Multiple Emulsions as a Biomaterial-based Delivery System for the Controlled Release of an Anti-cancer Drug

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Abstract. This paper focuses on developing multiple emulsions as a pH-responsive drug delivery system, for glioblastoma multiforme-GBM therapy, with reduced toxicity to healthy cells. The multiple emulsions with a stimuli-responsive biopolymer (CMC- sodium carboxymethylcellulose) were prepared in a Couette-Taylor flow contactor. As an external stimulus, the difference in pH of the cancer environment, and normal tissue, was investigated by adding salts as a triggering agent. The cancer cell lines of glioblastoma multiforme were investigated: U87MG, LN229, T98G, in order to verify emulsions’ components cytotoxicity to cells. Also normal (healthy) cells, K21-fribroblast, were analysed. Rhodamine B was used as a model drug instead of the clinically used chemotherapeutics (e.g. doxorubicin) in oncology. Results showed that multiple emulsions by themselves had no adverse effect on the viability of investigated cells, excluding one cell line: LN229. The control and modulated release rates of a model drug, by stimuli-responsive biopolymer, were established. Results confirmed the possibility of controlling the release rates of a drug in the acidic environment of the cancer cells. The proposed multiple emulsion could be explored for the potential delivery of chemotherapeutics in GBM therapy.

1. Introduction

In medicine, biomaterials play a key role mostly in designing and creating drug delivery systems, and substitutes for the extracellular matrix for cell proliferation and differentiation, to enhance their biological activities [1-3]. In addition to therapeutic applications, biomaterials are also useful in the progress of research and development of medicine and cell biology, including the engineering of biological functions of cells [4]. Various advanced biomaterials have so far been investigated and used in different ways. Among them, the increasing role in delivery systems is played by novel components i.e. stimuli-responsive materials, that trigger and enable the control of the release rates of drugs [5, 6]. Multiple emulsions are a potential biomaterial-based delivery system with several applications in pharmaceuticals, medical, and biological engineering applications. Multiple emulsions are liquid dispersed systems of at least three phases (W/O/W or O/W/O, W-water phases O- organic phase), wherein only one phase is continuous, while others occur in the form of drops of the internal and membrane phases dispersed in one another (Figure 1). The simplest structures of multiple emulsions are double emulsions, their more complex forms are triple, quadruple, or even higher-order structures. The coexistence of the aqueous and organic phases makes them suitable for co-encapsulation of various molecules either in the aqueous/oil phase, or in both phases, and carry polar and non-polar molecules. The encapsulation process includes different content e.g. drugs, living cells, nutrients, diagnostic agents, cosmetics, and food. That content can be released in a controlled manner through
drops sizes, composition of emulsions, and their rheological and physicochemical properties. The most recent scientific literature demonstrates that multiple emulsions, in both a liquid and solid form, as nano- or microparticles have been successfully used in controlled drug-release therapies, especially in cancer treatment including chemotherapy, detoxification, and antigen delivery [7-10]. They are also used in cellular therapies as carriers of living cells/stem cells for tissue engineering, regenerative medicine, and transplantation, or for the protection of probiotics before therapeutic application [11, 12]. The structure of multiple emulsions assists targeted drug delivery by surface modification i.e. deposition of the specific surface antibodies on the interface of oil or water drops of W/O/W or O/W/O emulsions that recognize the receptors of cancer cells (Figure 1). Consequently, the surface modified emulsion can selectively act on tumor cells, while the healthy cells are protected from toxic agents [13]. Furthermore, multiple emulsions are considered as a programmable microenvironment for cellular studies, and miniaturized bioreactors, used to improve the synthesis of DNA-nanocomplexes [14], detect single-cell enzyme activity [15], and protein expression levels [16]. In view of the increasing role of novel biomaterials and stem cell therapy [17-21], multiple emulsions can also be used for the development of an effective cell-banking method that can preserve the function and life of cells before clinical applications [22, 23].

