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Research Article

Improvement of *in vitro* antioxidant activity of kaempferol by encapsulation in copolymer micelles

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Abstract

Antioxidant capacity of poorly soluble natural antioxidant kaempferol, in particular free or loaded in two types of cationic micelles, was studied on non-enzyme induced lipid peroxidation (LPO) *in vitro*. The micelles were based on triblock copolymers - poly(2-(dimethylamino)ethyl methacrylate-b-poly(propylene oxide)-b-poly(2-(dimethylamino)ethyl methacrylate (PDMAEMA-PPO-PDMAEMA) and poly(2-(dimethylamino)ethyl methacrylate-b-poly(ε-caprolactone)-b-poly(2-(dimethylamino)ethyl methacrylate (PDMAEMA-PCL-PDMAEMA). The lipid peroxidation was induced by incubating of rat liver microsomes with iron sulphate and ascorbic acid (Fe^{2+}/AA). The effect of free and micellar kaempferol (at concentrations 25, 50 and 75 μg/ml) was assessed after 20 min incubation time. In the non-enzyme lipid peroxidation model, the kaempferol-loaded micelles significantly decreased the formation of malondialdehyde (MDA). The effect of kaempferol loaded in PDMAEMA-PCL-PDMAEMA micelles was more pronounced, showing an improved antioxidant activity in the conditions of oxidative stress and lipid peroxidation *in vitro*.

Keywords

kaempferol, *in vitro*, antioxidant, copolymer micelles

Introduction

Natural antioxidants are very attractive active substances because of their high potential to prevent or treat various diseases associated with oxidative stress. Oxidative stress occurs due to the lost balance between the prooxidant and antioxidant reactions in living organisms (Bhat et al. 2015). The overproduction of oxidative free radicals and reactive oxygen species (ROS) could attack and damage cellular proteins, lipids or DNA, inhibiting their normal function (Rani et al. 2016). Oxidative stress has been implicated in the pathophysiology in number of human pathologies, such as Alzheimer's and Parkinson's diseases (Halliwell, no date), hypertension (Montezano et al. 2015) and cardiovascular diseases (Mei et al. 2015), diabetes (Giacco, Brownlee and Schmidt, 2010), carcinogenesis (Andrisic et al. 2018) etc. Flavonoids, anthocyanins and carotenoids are natural antioxidants, widely distributed in fruits and vegetables, and are reported to exert multiple biological effects. The flavonoids possess antioxidant activity which could be explained by the ability to scavenge reactive oxygen and singlet oxygen species by single-electron transfer (Hirano et al. 2001), inhibiting several enzymes such as oxidase, lipoxygenase, protein kinase C, cyclooxy-

genase, glutathione S-transferase (Masella et al. 2005; Agrawal 2011) and transition metal ion chelating activity (Wang and Bi 2018).

The flavonoids (quercetin, rutin, kaempferol etc.) and hydroxycinnamic acids (caffeic, ferulic etc.) are intensively studied natural antioxidants. Kaempferol is a flavonol showing a variety of activities, including antioxidant, cytoprotective, anticancer etc. (Upadhyay et al. 2010; Chen and Chen 2013; Kashyap et al. 2017). However, the low water solubility and instability are closely related to its poor bioavailability. Different approaches could be considered for improvement of stability, bioavailability and activity of kaempferol. Deng et al. (2019) have reported an improvement of its solubility and antioxidant activity by synthesis of appropriate kaempferol derivatives (Deng et al. 2019). Tu et al. 2016 have synthesized kaempferol-zinc(II) complex, which showed two times stronger anticancer effect on human oesophageal cancer cell line (EC9706) than free kaempferol (Tu et al. 2016). Another innovative approach is to encapsulate kaempferol into micro- or nanoparticulate systems. It has been reported that incorporation of kaempferol in liposomes could increase its stability (Huang et al. 2017). Yoncheva et al. (2019) have developed polymeric nanoparticles based on poly(ε-caprolactone) and micelles based on its copolymer with poly(2-(dimethylamino)ethyl methacrylate) (Yoncheva et al. 2019). The results revealed that the micelles provide preservation of antioxidant activity of kaempferol during storage.

