ПОВЕРХНОСТНО ФУНКЦИОНАЛИЗИРОВАННАЯ ЭМУЛЬСИЯ
ДЛЯ СЕЛЕКТИВНОГО ТРАНСПОРТА ЛЕКАРСТВЕННЫХ СРЕДСТВ

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Представлены результаты функционализации поверхностей капель двойных эмульсий для селективного транспорта лекарственных веществ и селекции антител для таргетинга на опухолевые клетки головного мозга - мультиформную глиобластому (GBM). Функционализация включала физическую адсорбцию антител: анти-CD15 (маркер болезни Ходжкина) на каплях масла эмульсии. Взаимодействия между молекулами антител и соединениями капель масла измеряли с помощью изотермической титровальной калориметрии (ИТК). Двойные эмульсии получали одношаговым методом, в спиральном контакторе потока. Анализ ИТК показал, что антитела взаимодействуют с эмульсиями соединениями. Изменение тепловых скоростей, отражающих молекулярные взаимодействия, показало, что белки прилипают к интерфейсам капель масла. Кроме того, поверхностные белки (антитела) были выявлены и проанализированы на конкретных GBM для клеточных линий: U87MG, LN229, T98G. Многократные эмульсии создавались в условиях: размер кольцевого зазора между соосными цилиндрами составлял 1,5 мм, частота вращения внутреннего цилиндра 2162 об/мин, объемные скорости потока внутренней водной фазы в мембранную масляную фазу 1:1 и мембранной масляной фазы во внешнюю водную фазу 1:15. Способ приготовления двойных эмульсий более точно описан в наших предыдущих работах. Размеры капель внутренней фазы и капель мембранной фазы определяли на основе анализа изображений, полученных при микроскопическом наблюдении образцов двойной эмульсии.

Ключевые слова: эмульсия, селективный транспорт, молекулы антител

SURFACE FUNCTIONALIZED EMULSION FOR SELECTIVE DRUGS TRANSPORT

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This paper presents the results of functionalization of surfaces of double emulsions drops for selective drug transport and selection of antibodies for targeting brain tumor cells - glioblastoma multiforme (GBM). The functionalization involved physical adsorption of antibodies: anti-CD15 (Hodgkin’s disease marker) on oil drops of the emulsion. The interactions between molecules of antibodies and compounds of the oil drops were measured using isothermal titration calorimetry (ITC). Double emulsions were prepared by a one-step method, in a helical flow contactor. The ITC analysis showed that antibodies interacted with emulsion compounds. The change in the heat rates reflecting molecular interactions proved that proteins adhered to oil drops interfaces. Additionally, surface proteins (antibodies) were identified and analyzed for targeting specific GBM cell lines: U87MG, LN229, T98G. The multiple emulsions were created under conditions: the size of the annular gap between the coaxial cylinders was 1.5 mm, the rotational frequency of the inner cylinder was 2162 rpm, the volumetric flow rates of internal water phase to membrane oil phase 1:1 and membrane oil phase to external water phase 1:15. The method of double emulsions preparation has been described more precisely in our previous papers. The sizes of the internal phase drops and the membrane phase drops were determined based on analyzing images captured during microscopic observation of the double emulsion samples.

Key words: emulsion, selective transport, molecules of antibodies

INTRODUCTION

The drugs transport to targeted tissue is an important challenge in the treatment of cancer. This may be accomplished by developing drug delivery systems that can provide efficient target-specific transport to the diseased site and selective cellular uptake. Several approaches with different mechanisms have been proposed to enhance the selectivity of drug delivery systems. One possible approach would be the improvement in the properties of drug delivery systems attributed to their functionalization by surface modification. The methods of surfaces modification, in general, can be categorized into two groups, namely coating and film deposition as well as in-situ surface e.g. nano-functionalization. These two types of methods are often combined. The surface of drops can be functionalized by deposition/adsorption of protein molecules, antibodies, nucleic acids aptamers, peptides and other small molecules like folic acid recognized by tumor cells [1]. Emulsions and microemulsions with antibodies adsorbed on the surfaces of drops besides polymeric micelles are expected to have promising applications for drug delivery and cancer targeting [2]. Adsorption of proteins (antibodies) as the method of the surface of drops functionalization may occur as physical adsorption – physisorption [3-5] and chemical adsorption – chemisorption [6-8]. The methodology of the quantitative determination of binding affinity during molecular interactions providing the surface modification of emulsions drops uses different characterization techniques: the transmission electron microscope (TEM) observation [6-7], bicinchoninic acid assay (BCA) [6-7], enzyme-linked immunosorbent assay (ELISA) [5], two-dimensional gel electrophoresis (2D-PAGE) [8], high-performance liquid chromatography (HPLC) [4], and isothermal titration calorimetry (ITC) [9].

