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Analysis of a fusion between rat glioma cells and biomimetic liposomes with encapsulated diamond nanoparticles or curcumin

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Abstract: *Analysis of a fusion between rat glioma cells and biomimetic liposomes with encapsulated diamond nanoparticles or curcumin.* Liposomes are used as carriers for different bioactive agents, both hydrophilic, which are encapsulated in water core of the liposome, and hydrophobic, which are entrapped within liposome walls. The walls are built from phospholipid bilayer, therefore their structure resembles cell membrane. It was hypothesized that if the wall is made of set of lipids typical for a cell, the liposome will be eagerly consumed by the cell. We performed the experiments using C6 rat glioma cells as an example, since central nervous system cells are extremely rich in lipids, including the unique ones. Since all cancer cells have high proliferation potential, they need to absorb precursors to build cell membrane around new cells, therefore such biomimetic liposomes may be one of the most effective way to deliver anticancer agents into the cell. Analysis of physicochemical properties of obtained liposomes, as well as *in vitro* tests, showed that obtaining such liposomes is possible and that the liposomes are biocompatible, stable carrier both for hydrophilic and hydrophobic agents. Encapsulation of diamond nanoparticles did not affect the liposomes, whereas entrapping of curcumin, which is a spice known in traditional Asian medicine for its anticancer properties, significantly increased its activity. Obtained results showed that biomimetic liposomes can be effective, individually-tailored carriers for bioactive agents.

Key words: glioma, liposomes, diamond nanoparticles, curcumin

INTRODUCTION

Intensive proliferation is one of the most prominent feature of all cancer cells, what allows them to rapid growth and to invade within an organism (Szala 2009). This feature is inseparable from the changed metabolism, since cell division requires cell membrane synthesis for newly arising cells, as well as energy storage in a form of lipid droplets. Thus, the changes include enhanced biosynthesis of lipids and highly enhanced intake of their precursors – cholesterol and fatty acids within lipoproteins. The mechanisms is especially noticeable within tumors in central nervous system (CNS), where cells are extremely rich in lipids, including the unique ones. It is known that in neural and glial cells membranes gangliosides are abundant, for example sphingomyelin which builds myelin sheath (Kłyszejko-Stefanowicz 2002). The lipid composition differs between normal and cancer cells, where there is less cholesterol and ratio of less complex to more complex gangliosides is higher than in healthy tissue (Baenke et al. 2013), what is important during

designing active agents delivery systems based on lipids, such as liposomes. Liposomes are artificially created vesicles composed from phospholipid bilayer, resembling cell membrane, where both hydrophilic and hydrophobic molecules can be transported (Mozafari 2004). For that reason they have been employed for successful delivering encapsulated agents such as drugs (Abreu et al. 2011), vitamins (Mozafari et al. 2008), dyers or nucleic acids (Sikorski et al. 2002). Since liposomes can be created from naturally occurring phospholipids, isolated from tissues, they are biocompatible and non-toxic. Usually, the composition compromises from one or a few most common phospholipids (e.g. lecithin) and cholesterol in a defined ratio. Considering the uniqueness of CNS cells, it was hypothesized that liposomes made of a lipid set typical for the cells might be one of the most effective way to deliver an agent into the cell, showing affinity to tumor cells, as for they need rapid cell membrane building, they eagerly consume necessary precursors.

In order to evaluate biocompatibility of the created liposomes the experiments were performed on rat glioma cells *in vitro* using liposomes that contained only water ("empty liposomes"). Furthermore, their applicability as a carrier for hydrophilic compounds was investigated employing diamond nanoparticles (DN). Their biocompatibility and active surface creates possibilities to use them as a platform for different ingredients (Grodzik et al. 2013), however they have a tendency to aggregation within an organism (Strojny et al. 2015). Liposomes can be solution for this problem, ensuring effective DN dispersion.

For the evaluation of delivering efficacy of hydrophobic agents, water-insoluble compound, which is curcumin (Cur), was chosen. It is a major active ingredient isolated from *Curcuma longa* rhizome, popular in traditional Asian medicine. Various biological activities of Cur has been demonstrated, including anticancer properties (Fan et al. 2014), however its usage is still restricted due to poor bioavailability (Anand et al. 2007), therefore functional carriers which increase Cur performance are still in search (Yallapu et al. 2012).

MATERIAL AND METHODS

Rat glioma cell culture

Rat glioma C6 cell line was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco™, Thermo Scientific, USA) supplemented with 10% foetal bovine serum (Gibco™), penicillin (100 U/mL) and streptomycin (100 mg/mL) and were maintained at 37°C in a 5% CO_2 and humidified atmosphere.

