A pH-responsive biopolymer-based multiple emulsion prepared in a helicoidal contactor for chemotherapeutics delivery

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Abstract: Among biopolymers, pH-responsive ones have much potential for drug delivery systems by exploiting the pH gradient between physiological and pathological states. In this paper multiple emulsions with a pH-responsive biopolymer for controlled drug delivery in brain tumor treatment were discussed. The characteristic and rheological properties of multiple emulsions, biopolymer adsorption, zeta potential, and release processes were examined. The results showed higher drug release rates in the acidic tumor microenvironment compared to the simulated conditions of normal cells.

Keywords: pH-responsive biopolymer, multiple emulsions, helicoidal contactor, release process, biopolymer adsorption.

Emulsje wielokrotne z pH-czułym biopolimerem wytwarzane w kontaktorze helikoidalnym do dostarczania chemoterapeutyków

Streszczenie: Wśród biopolimerów, te reagujące na pH wykazują duży potencjał w systemach dostarczania leków dzięki wykorzystaniu gradientu pH między stanami fizjologicznymi i patologicznymi. W pracy omówiono emulsje wielokrotne z biopolimerem reagującym na zmiany pH, do kontrolowanego dostarczania leków w terapii guza mózgu. Zbadano charkterystykę i krzywe reologiczne emulsji, adsorpcję polimeru na kroplach, zeta potencjał i proces uwalniania. Wyniki wykazały wyższe szybkości uwalniania leku w kwaśnym mikrośrodowisku guza w porównaniu z symulowanymi warunkami normalnych komórek.

Słowa kluczowe: pH-czuły biopolimer, emulsje wielokrotne, kontaktor helikoidalny, proces uwalniania, adsorpcja biopolimeru.

Stimuli-responsive materials, particularly polymers and copolymers, are the materials that can respond to the fluctuation of environmental parameters thanks to changes in structural and macroscopic properties, such as chain conformation, surface activity, solubility, and configuration. These changes have made them suitable, especially in the last two decades, for scientific and technological applications [1, 2]. They are used as components of nano- and microdevices when particular internal (natural) or external (artificial) stimuli are present for effective therapeutics delivery, including site-specific delivery (selective transport), tissue engineering, sensing, in separation processes, membrane and surface functionalisation, and in the chemical, agriculture, and food industries [3–5]. The internal stimuli-responsive smart biomaterials include those that respond to specific enzymes, mechanical force, changes in microenvironment pH, or redox potential. The external stimuli exploit light, ultrasound, electric, magnetic or acoustic energy [2]. These stimuli have been used among others in the field of therapeutics delivery as mechanisms triggering their release. Much attention has been attracted by pH-responsive polymers (PRPs) for tailoring drug release kinetics, overcoming conventional therapy limitations and targeted drug delivery [6]. PRPs are used to encapsulate an active agent within a polymer lattice when preparing a drug delivery system, and then to control its release rates in response to the pH- gradient between normal and tumour cells. Extracellular environments of certain tumours and inflamed tissue have lower pH values (between 6.0–6.8 or 5.4–7.0) than homeostatic conditions (pH 7.4) [7–9]. The most commonly studied pH-responsive natural polymers are alginate, cellulose derivatives (carboxymethylcellulose or carboxymethyl dextran), hyaluronic acid, and chitosan [1, 10]. Drug carriers can be synthesised from different types of materials including stimuli-respon-

