Evaluation of physicochemical and antioxidant properties of nanosized copolymeric micelles loaded with kaempferol

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Abstract

The study was focused on the evaluation of two copolymers as micellar carriers for kaempferol delivery. The copolymers comprised identical hydrophilic blocks of poly(2-(dimethylamino)ethyl methacrylate) and different hydrophobic blocks of either poly(ε-caprolactone) (PDMAEMA₉-b-PCL₇₀-b-PDMAEMA₉) or poly(propylene oxide) (PDMAEMA₁₃-b-PPO₆₉-b-PDMAEMA₁₃). The calculation of Flory-Huggins parameters and determination of encapsulation efficiency showed that PDMAEMA-b-PCL-b-PDMAEMA copolymer possessed higher capacity for kaempferol loading. The diameter of the micelles before and after lyophilization was not changed, suggesting that the micelles could be lyophilized and redispersed before administration. The in vitro release of kaempferol from PDMAEMA-b-PPO-b-PDMAEMA micelles was faster than the release from PDMAEMA-b-PCL-b-PDMAEMA micelles, probably due to the higher affinity of kaempferol to this copolymer. Further, the higher affinity resulted in a retention of antioxidant activity of kaempferol in the presence of DPPH and KO₂ radicals. Thus, PDMAEMA-PCL-PDMAEMA was considered more appropriate carrier because of the higher encapsulation efficiency and preservation of antioxidant activity of the drug.

Keywords
copolymeric micelles, nanosized systems, kaempferol, radical scavenging activity

Introduction

Copolymeric micelles are core-shell nanoaggregates formed by amphiphilic block copolymers that self-assemble in an aqueous medium above certain concentration known as critical micellar concentration. Copolymeric micelles are intensively investigated drug delivery carriers due to the high potential for efficient loading of hydrophobic active molecules in their core (Kwon 2003; Miyata et al. 2011; Lu and Park 2013). Core-forming hydrophobic blocks are usually biodegradable polyesters, e.g. poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(propylene oxide) (PPO), copolymers of lactic and glycolic acids, etc (Meier et al. 2005; Lee et al. 2009; Somekawa et al. 2015). The hydrophilic shell of the micelles provides long blood circulation and physical stability in...
physiological fluids. The hydrophilic shell of micelles is typically based on poly(ethylene glycol) (PEG), poly(acrylic acid) (PAA) or their combination (Kwon 2003; Andre et al. 2005; Yoncheva et al. 2015a). In all cases, the composition of the copolymers is related to the physicochemical properties (size, dispersity and zeta-potential), loading capacity, stability and in vivo distribution of the resulted micelles (Kim et al. 2010).

Antioxidants are an important class of active substances related to the treatment of many diseases associated with oxidative stress. However, many of these substances, especially those with a natural origin, are highly unstable in vitro or in vivo. For example, flavonoids could be degraded during processing or storage at inappropriate conditions, e.g. light or oxygen exposure (Chaaban et al. 2017). Kaempferol is a well-known antioxidant but its hydrophobic properties hinder its formulation in typical dosage forms as well as limit its bioavailability. Thus, the development of drug delivery systems that are able to improve kaempferol dissolution, stability and bioavailability is highly required. Tzeng et al. (2011) showed that water dispersion of kaempferol loaded nanoparticles exerted stronger radical scavenging activity than similar dispersion of free kaempferol. A recent study has reported that encapsulation of kaempferol in chitosan nanoparticles provided higher scavenging activity and maintained this activity for longer storage compared to the non-encapsulated drug (Ilk et al. 2017).

The aim of the present study was to evaluate two amphiphilic copolymers as micellar carriers for kaempferol delivery. The copolymers comprised nearly the same shell-forming blocks of poly(2-(dimethylamino)ethyl methacrylate (PDMAEMA) and different hydrophobic core-forming blocks (PCL or PPO). Thus, the work was focused on assessing the main physicochemical properties of kaempferol loaded micelles prepared from the two copolymers as well as their potential as antioxidant delivery systems.

**Materials and methods**

**Materials**

Kaempferol, 1,4-dioxane, 2,2-diphenyl-1-picrylhydrazyl (DPPH), luminol and potassium superoxide were purchased from Sigma-Aldrich. The triblock copolymers PDMAEMA<sub>9</sub>-b-PCL<sub>69</sub>-b-PDMAEMA<sub>13</sub> and PDMAEMA<sub>9</sub>-b-PCL<sub>69</sub>-b-PDMAEMA<sub>13</sub> were previously synthesized as reported elsewhere (Petrov et al. 2008; Yoncheva et al. 2015b).