This paper focuses on multiple emulsions as biomaterial-based drug delivery systems with a pH-triggered mechanism to control chemotherapeutics’ release rates in the treatment of brain cancer-glioblastoma multiforme-GBM. GBM is the most aggressive form of primary brain tumor, shortening the lives of patients by up to 1 year after diagnosis. A pH-responsive biopolymer, carboxymethyl cellulose (CMC), was used as a biomaterial enabling control release of chemotherapeutic in the acidic environment of the tumour [24, 25]. This biopolymer was the component of the water-external phase of W/O/W emulsion. Also other parameters controlling the release process rate from multiple emulsions, i.e. the drop size and structure of emulsions, have been included to show the range of this proposed therapy. The scope of this paper also includes the study of the cytotoxicity of emulsions’ components to healthy cells and cancer cell lines - glioblastoma multiforme - to show the potential of the proposed and developed drug delivery system. In order to select the proper dose of the drug for patients, the influence of the drug concentration in the internal droplets of emulsions on the release rates has been investigated and discussed.

Figure 1. The structure of multiple emulsions (structured as double and triple emulsions) and their applications.
2. Materials and methods

2.1. Cell lines
The model lines of GMB cancer: U87 MG, T98G, LN229; the control (non-cancer) cell line: human K21 fibroblasts. The cells were derived from Institute of Biochemistry and Biophysics PAN, Poland.

2.2. 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 at 37°C and 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).

2.3. Preparation of multiple emulsions
The W/O/W emulsions with an encapsulated drug in the internal drops were prepared in a Couette-Taylor flow (CTF contactor). In the CTF contactor intensive mixing occurs, resulting from rotational and axial flows. The internal, membrane, and external phases were introduced at fixed-flow rates into the space between the coaxial cylinders of the CTF contactor. The drug was dissolved in the internal phase. After forming emulsions in steady-state hydrodynamic conditions, the acquired samples of emulsions were collected. The full description of the emulsification and encapsulation process in the CTF-contactor can be found in papers [26-30]. The composition of emulsions and preparation conditions in the CTF contactor are presented in Tables 1-2. All experiments were carried out in the laminar class II biological safety cabinet (TopSafe Biohazard), after the CTF biocontactor was sterilized with ethanol.

**Table 1.** The preparation conditions and composition of multiple emulsions for cytotoxicity experiments and the rheological measurements

| Emulsion | Composition of emulsion phases | Preparation conditions in the CTF contactor |
|----------|--------------------------------|-----------------------------------------|
|          | Internal phase | Membrane phase | External phase | Rotational frequency of inner cylinder (rpm) | Annular gap width (mm) | The ratio of volumetric flow rates of phases: |
|          |                |                |                | internal/external | membrane/external |
| 1A       | Distilled water + (2wt%) alginic acid + (0.25wt%) Poloxamer 407 | Soybean oil + (2wt%) Span 83 | Distilled water + (0.25wt%) Tween 80 + (0.20wt%) CMC + (0.25wt%) Poloxamer 407 | 2162 | 1.5 | 0.067 0.067 |
| 1B       | Distilled water + (2wt%) alginic acid + (0.25wt%) Pluronic P-123 | Soybean oil + (2wt%) Span 83 | Distilled water + (0.25wt%) Tween 80 + (0.20wt%) CMC + (0.25wt%) Pluronic P-123 | 2342 | 1.5 | 0.067 0.067 |
| 1C       | Distilled water + (2wt%) alginic acid + (0.25wt%) Poloxamer 407 | Soybean oil + (2wt%) Span 83 | Distilled water + (0.25wt%) Tween 80 + (0.20wt%) CMC + (0.25wt%) Pluronic P-123 | 2162 | 1.5 | 0.067 0.067 |

Highly purified sodium alginate (Sigma), CMC-sodium carboxymethyl cellulose (Merck), soybean oil (Sigma), Span 83 (Sigma), Tween 80 (Sigma), Poloxamer 407 (Sigma), Pluronic P-123 (Sigma)
Table 2. The preparation conditions and composition of multiple emulsions for release study

| Emulsion | Internal phase | Membrane phase | External phase | EE_RhB (%) |
|----------|----------------|----------------|----------------|------------|
|          | Distilled water + (2 wt%) alginic acid + (0.25 wt%) Pluronic P-123 + (0.1 wt%) Rhodamine B (model drug) | Soybean oil + (2 wt%) Span 83 | Distilled water + (0.25 wt%) Tween 80 + (0.20 wt%) CMC + (0.25 wt%) Pluronic P-123 | 83.7 |
| 2B       | Distilled water + (2 wt%) alginic acid + (0.25 wt%) Pluronic P-123 + (0.1 wt%) Rhodamine B (model drug) | Soybean oil + (2 wt%) Span 83 | Distilled water + (0.25 wt%) Tween 80 + (0.20 wt%) CMC + (0.25 wt%) Pluronic P-123 | 86.4 |