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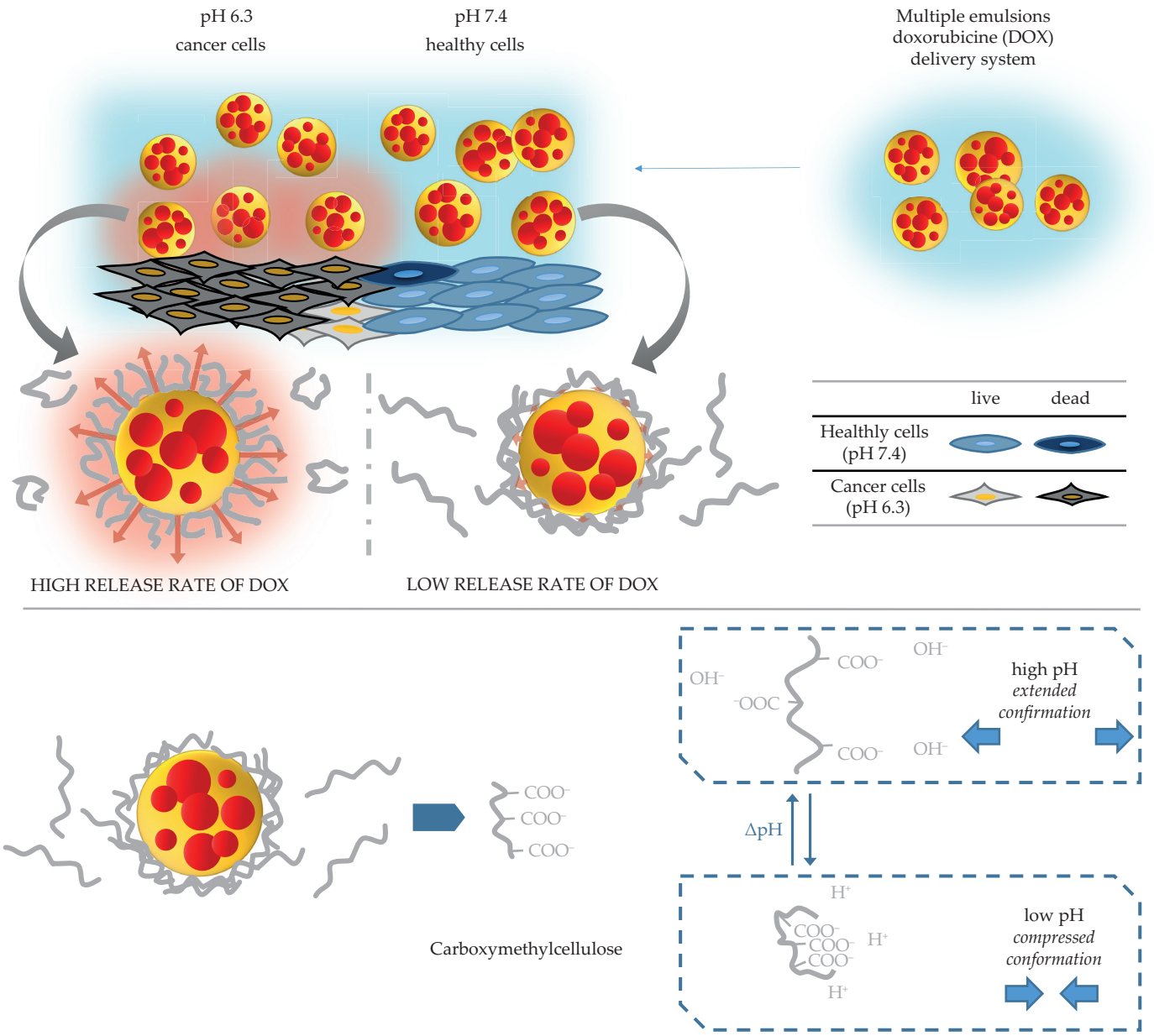

Fig. 1. The concept of chemotherapeutic delivery by double emulsions with pH-dependent chain conformation of biopolymer

sive polymer nano-microparticles, inorganic nano- and microparticles, and polymer/inorganic composites with different morphologies. Among nano- and microdevices for drug delivery, such as nano- microparticles, polymersomes, liposomes, and polymeric micelles, also nanoemulsions, microemulsions, and multiple emulsions have been found to be highly effective [12–14]. Multiple emulsions are hierarchically structured dispersed systems of "droplets in drops", composed of at least three phases, two dispersed: internal and membrane, and external-continuous phases. Three-phase emulsions are denoted as O/W/O (oil in water in oil) and W/O/W (water in oil in water). Such systems are also called double emulsions. There are also many complex multiple emulsions with more than two dispersed phases i. e. triple, quadruple and higher-order emulsions. These dispersed systems prepared mostly on the base of polymers offer a wide applications range in chemistry and chemical engineering (separation processes and environmental protection) and their interdisciplinary fields, such as pharmacy and medicine, especially for the encapsulation and controlled release of active ingredients (drugs, stem cells, nutrients, cosmetics, food) [15–18]. In medical engineering, they can be used as a chemotherapy drug carriers with reduced toxicity and increased selectivity when compared to conventional chemotherapeutic agents used clinically in targeting cancer [13, 15, 17]. This study aimed to present the concept (Fig. 1), experimental findings, and benefits of multiple emulsions with chemotherapeutic (doxorubicin) and pH-responsive biopolymer-carboxymethylcellulose as the drug delivery system in the treatment of brain tumour – glioblastoma multiforme (GBM).

T a b l e 1. The composition of the phases in double emulsions and preparation conditions in the CTF contactor