**Determination of Flory-Huggins parameter**

Flory-Huggins parameter $\chi_{sp}$ was calculated applying the equation:

$$\chi_{sp} = \frac{V_s (\delta_s - \delta_p)^2}{RT}$$

where $V_s$ is the molar volume of the drug, $\delta_s$ and $\delta_p$ are the Schatchard-Hildebrand solubility parameters of the drug and polymer block forming the core, $R$ is the gas constant and $T$ is the Kelvin temperature (Flory 1953). The solubility parameters were calculated applying the Fedors method that is based on the contribution of the chemical groups in the molecules to their cohesive energy (Fedors 1974).

**Preparation of kaempferol loaded micelles**

Kaempferol loaded PDMAEMA-b-PCL-b-PDMAEMA and PDMAEMA-b-PPO-b-PDMAEMA micelles were prepared by the solvent evaporation method. Briefly, the selected copolymer (10 mg) and kaempferol (1.5 mg) were dissolved in 5 ml of 1,4-dioxane. After incubation for 30 min. (700 rpm), 2 ml of purified water was added dropwise to the organic phase. Next, the dioxane was evaporated under reduced pressure (Buchi-144, Switzerland) and the resulted micellar dispersions were filtered (0.22 µm) to separate the micelles from non-encapsulated drug. The filter was rinsed with ethanol and this drug fraction was collected to determine the drug loading efficiency. The aqueous micellar dispersions were lyophilized using sucrose as a lyoprotector.

**Characterization of the drug-loaded micelles**

The size, dispersity and zeta potential of drug-loaded micelles were determined by dynamic and electrophoretic light scattering using a Zetasizer NanoBrook 90Plus PALS, equipped with a 35 mW red diode laser, ($\lambda = 640$ nm) at a scattering angle of 90°. The zeta potential was calculated from the obtained electrophoretic mobility. All samples were measured at 25 °C.

Atomic force microscopy (AFM) images were obtained using a Bruker NanoScope V9 Instrument operating at 1.00 Hz scan rate under ambient conditions. The micelle solution (0.5 mg/ml) was spin-casted (2000 rpm) on a freshly cleaned glass substrate. AFM measurements were performed in Peak Force Tapping mode.

Kaempferol encapsulation was calculated as a difference between the initial concentration of the drug and the concentration found in the ethanol fractions collected after the filtration of the fresh micellar dispersion. Kaempferol was determined by UV-Vis spectrophotometry at a wavelength of 266 nm (ThermoScientific) according to a standard curve (5–25 µg/ml, r>0.9992). The encapsulation efficiency (EE) was calculated using the following equation:

$$EE(\%) = \frac{\text{Total amount of drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100$$

**In vitro release study**

In vitro release of kaempferol from the micelles was examined in distilled water. The freshly prepared micellar dispersions were introduced into a dialysis membrane bag
(MW=6000–8000) that further was placed into 100 ml distilled water. The release medium was stirred (50 rpm) and the temperature was maintained constantly during the study (37 °C). At predetermined time intervals samples were withdrawn from the medium outside the dialysis bag and the concentration of the released kaempferol was determined by UV-Vis spectrophotometry as described above.

In vitro antioxidant activity

The antioxidant activity of free kaempferol and kaempferol-loaded micelles was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and potassium superoxide scavenging assay (KO2). DPPH assay was performed according to a previously reported procedure (Goupy et al. 2003). Shortly, an ethanol solution of DPPH radical (2 ml) was incubated with the solutions of pure kaempferol or its micellar dispersions at room temperature. After 60 min., the absorbance of the samples was measured at 517 nm and the results were presented as percentage from the control sample.

Potassium superoxide scavenging assay (KO2) was performed by detection of the luminol-dependent chemiluminescence in a system of KO2 produced O2 -●. The apparatus (LKB 1251 luminometer, BioOrbit, Finland) was connected with AT-type computer via serial interface and MultiUse program ver. 1.08 for the collection of the obtained experimental data. The tested solutions of pure and micellar drug were mixed with 1 ml phosphate saline buffer (pH=7.4) containing 0.1 mM luminol. In parallel, control solutions without the tested pure or micellar kaempferol were prepared. The chemiluminescence response was measured immediately after the addition of 20 µl KO2 solution in DMSO. The chemiluminescence was registered for 1 min. every 50 milliseconds after the addition of KO2. The chemiluminescent response was calculated by determination of the area under the obtained chemiluminescent curve. The chemiluminescent ratio in the presence/absence of the tested compounds in percentage was used for calculation of the scavenging properties of the samples (Hadjimitova et al. 2002).