Liposomes preparation

Lipid film preparation and Cur encapsulation

Following steps were performed in three replicates. After obtaining cell monolayer (approximately $10 \cdot 10^6$ cells per 75 cm^2 bottle) medium was removed and cells were washed twice with cold PBS, then 2 ml of fresh PBS were added to collect cells by scratching and they were transferred to a 2 ml tube. After centrifugation (5 min, 400 × *g*), supernatant was

removed, 1 ml of dissolvent was added (chloroform : methanol, 1 : 2, v/v) and the pellet was vortex for 20 min. Then, 200 µl of 0.9 % NaCl was added and the mixture was vortex for 5 min. before centrifugation (10 min, 500 × *g*). When two phases with protein interphase appeared, the lower phase containing total lipid extract was collected into glass round-bottom tube. To one of the tube 36.8 mg of Cur (LKT Laboratories, USA) was added and mixed well in order to encapsulate Cur within liposomes hydrophobic walls. All tubes were evaporated under N_2 to total dry (approximately 1 h) in order to obtain lipid film. To remove the rest of the solvent, the film was lyophilized and stored tightly capped in –20°C.

Hydratation of films

To obtain control liposomes containing only water ("empty liposomes") or Cur, 1 ml of sterile ultrapure water (60°C) was added to tubes containing either clean lipid film or Cur film, in 100 µl portions, mixing on vortex all the time (20 min). The same procedure was employed to encapsulate DN (Skyspring Nanomaterials Inc., USA); 1 ml of previously soniqated DN hydrocolloid (1,000 µg/ml) was use instead of water. Suspensions were transferred into 1 ml tubes to ultrasound bath for 10 min to reduce size and lamella number in vesicles. Liposomes were stabilized for 1 h (RT) and stored in 4°C.

Determination of actual Cur concentration

Standard curve was prepared by series of 10x dilutions of 0.1 M solution of Cur in methanol. To determine Cur concentration in liposomes, 10 µl of suspension was dissolved in 90 µl of methanol. Absorbance was measured at 500 nm by Infinite® 200 PRO microplate reader with i-control™ Software (Tecan Group Ltd., Germany).

Transmission electron microscopy (TEM)

A droplet of a liposome suspension was placed onto formvar-coated copper grids (Agar Scientific, UK) and 10 µl of 2% uranyl acetate was added for negative staining. After 2 min an excess was removed by filter paper. When totally dry, grids were inspected at 80 keV by TEM (JEOL, Japan).

Zeta potential and size distribution

The zeta potential and size distribution of liposome suspensions were determined after 120 s of stabilization at 25°C by the Dynamic Laser Scattering electrophoretic method with Smoluchowski approximation for zeta potential by Zetasizer Nano-ZS90 (Malvern, UK). The potential was also measured after 2 months of storage in 4°C to determine its stability in empty liposomes.

Fluorescent staining and fusion analysis

Stock of fluorescent dye [4-4--(didecyloamino)styryl] N-methylopyridinum iodide (DiA; Life Technologies, UK) was prepared by diluting in 99.99% ethanol (1 mg/ml). DiA shows affinity to phospholipid bilayer and fluorescence only in so bound form. Liposomes were stained by adding 1 µl of stock solution to 1 ml of suspension. To measure dynamics of fusion between liposomes and glioma cells, cells were seeded on 96-well white-bottom plate at a density

of $1 \cdot 10^4$ cells/ml. The next day, the medium was removed and replaced with fresh medium containing dilutions of Cur at concentration 25 μM, stained liposomes with respective Cur concentration and stained empty liposomes with respective lipid concentration. Mean fluorescence intensity (MFI) of cells was measured at 0, 15, 30, 90 and 240 min time points, washing cells with PBS before readings to avoid background signal (separate wells for each of the time point, 6 replicates). Reading were performed at Ex/Em = 456/590 nm.

Cell proliferation assay

Cells were seeded as previously described on 96-well transparent microplates (Nest Scientific, USA). The next day, the medium was removed and replaced with fresh medium containing dilutions of liposomes, DN and Cur (at concentrations 50–100 ppm for DN, 25–50 μM for Cur and equivalent volumes of lipids in empty liposomes). Cell proliferation inhibition was assessed after 72 h by XTT assay (Sigma Aldrich, USA), where transparent soluble tetrazolium salt is converted to purple formazan crystals. Spectrophotometer readings were performed at 450 nm. Results were expressed as the percentage of the control group, which was 100%, and analyzed by ANOVA with Tukey's post-test. Differences with $P < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Presented results demonstrated that obtaining biomimetic liposomes produced of lipids isolated from glioma cells is possible, what was confirmed by measurements of their physicochemical properties. TEM analysis showed typical images of liposomal vesicles, moreover, successful entrapping of DN in the water phase (the core of a liposome) was observed (Fig. 1). Size measurements also revealed that size of obtained liposomes was generally less than 1 μm and that all of the populations were not homogenic, what was expected due to lack of so-called calibration, the process which is needed for sizing liposomes. Most of the population for empty and Cur-entrapped liposomes has size around 100 nm, but for DN the size was rather around 1,000 nm (Fig. 2). However, this may result from free non-entrapped DN, which possibly may interference the measurements, since TEM analysis revealed that DN liposomes have similar size to the empty ones. Another parameter, zeta potential, was highly negative, about 40 mV, what clearly indicates on stable suspensions of liposomes without tendency to aggregation, even though DN have slightly negative impact, the potential still remained 14 mV. What is important, the potential of empty liposomes, measured after 2 months of storage in 4°C, remained ap-