	Double emulsion type			
	DE1-DOX	DE2-DOX	DE3-DOX	DE4-DOX
	Composition of double emulsions' phases			
Internal phase	69.9 μM DOX 2 wt.%. alginic acid, 0.25 wt.% Pluronic P-123, distilled water	170 μM DOX, 2 wt.%. alginic acid, 0.25 wt.% Pluronic P-123, distilled water	69.9 μM DOX 2 wt.%. alginic acid, 0.25 wt.% Pluronic P-123, distilled water	170 μM DOX, 2 wt.%. alginic acid, 0.25 wt.% Poloxamer 407, distilled water
Membrane phase	soybean oil, 2 wt.% Span 83			
External phase	**0.2 wt.% CMC**, 0.25 wt.% Tween 80, 0.25 wt.% Pluronic P-123, distilled water	**0.2 wt.% CMC**, 0.25 wt.% Tween 80, 0.14 wt.% Poloxamer407, 0.11 wt.% Pluronic P-123, distilled water	0.25 wt.% Tween 80, 0.25 wt.% Pluronic P-123, distilled water	0.25 wt.% Tween 80, 0.25 wt.% Poloxamer407, distilled water
	Preparation conditions of double emulsions in the CTF contactor			
N, rpm	2162	2162	2350	2580
V_{in}, cm^3min^{-1}	15	10	15	10
V_m, cm^3min^{-1}	30	10	30	10
V_{ext}, cm^3min^{-1}	60	150	60	150

DOX - doxorubicin hydrochloride; CMC – sodium carboxymethylcellulose; N – the rotational frequency of contactor rotor; V_{in}, V_m and V_{ext} – the volumetric flows of the internal, membrane, and external phases, respectively

EXPERIMENTAL PART

Materials and methods

Double emulsion (DE) composition

The composition of the internal, membrane, and external double emulsions phases W1/O/W2 with doxorubicin (DOX): DE1-DOX, DE2-DOX, DE3-DOX, and DE4-DOX was presented in Table 1. The composition of the emulsions DE1 and DE2 is the same as the emulsions DE1-DOX and DE2-DOX. The only difference is that they are without doxorubicin (DOX) in the internal droplets of emulsions. All compounds were supplied by Sigma Aldrich.

Double emulsion (DE) preparation

DEs were prepared using a helicoidal contactor also called a Couette-Taylor Flow contactor (CTF). The emulsion formation takes place in the intensive mixing zone

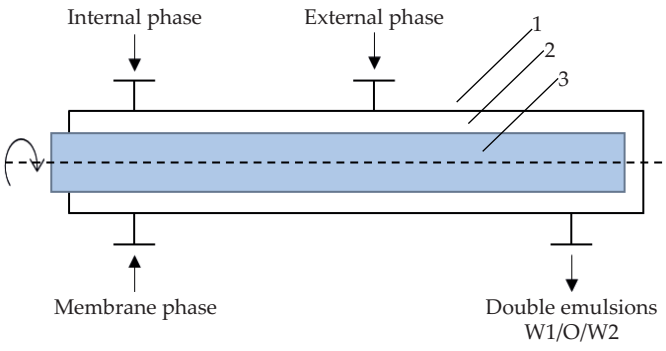

Fig. 2. A helicoidal (CTF) contactor for preparation of double emulsions: 1 – fixed external cylinder, 2 – annular gap, 3 – rotary cylinder

[19, 20] – the annular gap between the coaxial cylinders of the CTF contactor. Three liquid phases (internal, membrane, and external) are introduced into the apparatus as shown in Figure 2. The CTF contactor geometry: gap size: 1.5 mm, length: 0.4 m, inner diameter of the outer cylinder: 0.035 m. The preparation conditions are presented in Table 1. More detailed information on the method of multiple/double emulsions preparation can be found in the authors' earlier works [16, 21, 22].

Double emulsions' structure observation and characterization

Microscopic observations were conducted using a digital camera (SC50, Olympus) connected to an optical microscope (BX-60, Olympus). Microscopic observations of the obtained DE were carried out immediately after preparation and at specific time intervals up to 90 days for emulsions stored at room temperature. Images were analyzed using software – Image Pro Plus 4.5 (Media Cybernetics). For each DE sample, at least 800 drops of the membrane phase and 1000 drops of the internal phase were measured, and then the Sauter mean diameter of the membrane (D_{32}) and internal (d_{32}) phase drops were determined. The results obtained from observing the droplets for 90 days were used to assess the stability of the emulsions.

Rheological measurements

Rheological measurements were performed using a rotational rheometer RheolabQC (Anton Paar) at 37°C. The cylinder measuring system DG42 (double-gap systems) was used. A measuring system geometry: active length 139.5mm; cylinder length 78.7; inner diameter of

rotating cylinder 40.5; outer diameter of rotating cylinder 42mm; inner diameter of stationary cylinder 43mm, gap size 0.5mm. The range of the shear rates: 1–2500 s^{-1}.

ζ-potential measurements

ζ-potential of the droplets in the double emulsions with CMC (DE1-DOX, DE2-DOX) and without CMC (DE3-DOX, DE4-DOX) was calculated based on measurements of the electrophoretic mobility of the droplets (Zetasizer Nano S series, Malvern Instruments). The DTS1070 capillary cell was used. To avoid multiple scattering effects, the emulsions were diluted to approximately 0.005 wt.% using PBS buffer (pH 6.3 or 7.4).