Results and discussion

In the present study triblock copolymers containing blocks of PDMAEMA, in particular PDMAEMA13-b-PCL70-b-PDMAEMA, or PDMAEMA13-b-PPO69-b-PDMAEMA, were examined as micellar carriers of kaempferol taking in account the safety profile of copolymers containing short chains of PDMAEMA (Tzankova et al. 2016). Kaempferol loaded polymeric micelles were prepared by self-assembly of either PDMAEMA13-b-PCL70-b-PDMAEMA or PDMAEMA13-b-PPO69-b-PDMAEMA amphiphilic triblock copolymers in aqueous media using the solvent evaporation method. This procedure yielded nanosized carriers of monomodal size distribution (Fig. 1). The mean hydrodynamic diameter and zeta potential of blank and drug-loaded micelles are given in Table 1. In general, the drug-loaded micelles were larger than empty ones and the size distribution of all systems was relatively narrow. Both types of micelles were positively charged due to the polycationic nature of PDMAEMA shell. In addition, slightly higher zeta potential of kaempferol loaded micelles as compared to the blank micelles was registered for both systems. Taking into account the values of zeta potential, the micelles were supposed to be stable against aggregation. Noteworthy, the size of PDMAEMA13-b-PPO69-b-PDMAEMA micelles was definitely larger that PDMAEMA13-b-PCL70-b-PDMAEMA micelles. This fact is even more pronounced for the drug-loaded system (Fig. 1).

AFM analysis revealed that PDMAEMA13-b-PPO69-b-PDMAEMA13 copolymer formed a mixture of spherical and anisotropic (elongated structures) micelles, unlike the PDMAEMA13-b-PCL70-b-PDMAEMA copolymer which formed only spherical micelles (Fig. 2). One may consider two specific factors contributing to the obvious increase of hydrodynamic diameter of the micelles after kaempferol loading. Firstly, embedding drug molecules into micellar core itself enlarged micelle dimensions. Secondly, PPO is a soft amorphous polymer and therefore the aggregates comprising PPO core are considered dynamic systems. Thus, the loading of kaempferol might have more pronounced effect on the structural rearrangements for PDMAEMA13-b-PPO69-b-PDMAEMA13 micelles (transition from spherical to elongated structures), while the less distinctive size changes observed for PDMAEMA13-b-PCL70-b-PDMAEMA13 micelles can be attributed to the formation of “frozen” micelles, due to the crystallisation of PCL.

The physicochemical properties of the micelles after lyophilization and redispersion are very important for their in vivo administration, efficiency and safety. The main characteristics that could be changed by lyophilization are the micellar size and the tendency for aggregation (Di Tommaso et al. 2010). Thus, our further task was to evaluate the influence of the lyophilization on the mean diameter of both type

![Figure 1](image-url)
of kaempferol loaded micelles. The diameter of the micelles was determined after redispersion of the lyophilized micelles in distilled water or aqueous 0.9 % NaCl solution. The latter was selected as dispersion medium aiming to examine for eventual aggregation of the micelles in the presence of electrolyte. The comparison of the data for the micellar diameter in water before and after lyophilization revealed that there were not significant changes for both types of the micelles (not shown). These results suggested that the micelles could be lyophilized, stored and redispersed in a suitable aqueous medium without a change of their diameter. The latter is important because a development of homogeneous aqueous dosage form of kaempferol is practically very difficult because of its low solubility (Telange et al. 2016).

The two copolymers are similar regarding their macrochain architecture and the type and length of the hydrophilic segments; so their distinctive feature is the type of the hydrophobic block. Since kaempferol is a hydrophobic substance it is expected that drug molecules will be embedded into the hydrophobic micellar cores. Therefore, the affinity of the active molecule to the core-forming polymer is of a big importance for the efficient loading, release of the active substance and in vitro and in vivo stability of micelles (Nishiyama and Kataoka 2006; Cabral and Kataoka 2014). It is widely accepted for micellar systems that the determination of Flory-Huggins interaction parameter ($\chi_{sp}$), According to the theory, as the value of $\chi_{sp}$ is closer to zero, this indicates higher affinity between the drug and the hydrophobic segment forming micellar core. Table 2 represents the calculated values for drug-polymer compatibility ($\chi_{sp}$). As seen, kaempferol has a higher affinity (lower values for Flory-Huggins parameter) for PCL-hydrophobic segment. Indeed, these results correlated with the results found for encapsulation efficiency of kaempferol in both types of polymeric micelles (Table 2). In correlation with calculated Flory-Huggins parameters, higher efficiency was obtained in copolymeric micelles containing PCL segment. These results revealed that regarding loading capacity PDMAEMA$_{9}$-b-PCL$_{70}$-b-PDMAEMA$_{9}$ copolymer was more appropriate carrier than PDMAEMA$_{13}$-b-PPO$_{69}$-b-PDMAEMA$_{13}$.