The aim of the present study was to evaluate the potential of two polymeric micellar systems to improve antioxidant activity of kaempferol in the model of iron/ascorbic acid (Fe^{2+}/AA) induced lipid peroxidation in rat liver microsomes.

Materials and methods

Materials

Kaempferol (KF) was purchased from Sigma Aldrich (Germany). The triblock copolymers poly(2-(dimethylamino)ethyl methacrylate-b-poly(propylene oxide)-b-poly(2-(dimethylamino)ethyl methacrylate ($PDMAEMA_{13}$-PPO_{69}-$PDMAEMA_{13}$) and poly(2-(dimethylamino)ethyl methacrylate-b-poly(ε-caprolactone)-b-poly(2-(dimethylamino)ethyl methacrylate ($PDMAEMA_9$-PCL_{70}-$PDMAEMA_9$) were synthesized in the Institute of Polymers (Bulgarian Academy of Science) according to previously established procedure (Petrov, Tsvetanov and Jérôme, 2008; Yoncheva et al. 2015) which were converted into ellipsoidal onion-like micelles on mixing with the PEO45-block-P(DMAEMA8-co-MMA4. Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), nicotinamide adenine dinucleotide phosphate, ascorbic acid, iron sulphate, potassium hydrogen sulphate, potassium-sodium tartrate, glycerol, sodium carbonate and sodium hydroxide were obtained from Merck (Germany).

Preparation of drug loaded micelles

Kaempferol loading in PDMAEMA-PPO-PDMAEMA and PDMAEMA-PCL-PDMAEMA micelles was performed by solvent evaporation method. In a brief, the copolymer and kaempferol were dissolved in 5 ml dioxane. After their incubation for 30 min, 2 ml purified water was slowly added to the organic phase. Dioxane was evaporated under reduced pressure (Buchi-144, Switzerland) and the aqueous micellar dispersions were filtered (0.22 μm). The filter was rinsed with ethanol and these fractions were determined for non-encapsulated kaempferol.

Iron/ascorbic acid (Fe^{2+}/AA) induced lipid peroxidation in rat liver microsomes

The microsomes were isolated from the livers of male Wistar rats (200.0–220.0 g) purchased from the National Breeding Center, Sofia, Bulgaria. At least 7 days of acclimatization were allowed before the commencement of the study. The vivarium (certificate of registration of farm No. 0072/01.08.2007) was inspected by the Bulgarian Drug Agency to check the husbandry conditions (No. A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and made according Ordinance No. 15/2006 for humaneness behaviour to experimental animals.

The livers were thoroughly perfused with 1.15% KCl and homogenized with ice-cold 0.1 mol/L potassium phosphate buffer (pH = 7.4). The liver homogenate was centrifuged at 9,000×g for 30 min at 4 °C and the resulting postmitochondrial fraction was centrifuged at 105,000×g for 60 min at 4 °C. The microsomal pellets were dispersed in 0.1 mol/L potassium phosphate buffer, containing 20% glycerol. Microsomal protein content was determined according to the method of Lowry et al. (Lowry et al. 1951).