Preparation conditions in the CTF contactor

| Emulsion | Rotational frequency of inner cylinder (rpm) | Annular gap width (mm) | The ratio of volumetric flow rates of phases: internal/external | membrane/external |
|----------|-----------------------------------------------|------------------------|---------------------------------------------------------------|------------------|
| 2A       | 2162                                          | 1.5                    | 0.067                                                         | 0.067            |
| 2B       | 0.25                                          | 0.50                   |                                                               |                  |

Rhodamine B (Sigma), highly purified sodium alginate (Sigma), CMC sodium carboxymethyl cellulose (Merck), soybean oil (Sigma), Span 83 (Sigma), Tween 80 (Sigma), Pluronic P-123 (Sigma)

2.4. Characterization of multiple emulsions

The observations of the morphology of multiple emulsions were carried out by fluorescence microscope connected with a digital camera (Zeiss AxioImager.M2) and image analysis software Image Pro Plus 4.5 (Media Cybernetics). The distribution of drop sizes (diameters) and size of dispersed phases were measured for each emulsion sample concerning at least 2500 drops of the membrane phase and 5000 droplets of the internal phase. The stability study of emulsions based on microscopic observation of the changes in drops of internal and membrane phase morphology during the processes that are considered.

2.5. Rheological measurements

The rheological behavior of multiple emulsions 2 (Table 1) was investigated in the presence and absence of external stimulus - pH stimulus (triggering agent) in the form of ions of salts in 5 wt% PBS buffer (Sigma). The PBS solution was added to pH responsive biopolymer: sodium CMC-water solutions (0.1 wt% and 0.2 wt%) used as the component of the external phase of multiple emulsions. The rheological measurements were carried out using a rotational rheometer RheolabQC (Anton Paar) equipped with a measuring system of concentric cylinder geometry (gap length: 60 mm, gap size: 1.64 mm, cone angle: 120°, ratio of radii: 1.08) at room temperature.

2.6. Encapsulation efficiency of Rhodamine B and in vitro release study

To evaluate the encapsulation efficiency (EE_RhB) and the fraction of Rhodamine B (Sigma) (model drug for doxorubicin) released the filtered (syringe filters: Nylon, 0.45 µm; RC, 0.45 µm; Profill) samples of the external phase of emulsions was spectrophotometrically measured (UV-Vis spectrophotometer-Helios Alpha&Beta, Thermo Scientific) at wavelength of 554 nm [31]. The EE_RhB was calculated according to the difference between the mass of Rhodamine B in the stream of internal phase to CTF contactor and the non-encapsulated mass of Rhodamine B in the external phase of emulsion.

The release experiment were performed for emulsions 3 (Table 2) in a standard glass stirrer tank (diameter: 10 cm; volume: 976 cm³, rotational frequency of stirrer: 250 rpm) equipped with impeller (four plane blade turbine, blades width 0.5 cm, diameter: 5 cm; blades set an angle of 45° at 37°C. All materials and glassware used were sterile. Each experiment/measurement was performed in triplicate and repeated three times. Results are presented as a mean± standard error (SE).
3. Multiple emulsion as a pH stimulus biomaterial-based drug delivery system for GBM therapy- results and discussion

