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Formulation, drug release features and *in vitro* cytotoxic evaluation of nonionic mixed surfactant stabilized water-in-oil microemulsion loaded with doxorubicin

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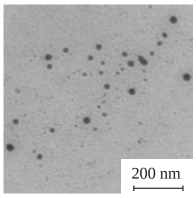
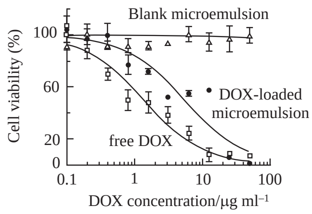
New water-in-oil microemulsion, based on paraffin oil, polyglycerol polyricinoleate and Tween 80, has been formulated and used as encapsulation matrix for doxorubicin. Drug release features and *in vitro* cytotoxicity for the formulation has been evaluated.





Doxorubicin (DOX) has been known as an important anticancer drug with a broad range of antitumor activity.[1,2] The intravenous injection is the most commonly used route of its administration.[3] Alternatively, the oral delivery may be beneficial compared to the intravenous one, since it modulates the drug toxicity and improves its therapeutic efficacy. At the same time, the oral administration of DOX is hampered by its low bioavailability due to the limited intestinal absorption. To enhance the bioavailability, various nanocarrier-based drug delivery systems such as liposomes, micelles, nanoparticles, nanocapsules and colloidal nanoformulations based on emulsions have been developed.[4–11] Among them, the microemulsions, *i.e.* isotropic thermodynamically stable colloidal systems which are composed of water, oil and a surfactant/co-surfactant mixture and which contain disperse phase droplets with less than 100 nm diameter, have found application in drug delivery[12–15] due to their stability, ease of preparation, protection of the loaded drugs against a hydrolysis in the gastric environment and finally due to an ability to incorporate a wide range of pharmaceuticals. It has been shown that microemulsions are able to improve the oral absorption of DOX, providing controlled release of the drug and positively influencing its pharmacokinetic parameters and biodistribution.[16]

The aim of this work was to develop water-in-oil (W/O) microemulsion based on paraffin oil, polyglycerol polyricinoleate (PGPR) and Tween 80 as the potential DOX delivery system with oral sustained release. PGPR having hydrophilic–lipophilic balance (HLB) value of 3.5 and Tween 80 with HLB value of 15 were chosen as the hydrophobic and the hydrophilic emulsifier, respectively. PGPRs represent a class of nonionic surfactants employed in the food industry for preparation of W/O and water-in-oil-in-water (W/O/W) emulsions.[17] Due to the absence of toxicity and low HLB value they are suitable for the fabrication of pharmaceutical oral dosage forms. At the same time, only few articles have described the preparation of PGPR-stabilized microemulsions loaded with biologically active substances.[18,19]

The four-component pseudo-ternary phase diagrams of systems containing paraffin oil, PGPR, Tween 80 and water were constructed[†] using water titration method of surfactant–oil mixtures to evaluate the microemulsion existence area. The screening of mixtures with various PGPR–Tween 80 mass ratios revealed that the microemulsion systems were formed at the ratios ranging from 2 : 1 to 7 : 1. The minimal concentration of the mixed surfactant, required for the microemulsion formation, was 72, 54, 63, 72 and 81% at the surfactant ratio of 2 : 1, 3 : 1, 4 : 1, 5 : 1 and 7 : 1, respectively. For further preparation of W/O microemulsion samples, the composition with 54% of emulsifiers and PGPR–Tween 80 ratio of 3 : 1 and hence the lowest viscosity, was chosen. The lowest viscosity composition is preferred for oral administration due to swallowing relief. It was shown that the blend of the surfactants at this ratio reduced the interfacial tension to more extent compared to the free surfactants.[‡] Thus, the PGPR–Tween 80 mixture revealed the synergistic effect in the efficiency of surface tension reduction (Figure 1).