The microsomes were preincubated with the solutions of the pure kaempferol or with micellar dispersions of kaempferol at 37 °C for 15 min. The lipid peroxidation was started with a 20 μmol/L solution of iron sulphate and 0.5 mmol/L of ascorbic acid (Mansuy et al. 1986). After 20 min incubation the reaction was stopped with a mixture of 25% TCA and 0.67% TBA. The quantity of malondialdehyde (MDA) was assessed as described by Deby and Goutier (1990) (Deby and Goutier, 1990). The absorbance of the samples was measured at 535 nm (Spectro UV-VIS Split). The amount of MDA was calculated using a molar extinction coefficient of 1.56×105 mol^{-1} cm^{-1}. The final results were presented as nmol/mg protein.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance analysis of variance with Dunnett's *post hoc* test. Differences were accepted to be significant when $P < 0.05$. All statistical analysis was carried out on Graph Pad 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results and discussion

Physicochemical properties of nanosized drug delivery systems are very important for their in vivo behaviour. In particular, the size is a key factor for cellular uptake ability and distribution. Here, the micelles having PPO-core were larger in size than those prepared with a copolymer possessing PCL-core (Fig. 1), which could be explained with the amorphous structure of PPO-core. Regarding zeta-potential, both types of micelles possessed positive zeta-potential (40–45 mV) due to the micellar shell of PDMAEMA chains.

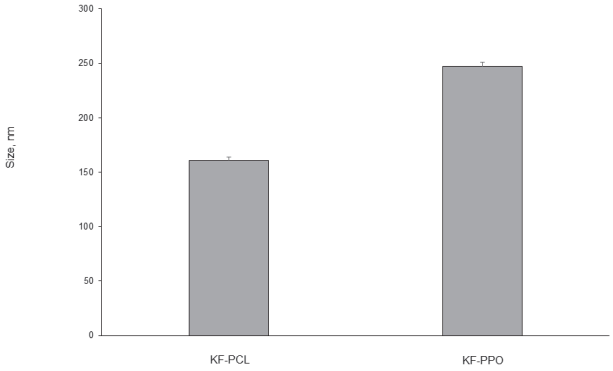

Figure 1. Mean diameter of both types of kaempferol loaded micelles – PDMAEMA-PCL-PDMAEMA (KF-PCL) and PDMAEMA-PPO-PDMAEMA (KF-PPO).

The objective of this study was to investigate the potential of free kaempferol and kaempferol loaded into two types of polymeric micelles to protect rat liver microsomes against iron/ascorbic acid (Fe^{2+}/AA) induced lipid peroxidation. Rat liver microsomes were selected as a relevant model for evaluation of antioxidant activity of natural antioxidants on microsomal lipid peroxidation (Afanas'ev et al. 1989). In fact, rat liver microsomes are one of the most commonly employed *in vitro* systems because they have good reproducibility and they are more readily available than human hepatocytes (Du et al. 2014). In the present study, the microsomes were incubated with a solution of iron sulphate and ascorbic acid (Fe^{2+}/AA) which were used for induction of non-enzyme lipid peroxidation model (Minotti and Aust, 1989).

Initially, we evaluated the effects of the empty and kaempferol loaded micelles (PDMAEMA-PCL-PDMAEMA and PDMAEMA-PPO-PDMAEMA) on non-treated rat liver microsomes. The microsomes were treated with three different concentrations (80, 160 and 240 μg/ml) of the empty polymeric micelles. The results showed no apparent induction of lipid peroxidation by empty micelles in non-treated rat liver microsomes (Fig. 2 and 3). A statistically significant increase in MDA was observed only after incubation of microsomes with the highest concentration 240 μg/ml of empty PPO-micelles (Fig. 3) However, no prooxidant effects were observed when kaempferol was loaded in PPO micelles at the same concentration. These results suggested that neither empty nor the kaempferol loaded micelles had prooxidant activity on the microsomes.