This paper was aimed at developing double emulsions with functionalized surface of emulsions drops, as alternative platforms for selective delivery of...
anti-cancer drug for glioblastoma multiforme (GBM) therapy. Furthermore, the scope of the study involved selection of antibodies for targeting brain tumor cells. Double emulsions represent the simplest multiple emulsions, which are dispersed systems having a hierarchical structure of droplets of an internal phase in drops of a membrane phase (Fig. 1). The structures of double emulsions allow one or more active agents to be encapsulated and then released in a controlled manner. The controlled release is achieved by drop sizes, volume packing of drops and physicochemical parameters of liquid phases of multiple emulsions. These dispersed systems offer a wide range of possible applications in chemistry and chemical engineering (separation processes and environmental protection) and its interdisciplinary fields such as pharmaceuticals, and medicine especially for the encapsulation and controlled release of active ingredients (drugs, living cells, cosmetics, food), [10]. The proposed functionalization of double emulsion included adsorption on drops surfaces the selected antibodies specific to the cancer cells surface receptors. Hence selective transport of drug to a tumor (Fig. 2) may be considered. The effect of bi-molecular interactions leading to functionalization of surfaces of emulsions drops was measured and characterized by the isothermal titration calorimetry (Fig. 3).

**RESULTS AND DISCUSSION**

**Double emulsion characteristics**

The structure of double emulsions considered for targeting GBM represented type W₁/O/W₂ (W – water phase, O – oil phase) emulsions with a single small drop of the internal phase inside bigger oil membrane phase drop. The double emulsions were prepared by a one-step method in helical flow contactor [11, 12]. The calculated Sauter mean diameter of internal phase drops was 7.1 μm and 11.0 μm for oil membrane phase drops for the emulsions samples just after preparation. The drop size distributions of created double emulsions are presented in Fig. 4. To evaluate the stability of emulsions after introducing antibodies, the microscopic observations were proceeded after the preparation of the emulsions and after the titration with the antibody (the isothermal titration calorimetry (ITC)). The calculated changes in the Sauter mean diameter after ITC experiments were less than 5% compared to the initial mean diameters of double emulsions drops.

**Fig. 1. The structure of double emulsions**

**Fig. 2. The double emulsions for drug delivery and targeted drug delivery**

**Fig. 3. The isothermal titration calorimetry (ITC) setup for measurement antibody – drops of double emulsions molecular interactions**

**Fig. 4. Double emulsion drop size distributions of the internal and membrane phases**

1 – inner phase drops; 2 – membrane phase drops
**Evaluation of protein (antibody) – drop of emulsions interaction**

The heat effect of all measurable interactions regarding the binding antibody (protein) to the surface of oil membrane phase drops of double emulsions is presented in Table and Fig. 5. The energy associated with binding antibody required the elimination of background from the heat measured by the isothermal titration calorimetry (ITC). Hence, the energy of proper interaction was calculated as algebraic combinations of the energy of all interactions according to the equation: energy (heat) of the interaction of an antibody and drop = energy of interactions: SET I – SET II + SET III – SET IV. The final heat effect i.e. after elimination of background of interactions, indicating the process of adsorption of an antibody onto the oil drops of double emulsions for this system, is shown in Fig. 6.

<table>
<thead>
<tr>
<th>SET</th>
<th>Liquid sample in the stirred cell</th>
<th>Liquid sample in the stirring syringe</th>
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<tbody>
<tr>
<td>I</td>
<td>The multiple emulsion</td>
<td>The antibody in suspension</td>
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<tr>
<td>II</td>
<td>The multiple emulsion</td>
<td>The antibody suspension buffer</td>
</tr>
<tr>
<td>III</td>
<td>The external phase of the emulsion</td>
<td>The antibody suspension buffer</td>
</tr>
<tr>
<td>IV</td>
<td>The external phase of the emulsion</td>
<td>The antibody in suspension</td>
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</table>

**Identification and selection of cell surface proteins for GBM therapy**

The results allowed an identification and selection of cell surface proteins (surface markers) for targeting GBM with double emulsion-based platforms delivering chemotherapeutics (doxorubicin). This involved analysis of the cell lines of glioblastoma multiforme (GBM) by protein expression to select specific surface protein expressed in excess by GBM cells. Three of GBM’s cell lines: U87 MG, T98G, LN229 were analyzed in comparison to non-cancer cells K21 used as a control.