Carboxymethylcellulose adsorption measurements

Emulsions with carboxymethylcellulose (CMC) and without CMC were compared to evaluate the degree of CMC adsorption on drops' surfaces. Emulsions with CMC (DE1-DOX and DE2-DOX) were diluted in PBS buffer 1:100 by volume. To measure CMC adsorption on drops of emulsions without CMC (DE3-DOX and DE4-DOX) they were also diluted with PBS buffer but in this case with dissolved CMC. It was important that the total CMC concentration in the whole volume of the solution should have been the same after dilution in both analyzed cases. Before dilution, the PBS buffer pH was adjusted to the value of 6.3 or 7.4 using HCl (0.1 mol dm^{-3}) or NaOH (0.1 mol dm^{-3}). After dilution, the emulsions were gently stirred for 1 min. The CMC adsorption amount was determined using the colorimetric method [23]. In brief, diluted emulsions were centrifuged (10min, 14 000 rpm) to obtain a supernatant. Then, 2 cm^3 of supernatant were mixed with 0.05 cm^3 of phenol (80%) and 5 cm^3 of sulfuric acid (98%). After 30 min, the absorbance of this coloured solution was quantified by measuring with a spectrofluorometer, Jasco Model FP-6500, at a wavelength of 490 nm. All measurements were made in triplicate. The amount of CMC adsorption on the emulsions' drops surfaces was calculated from a calibration curve according to the concentration difference in the adsorption tests for emulsions with and without CMC.

Cell culturing

In this study, human glioblastoma cell lines: LN229 and T98G were used derived from the IBB PAN (Poland). The cells were cultured on 10 cm cell culture dishes (BDFalcon) in a growth medium: DMEM high glucose with L-glutamine (HyClone), 10% fetal bovine serum-FBS (Gibco), and 1% penicillin/streptomycin (Life Technologies) in an incubator (37°C, 5% CO_2). When a cell reached about 80–90% confluence, the cells were washed in 1×PBS (phosphate-buffered saline) (Lab Empire) and passaged (0.25% trypsin + 0.1% EDTA) (HyClone).

Release kinetics of chemotherapeutic

The doxorubicin release rates were measured in the pH-responsive emulsion systems with DOX at a concentration of 0.1 mM for two glioblastoma cell lines LN229 and T98G. Glioblastoma cells were placed on 12-well microplates. The PBS buffer of pH 6.3 was used to simulate the acidic microenvironment of the GBM tumor. The environment of normal/healthy cells was simulated by PBS buffer of pH 7.4. In each well, a 1 cm^3 of emulsion solution was placed in PBS buffer (emulsion concentration 1%). The microplates were incubated at 37°C. The concentration of released DOX in the release medium was then measured at the appropriate time intervals of 98h. The release medium was separated from emulsion drops using a syringe filter (0.2 μm). To determine the DOX concentration, the spectrofluorometer FLUOstar OPTIMA (extinction: 488 nm/emission: 593 nm) (BMG Labtech) was used. The encapsulation efficiency (EE) of DOX in the emulsion was calculated based on the following equation:

$$EE = \frac{M_{DOX.0} - M_{DOX.1}}{M_{DOX.0}} \cdot 100\% \quad (1)$$

where:

$M_{DOX.0}$ – the DOX mass in the stream of the internal phase fed to a CTF contactor,

$M_{DOX.1}$ – the DOX mass in the external phase of the emulsions measured just after preparation- nonencapsulated DOX.

RESULTS AND DISCUSSION

The concept of using multiple emulsions as a pH-responsive drug delivery system in the treatment of glioblastoma multiforme includes carboxymethylcellulose as component of the emulsions external phase. This biopolymer as a bioadhesive substance may also be adsorbed on membrane phase drops of double emulsions. As a biopolymer with pH-induced conformation changes, it enables the control of the chemotherapeutic (doxorubicin) release rates in the acidic cancer cell environment (Fig. 1). Doxorubicin is encapsulated in the internal droplets of multiple emulsions to separate this aggressive chemotherapeutic from normal cells and thus reduce the side effects of chemotherapy, Fig. 1. The double emulsion is intended to be introduced as a biopolymer-based liquid implant after resection of the tumor [24]. The particular aims of this study focused on: (i) the determination of structures, drop sizes, and stability of pH-responsive multiple/double emulsions prepared at different conditions in a Couette-Taylor flow contactor, (ii) the rheological and electrokinetic behavior of emulsions, and adsorption amount of carboxymethylcellulose as pH-responsive biopolymer, (iii) the release kinetics of the chemotherapeutic in both acidic (pH=6.3) and practically neutral conditions (pH=7.4), and also in the presence