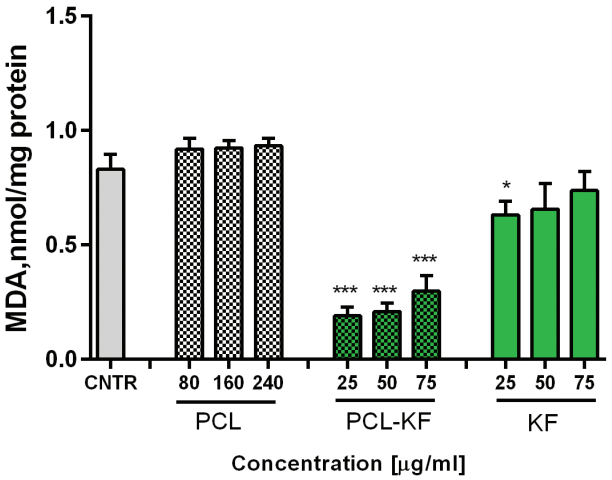

Figure 2. Effect of empty PDMAEMA-PCL-PDMAEMA (PCL) and kaempferol (KF) loaded PDMAEMA-PCL-PDMAEMA (KF-PCL) micelles (25, 50, 75 μg/ml) on the level of malondialdehyde (MDA) in non-treated rat microsomes.

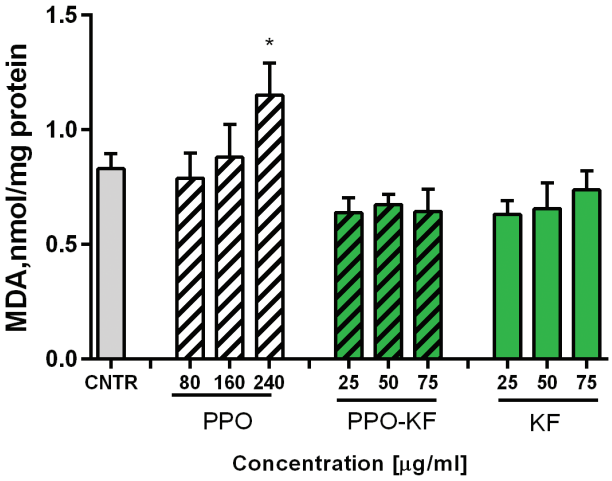

Figure 3. Effect of empty PDMAEMA-PPO-PDMAEMA (PPO) and kaempferol (KF) loaded PDMAEMA-PPO-PDMAEMA (PPO-KF) micelles (25, 50, 75 μg/ml) on the level of malondialdehyde (MDA) in non-treated rat microsomes.

The results are expressed as means ± SD of triplicate assays (n = 3). All groups were compared statistically *vs* untreated controls by one-way Anova with Dunnet's post-test. *$p < 0.05$; ***$p < 0.001$ *vs* control.

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In the next experiments, we evaluated the protective activity of free and micellar kaempferol (25, 50 and 75 μg/ml) in a lipid peroxidation model on iron/ascorbic acid treated microsomes. The results related to iron/ascorbic acid treated microsomes are presented on Fig. 4 and Fig.5. The treatment of rat microsomes with iron/ascorbic acid caused significant lipid peroxidation, increasing the level of MDA by 155%. It is also seen that the loading of kaempferol into

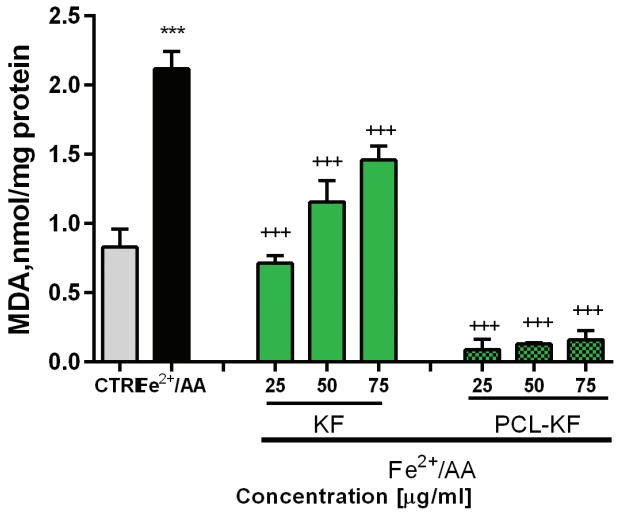

Figure 4. Protective effects of free kaempferol (KF) (25, 50, 75 μg/ml) and kaempferol loaded PDMAEMA-PCL-PDMAEMA (KF-PCL) micelles on the level of malondialdehyde (MDA) in iron/ascorbic acid (Fe^{2+}/AA) treated microsomes.