The compositions with water content 1–10 wt% were homogeneous, transparent and yellow-colored. Further increase in the

[†] For construction of each phase diagram, paraffin oil (Pionier, Hansen & Rosenthal Group, Germany) and blend of the surfactants [polyglycerol-3 polyricinoleate (Gobiotics BV, Netherlands) and Tween 80 (Sigma, USA) at weight ratios 1.5 : 1, 2 : 1, 3 : 1, 4 : 1, 5 : 1, 7 : 1 and 10 : 1] were mixed carefully at paraffin oil–surfactant blend weight ratios from 9 : 1 to 1 : 9. The mixture at each weight ratio was titrated with distilled water. The equilibration time period between the additions of successive aliquots was from 1 to 24 h. The systems were observed visually. Among the several types of systems formed, a monophasic region corresponding to the existence of homogeneous, clear, optically transparent and fluid W/O microemulsions was detected. Each experiment was repeated three times.

[‡] Interfacial tension was measured by the drop-weight method using a Harvard Apparatus model 55-2222 stalagmometer (USA) at room temperature in triplicate and calculated on the basis of Tate's law.[20] The highest value of the relative standard deviation did not exceed 10% (n = 3).

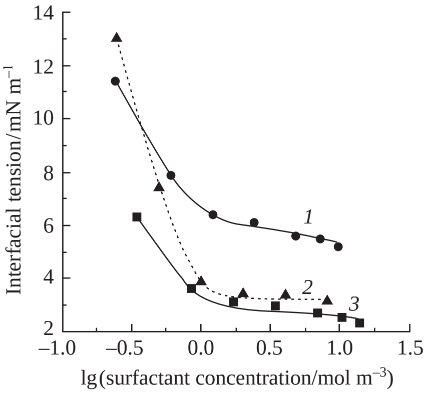


Figure 1 The interfacial tension between water and paraffin oil containing (*1*) PGPR, (*2*) Tween 80 and (*3*) PGPR–Tween 80 blend with 3 : 1 weight ratio *vs.* logarithm of the surfactant concentration.

aqueous phase content resulted in the turbidity appearance followed by the phase separation. According to the principle of water content maximization in the microemulsion system, the formulation composed of 36 wt% paraffin oil, 54 wt% PGPR–Tween 80 mixture with 3 : 1 weight ratio and HLB value of 6.4 as well as 10 wt% aqueous phase was chosen for further investigation.

This formulation exhibited kinetic stability after 62 days of storage in glass vial at room temperature keeping transparency and optical isotropy without phase separation. Microemulsion loaded with DOX was prepared by introducing 20 mg ml^{-1} of the drug aqueous solution into the mixture of the surfactants and oil at appropriate weight ratio. The loaded microemulsion had the overall concentration of drug of 2 mg ml^{-1}. The mean droplet size *d* of the samples was determined using transmission electron microscopy (TEM).[§] In the presence of DOX the droplet size slightly increased (Figure 2). The polydispersity indices for blank and drug-loaded microemulsions were 1.7 and 1.3, respectively. Since liquid drug formulations such as microemulsions are typically diluted with the gastrointestinal fluid after oral administration, the average droplet diameter for the unloaded and DOX-loaded microemulsions after dilution was also evaluated[¶] and found to be 200 ± 20 nm.

The *in vitro* drug release profile[††] from the DOX-loaded microemulsion and the control free DOX solution sample (Figure 3) demonstrated a reduction in release rate as a result of incorporation of the drug into the microemulsion. The DOX-loaded microemulsion exhibited a lower burst effect and released *ca.* 13% of the active substance for 48 h. The prolonged drug release can be explained by the barrier to the diffusion of drug molecules into the external medium, which was created by the layers of surfactants adsorbed at the surface between the dispersed phase droplets and the viscous continuous phase.