Protein expression study covered examinations of cancer stem cells within GBMs – cancer surface markers: CD133, CD97, CD44 and CD15 [13-15]. The β-actin was used as a loading control in Western blot experiments. An analysis of protein expression data indicates that the CD97 and CD44 expression was found both in GBM (U87 MG, T98G, and LN229) and fibroblast K21 cell line (Fig. 7a). The expression levels of these proteins were similar for all studied samples. The similar level of CD97 and CD44 in cancer and non-cancer cells eliminated these proteins as good selective GBM surface markers. The CD133 and CD15 expression were not detected (Fig. 7a). This result may suggest that in monolayer cell culture cancer stem cells constitute a very small fraction [15]. RTK receptors: receptors of tyrosine kinases (RTKs) are a group of cell surface proteins, which are often overexpressed in glioblastoma tumors [16, 17]. RTKs are one of the most frequent targets in cancer therapy [17, 18]. Here expression of EGFR, PDGFR alpha and PDGFR beta was examined. We have found a low level of PDGFR beta in three GBM lines was compared to K21 non-cancer cell line (Fig. 7b). The PDGFR alpha was not detectable in GBM cells (Fig. 7b). In the LN229 GBM cell
line, the RTK expression was not detected at all in contrast to non-cancer fibroblast line, which expressed all studied markers (Fig. 7b). That is why RTK receptors could not be used as GBM surface marker target.

Selected CD class proteins: The selected CD protein (CD338, CD325 as well as CD82) expression was examined. The western blot analysis showed a low level of all three selected surface proteins in a K21 non-cancer cell line, while expression of CD388 and CD325 (N-cadherin) was higher in U87 MG, LN229 and T98G compared to the control cell lines. A very high level of CD82 protein in U87 and T98G GBM lines was observed. The level of CD82 was low in LN229 (Fig. 7c). These data may suggest, that CD (cluster of differentiation) proteins, particularly the CD82, are a good target for GBM therapy [13, 19]. CD325(N-cadherin) can also be used for immobilization/adsorption at surfaces of emulsions drops in combination with CD82 for a GBM cell surface protein target. It is worth to note that CD325 (N-cadherin) might be used for other types of cancer, since it frequently replaces E-cadherin, a component of the cell membrane in non-cancer cells, during carcinogenic process.

**EXPERIMENTAL PART**

**Antibody suspension and a double emulsion composition**

Antibody chosen for the experiment was anti-CD15 (SantaCruz Biotechnology, USA) suspended in the buffer recommended by a supplier. Buffer composition: PBS with < 0.1% sodium azide and 0.1% gelatin. The internal phase of the emulsion was distilled water, 2 wt.% alginic acid, 0.25 wt.% Poloxamer 407. The membrane phase of the double emulsion was soybean oil, 2 wt.% Span 83. The external phase of the emulsion was distilled water, 0.25 wt.% Tween 80, 0.25 wt.% Poloxamer 407, 0.2 wt.% sodium carboxymethylcellulose (CMC). All compounds were products of Sigma Aldrich.

**Double emulsion preparation and characterization**

The one-step process of double emulsion formation was carried out in a helical flow contactor, which enables the formation of stable dispersed systems and intensive mass transfer in the multiphase flow [20-22]. The multiple emulsions were created under conditions: the size of the annular gap between the co-axial cylinders was 1.5 mm, the rotational frequency of the inner cylinder was 2162 rpm, the volumetric flow rates of internal water phase to membrane oil phase 1:1 and membrane oil phase to external water phase 1:15. The method of double emulsions preparation has been described more precisely in our previous papers [11, 12, 23]. The sizes of the internal phase drops and the membrane phase drops were determined based on analyzing images captured during microscopic observation of the double emulsion samples. Microscopic observation set up contained a digital camera (SC50, Olympus, Japan) connected to an optical microscope (BX-60, Olympus, Japan). Image analyze were made with software - Image Pro Plus 4.5 (Media Cybernetics, USA). For each emulsion sample, at least 1000 drops of the internal phase and 800 drops of the membrane phase were measured. Based on the measured drop sizes the Sauter mean diameter of the internal and membrane phase drops were calculated.
**Isothermal titration calorimetry (ITC)**