The in vitro release of kaempferol from the micelles was performed in distilled water. The study showed the presence of initial burst effect and sustained release in the second phase (Fig. 3). The release of kaempferol from PDMAEMA$_{9}$-b-PPO$_{69}$-b-PDMAEMA$_{13}$ micelles was faster than the release from PDMAEMA$_{13}$-b-PCL$_{70}$-b-PDMAEMA$_{13}$ ones. In particular, in the case of PDMAEMA$_{13}$-b-PPO$_{69}$-b-PDMAEMA$_{13}$, 100 % release was achieved in 24 h, whereas the complete release of kaempferol from PDMAEMA$_{9}$-b-PCL$_{70}$-b-PDMAEMA$_{9}$ micelles occurred after 48 h. These results could be explained with the higher affinity of kaempferol to the copolymer containing PCL-segment, which probably sustained its release.

### Table 1. Physicochemical properties of empty and kaempferol loaded micelles. Mean ± SD.

| Micelles | Mean diameter (nm) | Dispersity | Zeta-potential (mV) |
|----------|--------------------|------------|---------------------|
| PDMAEMA$_{9}$-b-PCL$_{70}$-b-PDMAEMA$_{9}$ | 134 ± 7 | 0.13 | 36.5 ± 5 |
| KF-PDMAEMA$_{9}$-b-PCL$_{70}$-b-PDMAEMA$_{9}$ | 161 ± 3 | 0.14 | 45.6 ± 2 |
| PDMAEMA$_{13}$-b-PPO$_{69}$-b-PDMAEMA$_{13}$ | 170 ± 4 | 0.15 | 34.9 ± 4 |
| KF-PDMAEMA$_{13}$-b-PPO$_{69}$-b-PDMAEMA$_{13}$ | 247 ± 4 | 0.17 | 40.3 ± 2 |

**Figure 2.** AFM images of kaempferol loaded PDMAEMA$_{9}$-b-PCL$_{70}$-b-PDMAEMA$_{9}$ (left) and PDMAEMA$_{13}$-b-PPO$_{69}$-b-PDMAEMA$_{13}$ (right) micelles.
Table 2. Calculated values for solubility parameters (δ), drug-polymer compatibility (χ_sp) for PCL- and PPO-containing copolymers and encapsulation efficiency (EE).

| Kaempferol / Copolymer                  | δ (MPa^{1/2}) (Fedors method) | χ_sp | EE (%) |
|----------------------------------------|-------------------------------|------|--------|
| Kaempferol                             | 34.2                          |      |        |
| PDMAEMA-b-PCL-b-PDMAEMA                | 19.7                          | 14.5 | 66     |
| PDMAEMA-b-PPO-b-PDMAEMA                | 16.1                          | 22.4 | 61     |

In addition, the crystallisation of PCL-core compared to the soft amorphous nature of PPO also might contribute to slower release of kaempferol. A recent study reported faster release of aniline pentamer from PEG- poly(ε-decalactone) micelles with amorphous core compared to PEG-poly(ε-caprolactone) micelles with semi-crystalline core (Glavas et al. 2015).

The antioxidant activity of free and micellar kaempferol was evaluated in two model systems, in particular systems containing stable DPPH radicals or superoxide radicals (KO₂). It is known that superoxide radicals participate in the formation of peroxynitrite, which is the most reactive form of the active forms of nitrogen. The radical scavenging activity of kaempferol loaded micelles and free kaempferol are presented in Fig. 4. The presence of empty micelles did not change the concentration of the radicals in both model systems (not shown). The results for kaempferol loaded into PPO-containing micelles revealed loss of its scavenging activity compared to the free drug (Fig. 4a). This tendency was more pronounced in the system with superoxide radicals, in particular the concentration of the radicals scavenged by micellar kaempferol was 36 % vs. 80 % by free kaempferol. The studies with kaempferol loaded into PCL-containing micelles showed retention of antioxidant activity of the drug in the presence of DPPH and KO₂ radicals (Fig. 4b). These results would be considered advantageous taking in account that the effect was achieved with aqueous dispersion, whereas the referent kaempferol was in the form of hydroalcoholic solution, which is not appropriate for in vivo administration.

**Conclusion**

The data in the present study suggest that micelles formed by PDMAEMA-PCL-PDMAEMA copolymer are appropriate system for delivery of kaempferol as the drug was well dissolved in aqueous media with the aid of micelles. The good compatibility between PCL block and kaempferol favoured a sustained drug release profile and contributed to preserve its antioxidant activity. In addition, the micelles maintained their structural integrity and nanosized dimensions after lyophilization and redispersion that encourage their further evaluation as drug delivery system of kaempferol.

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