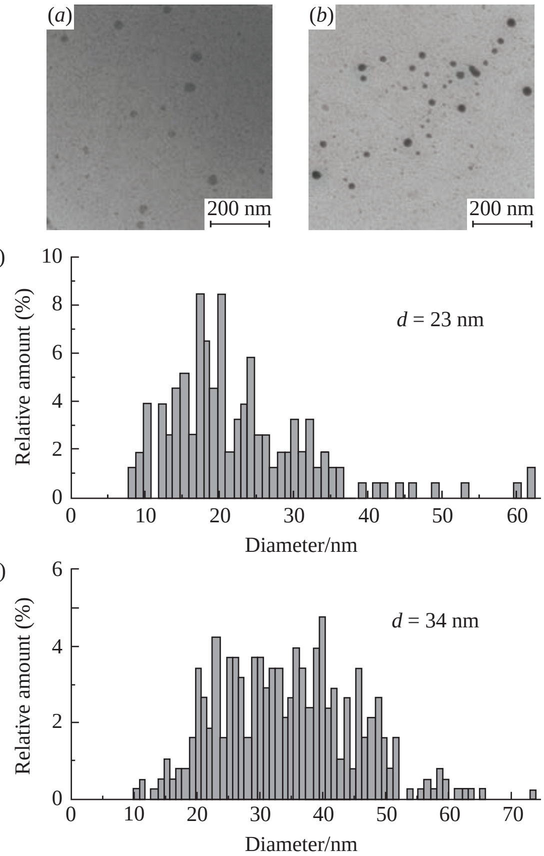


Figure 2 TEM images of (*a*) blank microemulsion, (*b*) DOX-loaded microemulsion; droplet size distribution for (*c*) blank microemulsion, (*d*) DOX-loaded microemulsion.

The toxicity of DOX aqueous solution, drug-loaded microemulsion and unloaded microemulsion to human breast adenocarcinoma cell line MCF-7 was investigated at different DOX concentrations for 48 h.[‡‡]

The increase in DOX concentration resulted in the decrease of viability of the cells treated with the DOX-containing samples (Figure 4). When the drug concentration increased from 0.4 to 12.5 μg ml^{-1}, MCF-7 cells treated with DOX-loaded formulation (Figure 4, curve *1*) had higher cell viability (p < 0.05) as

[§] The droplets of blank and DOX-loaded formulations were stained with 2% aqueous solution of phosphotungstic acid and dried at room temperature. Then the microemulsions were analyzed using a LEO 912 AB OMEGA TEM microscope (Carl Zeiss, Germany). The droplet size and size distribution were calculated using an ImageScope M software (SMA Ltd., Russia).

[¶] The microemulsions were used at 2000-fold dilution with water for injection (Solopharm, Russia). The mean particle diameter of the diluted samples was measured using a Multi-angle dynamic and static light scattering instrument Photocor Complex (Russia) with avalanche photodiode photon counting system at 25 °C at the scattering angle of 90°.

[††] To estimate *in vitro* drug release of the DOX-loaded microemulsion, an aliquot of each DOX aqueous control solution and DOX-loaded formulation was placed in a dialysis bag (MWCO 10000, Sigma, USA) and immersed in 0.01 M phosphate-buffered saline pH 7.4 (50 ml) in a shaking incubator at 37 °C and 180 rpm. At predetermined time intervals, the aliquots of release medium were withdrawn. Each aliquot was replaced by equal volume of fresh PBS to compensate for the loss of volume. DOX concentration was determined by UV-VIS spectrophotometry at 480 nm using a Multiskan FC apparatus (Thermo Fisher Scientific, USA).