The heat effect of interaction between an antibody in suspension and the membrane phase drops of multiple emulsion was measured by the isothermal titration calorimetry. The ITC experiments were performed at 37 °C in the Nano ITC Standard Volume (TA Instruments, USA), the cell volume – 950·10⁻⁶ dm³ and the syringe volume – 250·10⁻⁶ dm³ (Fig. 3), the rotational frequency of stirring syringe 250 rpm. The reference cell was filled with distilled water. All samples were vacuum degassed just before the experiments. The complex composition of each solution in ITC experiments required the elimination of background of molecular interactions hidden in emulsion titration by the antibody suspension (Set I – IV in Table I). First smaller volume injection allowed stabilization of the ITC equipment and then eighteen 13.14·10⁻⁶ dm³ aliquots of 3.39·10⁻⁴ mM antibody solution or antibody solution buffer were injected sequentially to the sample cell containing double emulsion and then double emulsion external phase only.

**Cell lines**

Identification of tumor markers was performed for the model lines of GMB cancer: U87 MG, T98G, LN229. As the control sample non-cancer cell line: human K21 fibroblasts were used. The cells were derived from Institute of Biochemistry and Biophysics PAN, Poland.

**Cell culturing**

The cells were grown in grown medium (DMEM with glucose, L-glutamine and sodium pyruvate (HyClone), 10% FBS (Gibco), 1% Penicillin/Streptomycin (Life Technologies)) in incubator (37 °C, 5% CO₂) on 10cm cell culture dishes (BD-Falcon) for 80-90% confluence and next were passaged using 0.25% trypsin with 0.1% EDTA (HyClone).

**Antibody**

*Primary antibody:* Anti-beta-Actin (Santa Cruz Biotechnology); Anti-CD133 (Millipore); Anti-CD97 (Santa Cruz Biotechnology), Anti-CD44 (Abcam), Anti-CD15 (Santa Cruz Biotechnology); Anti-EGFR (BD), Anti-PDGFR alpha (Santa Cruz Biotechnology), Anti-PDGFR beta (Santa Cruz Biotechnology); Anti-CD338 [ABC2G2, BCRP1] (Millipore), Anti-CD325 [N-cadherin] (Thermo Fisher Scientific), Anti-CD82 (Cell Signaling).

*Secondary antibody:* Goat anti-rabbit (Santa Cruz Biotechnology), Goat anti-mouse (Santa Cruz Biotechnology).

**Western blot analysis**

To select GBM cell surface markers the scapped pellet of the cells was lysed in denaturing buffer (9 M urea, 150 mM β-mercaptoethanol, 50 mM Tris-HCl pH 7.4) (Millipore) before the sonication. After cell lysates preparation, the protein concentration of each sample was measured by the standard procedure of Bradford protein assay using reagent from Bio-Rad company. Finally, 30 μg of protein from each sample was mixed with Laemml Buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) (Millipore) and denatured by heating in thermo block. The samples were separated by SDS-PAGE electrophoresis in 10% acrylamide gel with SDS. Electrophoresis was run (1h, 90V) in 1x SDS-PAGE buffer (pH 8.5) (Millipore). Next, proteins were transferred (2h, 300 mA) on PVDF membrane (Millipore), using Tris-Glycin Buffer with methanol. After samples transfer, a membrane with proteins from lysates was blocked 1h by incubation in 5% milk in 1xTBST (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Tween 20). Next, the membrane was incubated overnight in cold room with primary antibodies, then 1h with secondary antibodies and visualized using Chemiluminescent HRP substrate (Millipore) according to manufacturer’s instructions.

**CONCLUSIONS**

The double emulsions with functionalized drops surfaces were proposed as platforms delivering anti-cancer drug for selective transport in the therapy of glioblastoma multiforme (brain tumor). The functionalization involved binding proteins to the surfaces of emulsions drops via physical adsorption. The isothermal titration calorimetry (ITC) was found to be a useful method to measure the effect of the molecular interactions leading to the physical adsorption of antibody in this system. The double emulsions proposed in this paper may increase the effectiveness of cancer chemotherapy as more efficient than conventional drugs, due to the selective transport of a drug. One approach to developing a selective drug delivery requires specific molecules – cancer surface markers, that would be recognized by a drug platform. In this study, we have found such specific proteins i.e. cancer markers of three glioblastoma cell lines namely CD325 (N-cadherin) and CD82, which can be used as targeting molecules. These study showed the potential of functionalized double emulsions for a brain cancer treatment.

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