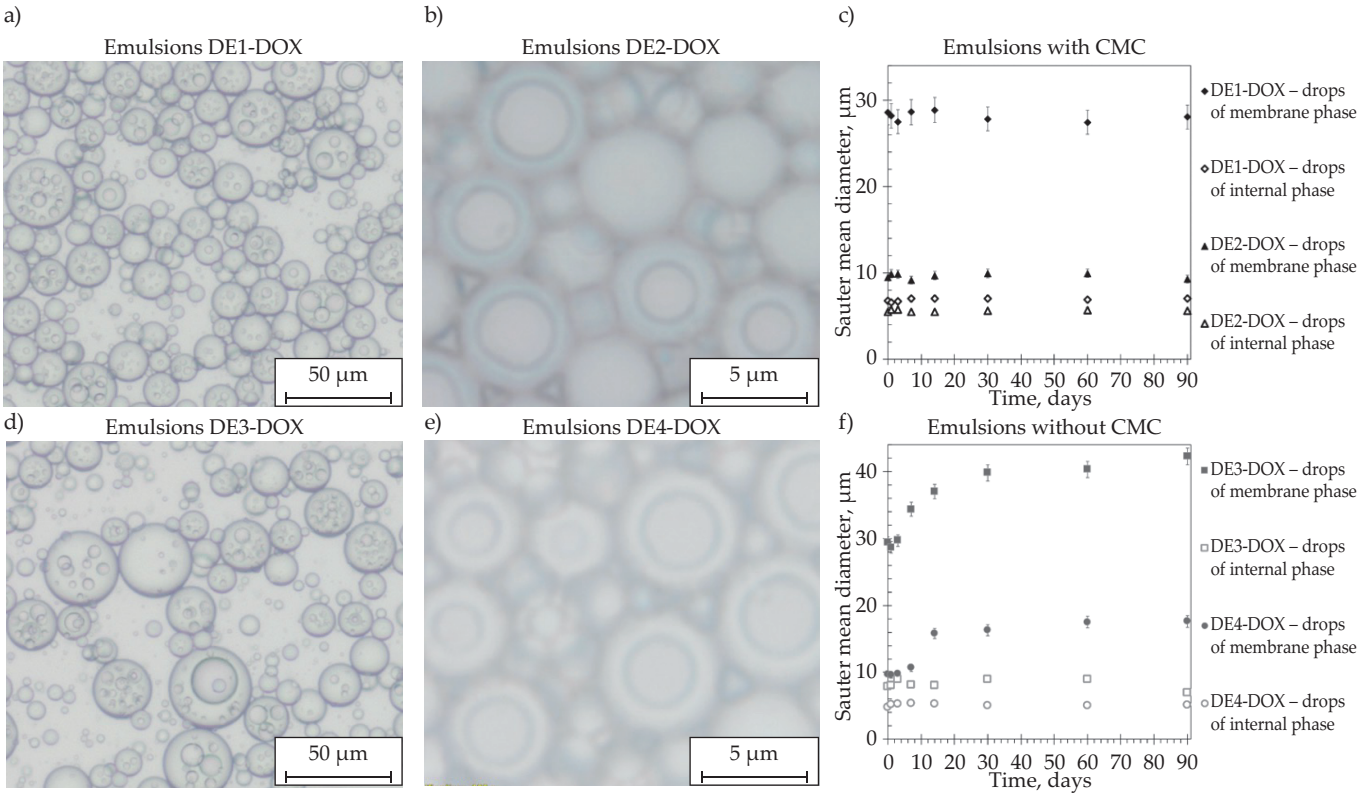

Fig. 3. Characteristics of double emulsions; structures of pH-responsive double emulsions: a, b with DOX; d, e without DOX; c, f stability

