M+UDD – melittin with nanodiamond, M+nGO – melittin with nanographene oxide

which undergo apoptosis, were in the complex of melittin with UDD, while there was low level of necrosis (12%), what may indicate that UDD could be useful nanocarrier for drug delivery of the substances. The melittin itself added to the cells resulted in more necrotic cells (Fig. 5). The complexes of melittin with GN and nGO gave similar results to UDD. Present results are preliminary and at this stage they indicate that carbon nanoparticles increase the efficiency of absorption of melittin by the cells and cell adhesion. Consequently, a dose of melittin in a drug delivery system may decrease.

CONCLUSION

Results of the work prove the effectiveness of carbon nanoparticles as nanocarriers, especially UDD, GN and nGO. They effectively transport the targeted melittin and help with adhesion of it to the glioma cells. Furthermore, they cause the apoptotic way of cell death. Such complexes can be used in future treatment of cancer, although further *in vivo* experiments are needed.

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Streszczenie: *Nanocząstki węglowe jako transportery melityny do komórek glejaka IV stopnia linii U87 w modelu* in vitro. Melityna jest jedną z naturalnie występujących substancji w przyrodzie, jest składnikiem jadu pszczelego. Jest cytotoksyczna i ma silne właściwości lityczne, które niszczą każdą błonę komórkową, co może mieć zastosowanie w zwalczaniu komórek nowotworowych. Aby można było ją wykorzystywać w leczeniu, wymagane jest zastosowanie dodatkowego składnika, który pokierowałby ją w odpowiednie miejsce. Celem jest stworzenie systemu kontrolowanego dostarczania leków, z wykorzystaniem nanocząstek węglowych, które mają małe rozmiary oraz są uważane za biokompatybilne. W badaniach użyto pięć rodzajów nanocząstek: grafenu, nanotlenku grafenu, nanodiamentu, grafitu oraz hierarchicznych nanoporowatych nanocząstek. Do wizualizacji powstałego kompleksu nanocząstek z melityną użyto elektronowego mikroskopu transmisyjnego, a do sprawdzenia wpływu melityny oraz jej kompleksu z nanocząstkami na morfologię oraz strukturę komórek użyto elektronowego mikroskopu skaningowego. W celu sprawdzenia żywotności komórek poddanych działaniu melityny oraz jej kompleksów z nanocząstkami wykonano test PrestoBlue™, a w celu specyfikacji drogi śmierci komórek test z jodkiem propidyny i aneksyną V. Wyniki wskazują, że różne rodzaje nanocząstek węglowych mogą w inny sposób oddziaływać z melityną. Kompleksy melityny z nanodiamentem, grafenem oraz nanotlenkiem grafenu spowodowały większą śmiertelność komórek niż sama melityna. Tworzenie oraz zastosowanie w praktyce kompleksów melityny z nanocząstkami węglowymi może skutkować efektywniejszym leczeniem glejaka.

Słowa kluczowe: nowotwory mózgu, nanocząstki, melityna, system kontrolowanego dostarczania leków.

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