3.1. Cytotoxicity of multiple emulsions to GBM and control cell lines.

The cell lines of GBM: U87 MG, T98G, LN229 and K21 (non-cancer) fibroblast cell line as control were analyzed. The effect of the composition of emulsions 1A, 1B and 1C themselves (without drug), (Table 1) on the viability of cell lines: (i) fibroblast - K21, and glioblastoma (ii) U87 MG, (iii) T98G, and (iii) LN229 was investigated by Alamar Blue assay [32, 33]. The control sample it means sample without adding emulsion. The cell viability experiment was used as a tool for evaluation of the cytotoxicity of the different emulsion compositions used in the current research. The control sample was a cell in grown medium. Cytotoxicity assay showed harmless effect of addition of multiple emulsions to growing medium 1:20 (v/v) and 1:10 (v/v) for examined glioblastoma cell lines (Figure 2). Moreover, the addition of emulsion stimulated cell growth. Comparing between the two cell lines (U87 MG and T98G) the composition of emulsions 1A, 1B and 1C exerted more stimulating effect on the growth of cell line U87 MG than T98G. The addition of emulsion 1A and 1C to K21 cells in growing medium (1:20 (v/v)) was harmless to cells (Figure 2). At the same time, the addition of 1:10 (v/v) double emulsions 1A, 1B and 1C as well as 1:20 (v/v) emulsions 1B to growing medium decreased K21 cells viability (Figure 2). In the K21 cell line, compositions of emulsions 1A and 1C were more biocompatible than emulsion 1B. As demonstrated in Figure 2, the composition of multiple emulsions was toxic to the cancer cell line LN229. The results showed that the composition of multiple emulsions 1A and 1C (emulsion to the growth medium with cells=1:20 v/v) had no adverse effect on the viability of the investigated cell lines, excluding the LN229 cell line. This cell line is the least resistant to chemicals as compared to others investigated. This is due to the natural defensive biological function of the LN229 line.

![Figure 2](image-url) Cytotoxicity of potential multiple emulsion-based drug delivery system to (a) K21, (b) U87 MG, (c) T98G and (d) LN229 cell lines used in this study (after 24h incubation with Alamar Blue).
3.2. Rheological behavior of multiple emulsion platforms with a stimulus -pH responsive polymer.

The rheological tests of emulsions 1A, 1B, 1C (Table 1) and their external phase as solitary phases were carried out to investigate the possible change in the viscosity of emulsions i.e. decrease after adding the triggering agent (ions of salts in the PBS buffer). The external phase of emulsion constituted solutions of sodium carboxymethylcellulose (CMC) in the absence and presence of PBS buffer. The apparent viscosity as a function of shear stress for the examined systems is demonstrated in Figure 3.

An explanation of decreasing in the viscosity of systems with PBS lays in the interaction between ions of salts presented in the solution of PBS and polymer chains. In the absence of PBS and so thus salts, the interaction between the CMC and water (solvent) is a dominant factor [24] and the chains of CMC are stretched to maximum. In the case of the presence of salts, the interactions between the polymer chains are not limited and the CMC chains are “curled up” (Figure 4). The salts induce changes in the conformation of the polymer molecules resulting in lowering the intrinsic viscosities of CMC-PBS solutions [24]. Therefore, the viscosity of emulsions 1A with PBS was lower than the emulsions 1A, 1B and 1C without the addition of PBS. As mentioned previously, the viscosity increased with the concentration of CMC and decreased after the contact with the salts. The salts can be used as the external stimuli -triggering agent to modulate the release rates of the drug as shown in Figure 5.

**Figure 3.** Apparent viscosity of emulsion platform and the solitary external phase of emulsion (points represent mean value ±standard error (SE), the SE was smaller than the size of symbol).

**Figure 4.** The change in conformation of CMC chains.
3.3. In vitro release study.

To determine the possibility of modulating and controlling the release process, we investigated the rates of the model drug release from multiple emulsion containing a stimuli-responsive biomaterial e.g. sodium carboxymethylcellulose (CMC) with salts (triggering agent) in the external phase and emulsions with different structures. Emulsions characterized by different structures and drop sizes were examined as they are basic factors influencing the release process (emulsions 2A, 2B). The ions from salts in the PBS buffer were considered as the external stimuli (triggering agent). Rhodamine B was used as a model chemotherapeutic drug to doxorubicin clinically used in oncology. The characteristics of emulsions delivery system 2A and 2B with the encapsulated model drug used in the in vitro release experiments are presented in Table 2 and Figure 6.