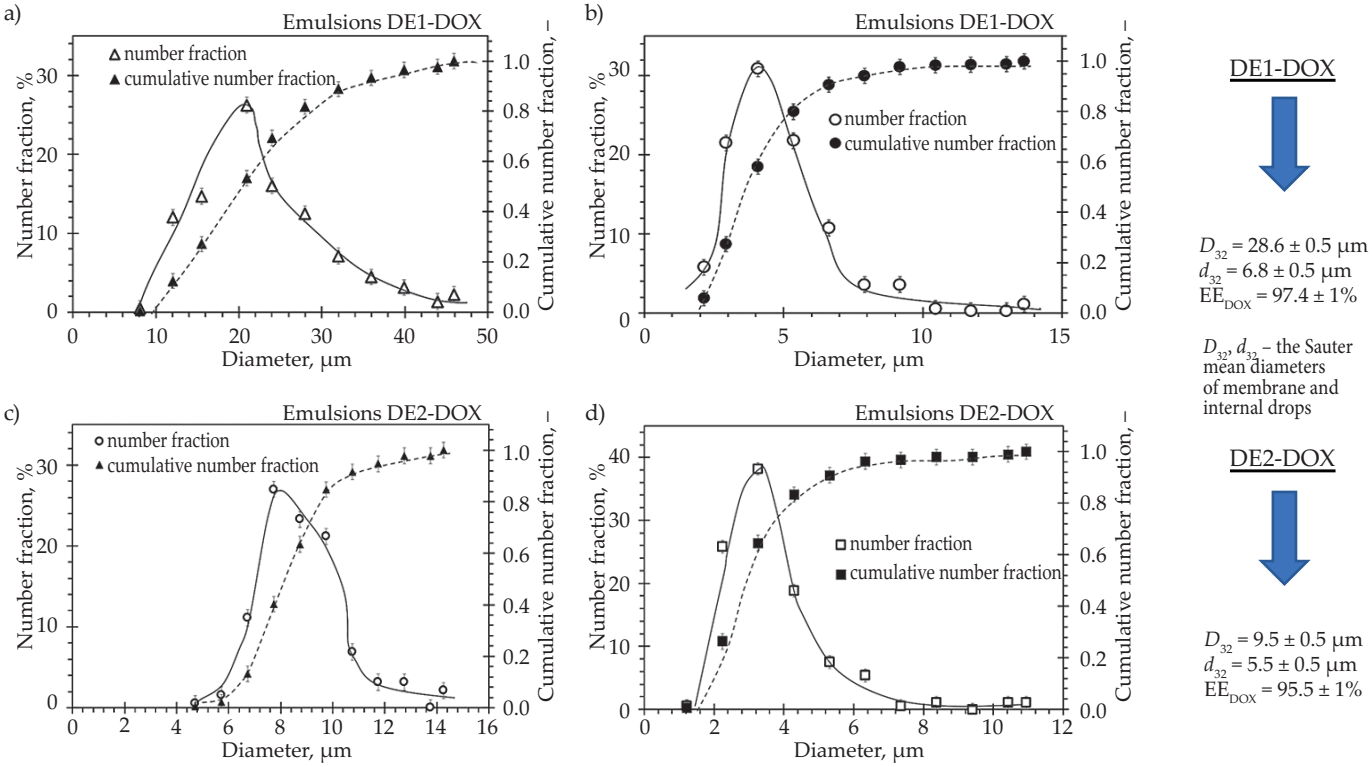

Fig. 4. The drop size distributions of the pH-responsive multiple emulsions with doxorubicin: a, b membrane and internal phase drops for emulsion DE1-DOX; c, d- membrane and internal phase drops for emulsion DE2-DOX

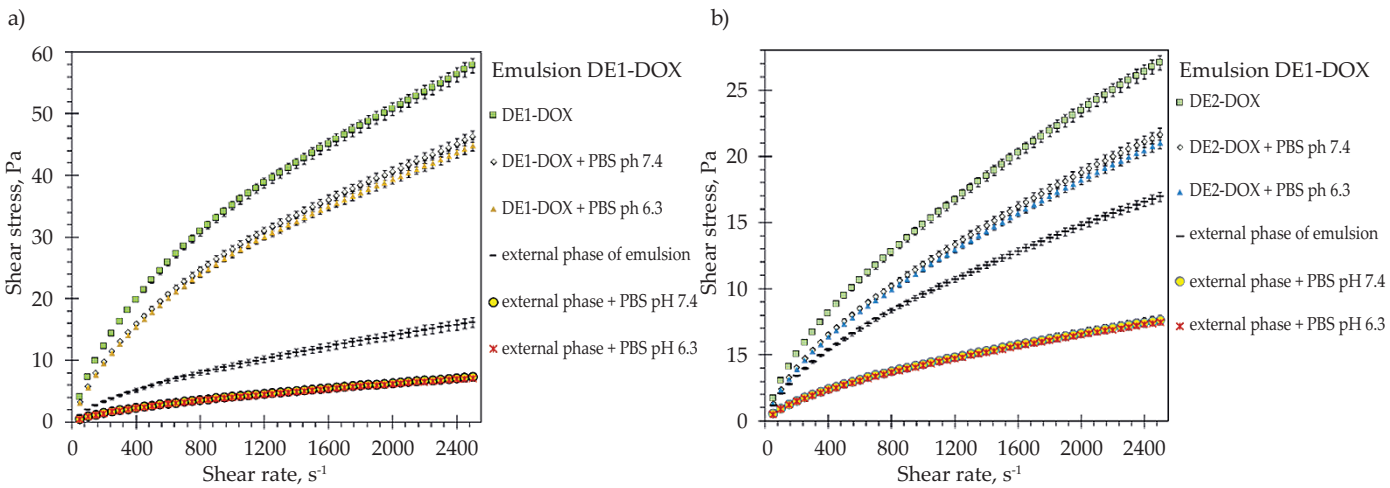

Fig. 5. The rheological curves for pH-responsive emulsions with doxorubicin and for their external phases at differing pH values

of glioblastoma multiforme cells LN229 and T98 G. The structures and drop size distributions of pH-responsive multiple/double emulsions obtained in the helicoidal contactor are presented in Figs. 3 a, b, d, e, and 4. Double emulsions with DOX were stable within 90 days, Fig. 3 c, whereas emulsion systems without CMC were unstable, especially within the first 10 days, Fig. 3 f. A criterion of emulsion stability assumed the changes in drops Sauter diameters with a time less than 15% in comparison to the drop sizes of emulsions after preparation.