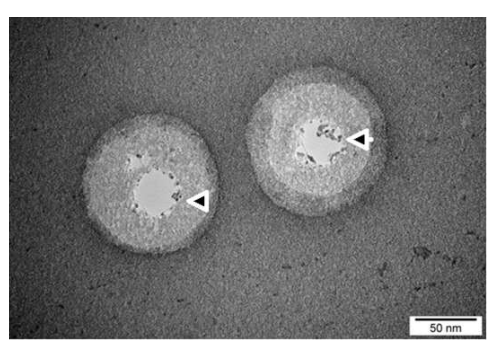

FIGURE 1. TEM photography of example liposomes. DN visible inside of the vesicles (arrows). Scale bar 50 nm

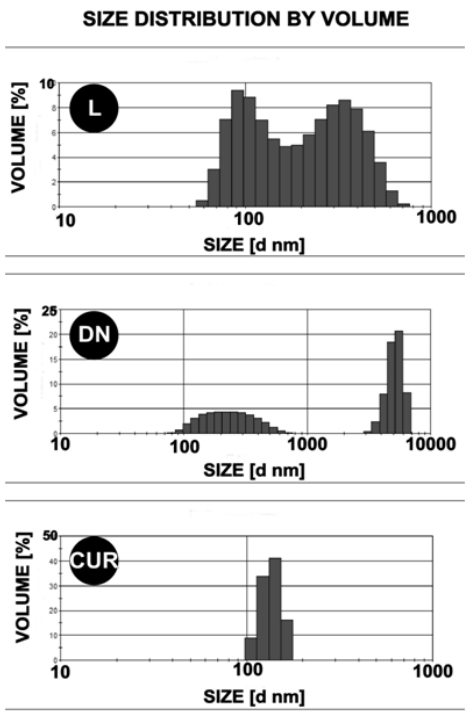

FIGURE 2. Size distribution of liposomes: L – empty, DN – with DN and CUR – with Cur

proximately the same, confirming liposomes stability.

In order to determine if liposomes are consumed by their source cells time-dependent analysis of fusion between the cells and fluorescently marked liposomes was performed. Since Cur is hydrophobic, it was entrapped within the liposome wall, which consist of phospholipid bilayer. Theoretically, it might have negative impact on fusion between the cells and liposomes, since it is the wall which has first contact with the cell membrane. Therefore, also fusion of Cur-entrapped liposomes was also compared. The analysis revealed very rapid increase of fluorescence in C6 glioma cells, what was clearly visible 30 min since the treatment. Importantly, Cur encapsulation did not affect the dynamics of fusion (Fig. 3).

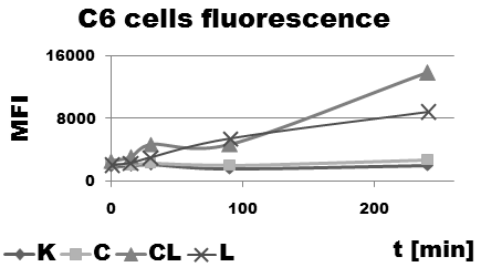

FIGURE 3. Mean fluorescence intensity (MFI) of C6 cells after treatment with dyed empty liposomes (L) and with Cur (CL). K – control cells, C – control cells treated with Cur

Finally, it was determined if the liposomes are biocompatible, meaning that the empty ones should not affect the cells, and if they are feasible as a carrier for hydrophilic and hydrophobic agents. Proliferation assay did not show any important impact of empty liposomes on C6 cells. There was also no visible difference between DN-treated cells and cells treated with DN-liposomes (Fig. 4), what showed that DN encapsulation has no negative impact hereby, since DN were proven previously to be