![Figure 5](image)

**Figure 5.** The concept of using an external stimuli (triggering agent) for modulating drug release profiles.

![Figure 6](image)

**Figure 6.** The drop size distribution of multiple emulsions with encapsulated model drug (Rhodamine B): (a) emulsion 2A, (b) emulsion 2B.

The process of drug release involved diffusion of Rhodamine B from the internal aqueous phase (W1) through the oil membrane phase (O) to the external (continuous) medium (W2) of the multiple emulsion. The profiles of the cumulative mass fraction of released Rhodamine B (0.1wt%) from multiple emulsions 2A and 2B without a triggering agent and for 2B with this agent are shown in Figure 7.
Figure 7. The profiles of a model drug release from multiple emulsions with a pH-responsive biopolymer and triggering agent. The points represent mean value±standard error (SE), the SE was smaller than the size of the symbol.

The in vitro release profiles exhibited an initial rapid release phase and then a slower release of the model substance. As shown in Figure 7, almost 50% of the model drug was released by diffusion within the first 3 hours for emulsions 2A and 10 hours for 2B respectively. The release of Rhodamine B was completed within approximately 72 hours for emulsion 2A and 96 hours for emulsion 2B. The diffusion rate of Rhodamine B for emulsion 2A was faster than that for emulsion 2B as shown in Figure 7, which is related to the size of the membrane phase drops. The drops of emulsion 2A are smaller ($D=11.4\pm0.5\mu m$) than emulsion 2B ($D=23.3\pm0.5\mu m$) and therefore the diffusion path of the substance from the internal droplets to the external environment is shorter.

The results also demonstrated the influence of the structure of multiple emulsions themselves on the release rate of the model drug. The emulsion 2A represents structures with single internal droplets dispersed within membrane phase drops, whereas emulsion 2B exhibits multiple internal droplets. Therefore, the release rates of a drug from emulsion 2A were found to be higher than those from emulsion 2B. When the release from emulsion platforms was stimulated by the triggering agent (small cations) a faster release of the drug was observed from emulsion 2B. The effect of the triggering agent on the drug release rates resulted from the decrease in the viscosity of emulsions when PBS was added to the external phase. Since the viscosity of the emulsion-based delivery system is the parameter controlling the transport rate of the drug, the use of stimuli-responsive biomaterials provide the ability to modulate release profiles. The influence of the concentration of a model drug in the internal phase of W/O/W (expressed also by the encapsulation efficiency - see table 2) for emulsions 2A, on the release rates, is presented in Figure 8. The increase in drug concentration was observed as a factor not influencing the structure and drop size. The higher the concentration, the higher the driving force, resulting in faster release of the drug. Different concentrations was considered to select proper doses of the drug in the potential therapy.
Figure 8. The profiles of drug release from multiple emulsion with different concentration of model drug: Rhodamine B in the internal phase of W/O/W emulsions. The points represent mean value±standard error (SE), the SE was smaller than the size of the symbol.

4. Conclusions
Specific materials are needed to meet the challenges of modern medicine, particularly in cancer therapy, which uses artificial or natural biomaterials to achieve the best therapeutic effect. We proposed a model of a biomaterial-based delivery system with controlled release of chemotherapeutics and reduced toxicity to healthy cells. The proposed delivery system is in the form of multiple emulsions with an encapsulated drug in the internal droplets. We have shown that multiple emulsions by themselves had no adverse effect on normal cells and on most of the investigated cancer cells of glioblastoma multiforme. Low cytotoxicity was ensured by appropriate selection of the emulsion composition. The encapsulation of the drug in the internal droplets of emulsions was intended to create a patient-friendly drug delivery system. The release rates of the drug from multiple emulsions as a delivery system can be controlled through the components of emulsions i.e. pH-responsive biopolymer, which enables the physicochemical properties to change i.e. the viscosity in the acidic environment of the tumour. Also the structure of emulsions, drop size, and the encapsulation efficiency of a drug (drug concentration in the internal droplets of emulsions) were found to be rate-controlling parameters. These studies demonstrated the potential of multiple emulsions in a wide spectrum of medical sciences, from treating cancer to regenerative medicine.

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