As shown in Figs. 3 a, b, d and e the obtained pH-responsive double emulsions were structured with many and singular drops. Additionally, emulsions with DOX, namely DE1-DOX, were characterized by bigger membrane phase drops than DE2-DOX, whereas both systems internal droplets were of comparable diameters. Also, the encapsulation efficiencies of DOX were similar and high (above 95%) for both emulsions, Fig. 4.

The rheological measurements showed the non-Newtonian behavior of shear-thinning fluids for all obtained emulsions and the external phases (Figs. 5 a, b).

The release kinetics of DOX from pH-responsive double emulsions were presented as the cumulative mass fraction of the drug (DOX) released in time. The results of DOX release from double emulsions at pH 6.3 and 7.4 simulating acidic microenvironments of the tumor and normal cells, proved that drug release rates were pH-dependent and controlled (Fig. 6 a). The release rates in the acidic microenvironment were almost twice as high as for the same dose of DOX at lower pH.

To explain the results of the drug (doxorubicin) release in response to the low extracellular pH, CMC adsorption on the surfaces of drops and ζ-potential measurements were examined. The results from Tabs. 2, 3 confirmed the adhesive properties of CMC.

The measured adsorption amount of CMC presented in Tab. 2 indicated that CMC was adsorbed on the drops' surfaces of the emulsions. The presence of CMC on the drops' surfaces was also confirmed by a decrease in the zeta potential measured for emulsions with CMC compared to emulsions without CMC (Tab. 3) – these data are consistent with references and confirmed the adhe-

T a b l e 2. The amount of CMC adsorption on the emulsion drops surfaces at pH 6.3 and 7.4 (mean ± SD, n = 3)

Emulsion type	D_{32}, μm	Amount of adsorbed CMC, mg · m^{-2}		a^*_{max}, mg · m^{-2}
		pH 6.3	pH 7.4	
DE1-DOX	28.6 ± 0.5	0.009 ± 0.0004	0.009 ± 0.0006	0.013
DE2-DOX	9.5 ± 0.5	0.014 ± 0.0007	0.013 ± 0.0009	0.017

* max amount of adsorbed CMC

T a b l e 3. Effect of emulsion type, the drop size of membrane phase of the emulsions, and pH on zeta potentials (mean ± SD, n = 3)

	Emulsion type	D_{32}, μm	Zeta potential, mV	
			pH 6.3	pH 7.4
Emulsions with CMC	DE1-DOX	28.6 ± 0.5	-31.7 ± 0.9	-38.6 ± 0.8
	DE2-DOX	9.5 ± 0.5	-45.3 ± 1.7	-51.8 ± 1.5
Emulsions without CMC	DE3-DOX	29.4 ± 0.7	-9.7 ± 0.7	-13.7 ± 1.2
	DE4-DOX	9.7± 0.6	-14.3 ± 0.8	-18.4 ± 1.6