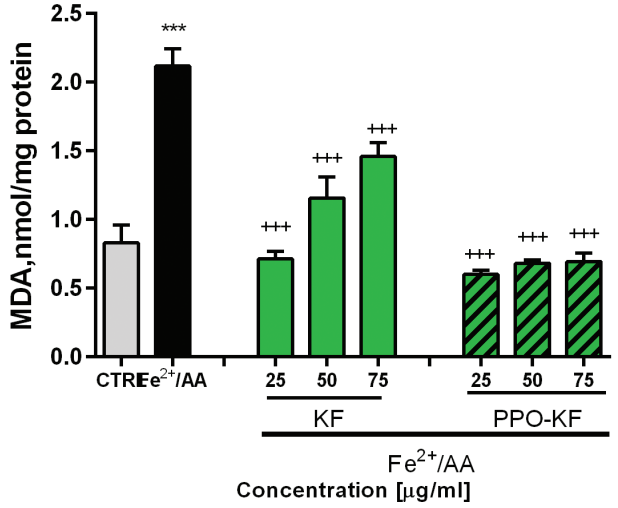

Figure 5. Protective effects of free kaempferol (KF) (25, 50, 75 μg/ml) and kaempferol loaded PDMAEMA-PPO-PDMAEMA (PPO-KF) micelles on the level of malondialdehyde (MDA) in iron/ascorbic acid (Fe^{2+}/AA) treated microsomes. Mean values ± SN (n = 6). *** $p < 0.001$ compared to untreated control group; $^{+++} < 0.001$ *vs* Fe^{2+}/AA is considered to be statistically significant.

both types of the micelles led to significantly higher potential for inhibition of lipid peroxidation than pure kaempferol. We suppose this could be explained with facilitated membrane transport of kaempferol when loaded into the nanosized micelles. In fact, our previous study with curcumin loaded polymeric PDMAEMA-PCL-PDMAEMA micelles showed a facilitated curcumin uptake by chronic myeloid leukemia derived K-562 cells and human multiple myeloma cells U-266 (Yoncheva et al. 2015). The facilitated membrane permeability is most probably due to an interaction between the cationic micelles and the negatively charged membrane surfaces. This suggestion correlated with other reports, showing better intracellular uptake of cationic nanoparticles in nonphagocytic cells than anionic ones (Mailander and Landfester, 2009).

Mean values ± SN (n = 6). *** $p < 0.001$ compared to untreated control group; $^{+++} < 0.001$ *vs* Fe^{2+}/AA is considered to be statistically significant.

The comparison between the protective activity of micellar kaempferol revealed a significant differences depending on the different polymeric micelles. When kaempferol was loaded into PDMAEMA-PCL-PDMAEMA micelles, the protective effects on Fe^{2+}/AA induced lipid peroxidation were more evident compared to free kaempferol and PDMAEMA-PPO-PDMAEMA micelles. For example, at 75 μg/ml kaempferol loaded PDMAEMA-PCL-PDMAEMA micelles (KF-PCL) decreased the level of MDA by 94% (Fig. 4), while kaempferol loaded PDMAEMA-PPO-PDMAEMA micelles (KF-PPO) decreased it by 66% (Fig. 5). The different antioxidant activity might be due to the different affinity of kaempferol to the core of the micelles. It might be suggested that the higher affinity is related to higher stability and more efficient intracellular transport of kaempferol.

Conclusion

The data in the present study suggest that the loading of kaempferol in micelles, especially based on PDMAEMA-PCL-PDMAEMA copolymer, might improve its antioxidant activity in the conditions of oxidative stress and lipid peroxidation.

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