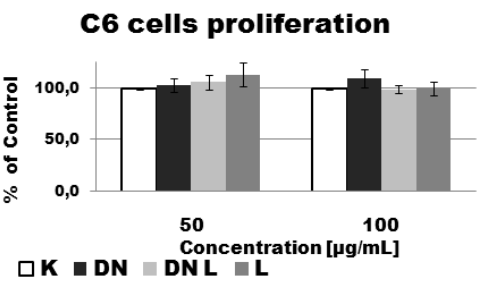

FIGURE 4. Mean proliferation of C6 cells after the treatment with: DN – diamond nanoparticles DN L – liposomes with DN, L – empty liposomes. No statistically significant differences between the groups (P >0.05), ANOVA, Tukey post-test

non-toxic (Kurantowicz et al. 2015). It creates a possibility to employ the liposomes as a dispersing carrier for DN, which can serve as a platform for different chemicals. As for Cur, inhibition of cancer cells proliferation is one of Cur known activity, although observed mostly *in vitro* due to poor availability within living organism. When comparing Cur suspended in water and entrapped in liposomes in identical concentrations, we observed significant effect on C6 cells. Cur-encapsulated liposomes with 50 μm of Cur significantly decreased the cells proliferation, comparing to 50 μM of bare Cur and to control cells (Fig. 5). The observed effect is important, since it indicates on Cur bioavailability increase after entrapping in liposomes walls, making the liposomes promising carrier of Cur or any other hydrophobic agent within a living organism.

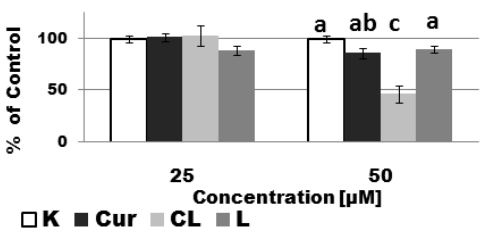

FIGURE 5. Mean proliferation of C6 cells after the treatment with: K – control (no treatment), Cur – curcumin; CL – liposomes with Cur, L – empty liposomes. Different letters indicate significant differences between the groups (P <0.05). ANOVA, Tukey post – test. No significant differences for concentration 25 μM (P >0.05)

CONCLUSIONS

In the presented studies possibility of creating biomimetic liposomes from total lipids isolated from rat glioma cells was confirmed. The liposomes are biocompatible and can serve as a carrier both for hydrophilic and hydrophobic agents. Encapsulation of DN did not have negative impact on liposomes, what makes them good dispersive carrier for those non-toxic nanoparticles. Entrapping Cur within liposome walls significantly increased Cur activity and did not affect liposomes sorption by cells. Obtained results creates a possibility of production of personally tailored carriers for bioactive agents, which are biocompatible, effective and safe due to isolation of ingredients from host tissue biopsies.

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Streszczenie. *Analiza fuzji pomiędzy komórkami glejaka szczurzego i liposomami biomimicznymi z enkapsulowanymi nanocząstkami diamentu lub kurkuminą.* Liposomy są wykorzystywane jako nośniki dla biologicznie aktywnych związków, zarówno hydrofilowych, które są enkapsulowane w wodnym rdzeniu liposomy, jak i hydrofobowych, które mogą być zamykane w jego ścianie. Ściany liposomów zbudowane są z dwuwarstwy fosfolipidowej, przez co strukturalnie przypominają błonę komórkową. Założono, że jeżeli taka ściana zostanie stworzona z zestawu lipidów charakterystycznych dla danej komórki, liposomy będą chętnie pobierane przez taką komórkę. Wykonano doświadczenie, w którym jako przykład użyto komórek glejaka szczurzego linii C6, ponieważ wiadomo, że komórki centralnego systemu nerwowego są wyjątkowo bogate w lipidy, włączając w to także te nietypowe. Ze względu na to, że wszystkie komórki nowotworowe charakteryzuje wysoki potencjał proliferacyjny, muszą one pobierać prekursory niezbędne do budowania błony komórkowej wokół nowopowstających komórek. Liposomy biomimiczne mogą być więc jednym z najbardziej skutecznych sposobów na dostarczenie związków przeciwnowotworowych do takich komórek. Analiza właściwości fizykochemicznych, a także testy *in vitro* pokazały, że otrzymanie takich liposomów jest możliwe i że liposomy te są biozgodnym, stabilnym nośnikiem zarówno dla związków hydrofilowych, jak i hydrofobowych. Enkapsulacja nanocząstek diamentu nie wpłynęła na wytworzone liposomy, podczas gdy zamknięcie w ścianie kurkuminy, która jest znana w tradycyjnej medycynie azjatyckiej ze względu na swoje właściwości przeciwnowotworowe, istotnie podniosło jej aktywność. Otrzymane wyniki pokazały, że liposomy biomimiczne mogą być efektywnymi, indywidualnie dopasowanymi nośnikami dla związków biologicznie czynnych.

Słowa kluczowe: glejak, liposomy, nanocząstki diamentu, kurkumina

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