[‡‡] MCF-7 cells were cultured in growth medium comprising DMEM/F-12 (Gibco), 10% FBS (Gibco), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Gibco) at 37 °C in an atmosphere of 5% CO_2. Toxicity of DOX solution, DOX-loaded and blank microemulsions to MCF-7 cells was evaluated by PrestoBlue (Thermo) assay. The cells were seeded onto a 96-well plate at a seeding density of 10 × 10^3 cells/well and cultured for 24 h. Then, the culture medium was replaced by DOX microemulsion at 40–20000-fold dilution (final concentrations 0.1–50 μg ml^{-1}), DOX solution (concentrations 0.1–50 μg ml^{-1}) and blank (unloaded) microemulsion at 40–20000-fold dilution. All drug dilutions were carried out with growth medium. Blank growth medium was employed as a negative control. Each assay was repeated five times. After 48 h incubation, the culture medium was replaced with addition of PrestoBlue assay reagent according to the manufacturer's instructions and the plate was incubated for another 0.5 h at 37 °C in an atmosphere of 5% CO_2. Fluorescence measurements were carried out with a Clariostar fluorimeter (BMG LABTECH) with the excitation/emission wavelengths set at 545/600 nm. Statistical analysis was performed utilizing a GraphPad Prism 7.00 program (GraphPad Software, La Jolla, CA, USA) by two-way ANOVA methods with a Bonferroni post-hoc test, Shapiro-Wilk's normality test and four-parameter sigmoidal dose-response curve to measure IC_{50} values. The data are presented as means ± standard deviation (SD), the differences were considered statistically significant at p < 0.05.

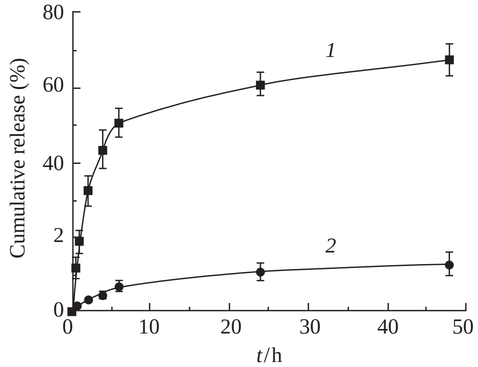


Figure 3 Time profile of *in vitro* DOX release: (*1*) from solution and (*2*) from drug-loaded microemulsion at 37 °C in 0.01 M PBS, pH 7.4. Data represent the mean ± SD (n = 3).

compared to those treated with DOX solution (curve *2*) owing to the sustained drug release from the microemulsion. For the same reason, IC_{50} values exhibited the higher cytotoxic effect for free DOX than for DOX-loaded microemulsion. At the same time, there were no statistically significant differences observed between the DOX-containing samples at low (0.1–0.2 μg ml^{-1}) and high (25–50 μg ml^{-1}) DOX concentration. The latter reveals that the microemulsion loading enhanced DOX delivery into MCF-7 cells compared to the cellular uptake of the free DOX.

The cell viability of the blank PGPR-stabilized microemulsion was 95.3 ± 5.6% during 48 h test on all concentrations indicating no cytotoxicity to MCF-7 cells.

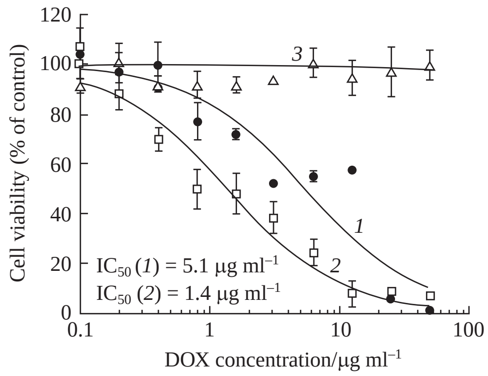


Figure 4 Dose-dependent response of MCF-7 cells to (*1*) DOX-loaded microemulsion, (*2*) free DOX and (*3*) blank microemulsion. The drug concentration was 0.1–50 μg ml^{-1} and the incubation period was 48 h. Data represent the mean ±SD (n = 5).

In conclusion, a new drug-loaded water-in-oil microemulsion stabilized by PGPR and Tween 80 mixture has been obtained and characterized. The results demonstrate that this microemulsion can be considered as a promising carrier for the prolonged delivery of doxorubicin.

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