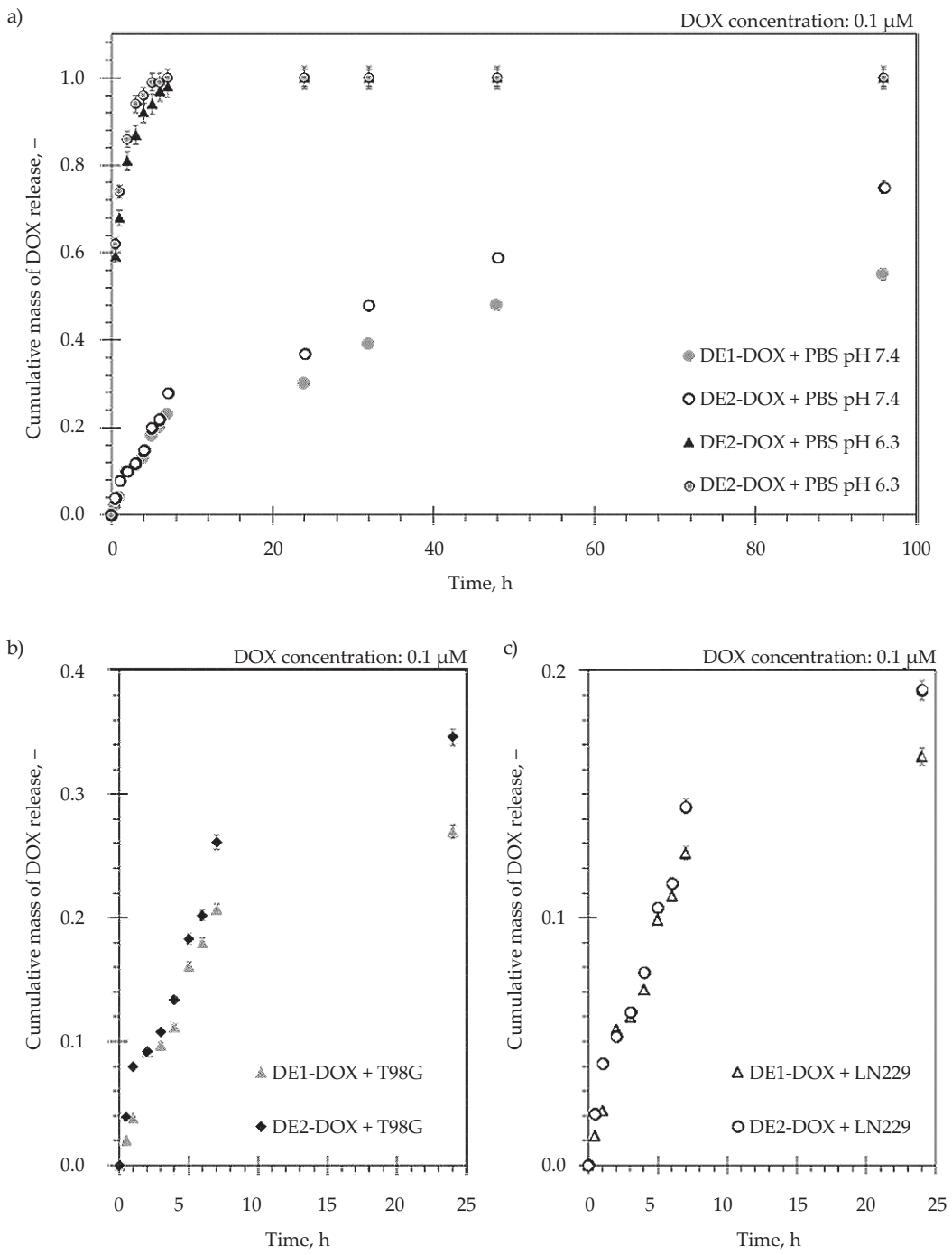

Fig. 6. The release kinetics of DOX from pH-responsive double emulsions: a) at acidic and almost neutral microenvironment; b, c) at the presence of glioblastoma multiforme cell lines: T89G and LN229

sive properties of the used biopolymer important for controlling the drug release process [25]. As biopolymer is adsorbed on the drops of emulsions, the conformation of its chains may change with the pH of the environment, and then the release at different rates (Fig. 1). Also, its presence in the continuous phase of double emulsions favours faster drug transport accomplished with extended chain conformation at the acidic pH. The results also proved the advantages of multiple emulsions as a drug delivery system with unchanged drop diameters with pH gradients (Tab. 3). Additionally, the proposed drug delivery system in the form of double emulsions enables the controlling of the release process through drop sizes of the membrane phase and the structure of emulsions. Emulsions systems with smaller membrane phase drops-DE2-DOX released the drug faster than emulsions DE1-DOX (Figs. 6 b, c). This was due to the higher interfacial area of DE2-DOX emulsion and its structure with singular internal droplets, which shorten the diffusion path of the drug as compared to DE1-DOX emulsion structured with many internal droplets and bigger membrane phase drops (Figs. 3 a, b and 4).

As shown in Figs. 6 b, c, the release rates of doxorubicin in the presence of LN229 and T98G glioblastoma cell lines are lower than the release profiles in the cell-free environment (pH 6.3) (Fig. 6 a). The differences indicated drug (DOX) consumption by LN229 and T98G cancer cells through, among others: the penetration of drug molecules through cell membranes, its adsorption on the cell surfaces, or entrapment in cell membranes, degradation and metabolism by cells [26, 27].

CONCLUSIONS

This paper addressed the selective drug delivery to the brain tumor by the use of advanced biomaterials in the form of multiple/double emulsions with pH-responsive biopolymer-carboxymethylcellulose. Biopolymer was a component of the external phase of double emulsions and, due to its bioadhesive properties, was also present on drop surfaces. Through its pH-dependent chain conformations drug-doxorubicin release from double emulsions was faster at pH 6.3, characteristic for the tumor microenvironment as compared to a pH of 7.4 – the normal physiological state of cells. Also, the structure of multiple emulsions and drop sizes influenced the release rates, and thus can be factors controlling drug delivery. This paper proposes an efficient method to prepare stable pH-responsive multiple (double) emulsions in a helicoidal contactor. Depending on the preparation conditions, this method enables emulsions with different characteristics (internal structure, encapsulation efficiency and drop sizes) to be obtained [21, 22]. Thus, it gives the possibility to create a multiple/double-based carrier of a chemotherapy drug with a specific release profile. All coupled factors may provide insight into designing liquid implants in the form of multiple emulsions with pH-responsive biocompatible polymers like carboxymethylcellulose for the controlled and selective delivery of chemotherapeutic to brain tumors.

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