Formulation and \textit{in vitro} Evaluation of Quercetin Loaded Polymeric Micelles Composed of Pluronic P123 and TPGS

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Abstract

The objective of this study was to develop a polymeric delivery system to improve the solubility and biological activity of Quercetin (QT). QT loaded mixed micelles, composed of Pluronic P123 (P123) and D-a-tocopheryl polyethylene glycol succinate (TPGS) with proportion of 7:3 (QT-P/T), were prepared by thin-film hydration method. The average size of the mixed micelles was 18.43 nm, and the encapsulating efficiency for QT was 88.94 ± 3.71%, drug-loading was 10.59 ± 0.38%. The solubility of QT in QT-P/T was 5.56 mg/mL, which was about 2738-fold that of crude QT in water. Compared with the QT propylene glycol solution, the in vitro release of QT from QT-P/T presented the sustained-release property. The in vitro cytotoxicity assay showed that the IC_{50} values on MCF-7 cells for QT-P/T and QT loaded P123 micelles (QT-P123) were 7.13 μg/mL and 10.73 μg/mL, respectively, while 7.23 μg/mL and 14.47 μg/mL on MCF-7/ADR cells. It could be concluded from the results that P123/TPGS mixed micelles might serve as a pharmaceutical nanocarrier with improved solubility and biological activity of QT.

Keywords: Quercetin, Polymeric Micelle, Pluronic P123, TPGS, Cytotoxicity
INTRODUCTION

Quercetin (QT, Fig.1), extracted and isolated from *Sophora japonica* L, is the major representative of the flavonoid, and has a broad range of biological activities and pharmacological actions such as anti-oxidant activity,\(^1\)\(^-\)\(^2\) anti-inflammatory,\(^3\)\(^-\)\(^4\) anti-diabete,\(^5\) anti-neural disorders,\(^6\) anti-tumor and anti-proliferative effects on a variety of human cancer cell lines.\(^7\)\(^-\)\(^8\) The *in vitro* and *in vivo* studies have demonstrated that QT may inhibit cancer cell growth by binding to type II receptors, which are over-expressed in a wide range of tumor tissues.\(^9\) In spite of this wide spectrum of pharmacological properties, the clinical studies of QT have been hampered due to the water insolubility (0.17-7.7 μg/mL).\(^10\)\(^-\)\(^12\) In order to improve its solubility, QT has been encapsulated in cyclodextrins,\(^13\) liposomes,\(^14\) and chitosan nanoparticles.\(^15\)

Recent studies show that encapsulation of hydrophobic drugs inside polymeric micelles is one of the most attractive alternatives.\(^16\) Due to the nanosize and a core-shell structure, polymeric micelles have been developed as drug delivery systems for various agents in therapeutic and diagnostic applications,\(^17\)\(^-\)\(^19\) and as one promising nanomedicine based technology, polymeric micelles as carriers for anticancer drugs have been evaluated in several clinical trials.\(^20\)\(^-\)\(^21\) For example, SP1049C containing doxorubicin in the mixed micelles of Pluronics L61 and F127 is the first anti-cancer micellar formulation to reach clinic evaluation and is undergoing Phase II clinical trials.\(^22\)\(^-\)\(^23\)

Pluronic P123 (P123), composed of PEO\(_{20}\)-PPO\(_{68}\)-PEO\(_{20}\), is one of the most common representatives of Pluronic copolymer.\(^24\) Here EO denotes oxyethylene OCH\(_2\)CH\(_2\), and PO denotes oxypropylene OCH\(_2\)CH(CH\(_3\)). It is a prominent feature for P123 that can self-assemble into spherical micelle structure constructed by EO as a hydrophilic outer shell and PO as a hydrophobic inner core.\(^25\) The PO core can serve as a ‘pool’ and the hydrophobic drug can be incorporated into the hydrophobic PO core, while the hydrophilic corona maintains the dispersion stability of Pluronic
micelles. It was also demonstrated that P123 had a significant cytotoxicity in the multidrug resistant (MDR) cell lines to doxorubicin due to inhibition of the P-glycoprotein (P-gp) drug efflux transport system that was over-expressed in these cells.26

D-a-tocopheryl polyethylene glycol succinate (TPGS) has been approved by FDA as a water-soluble vitamin E nutritional supplement and drug delivery vehicle.27 It is reported that TPGS can enhance the solubility of poorly soluble drugs by micellar solubilization.28 Recently, it is discovered that TPGS is one of P-gp inhibitory excipients.29-30 Co-administrating with TPGS, the cellular uptake of doxorubicin on Caco-2 cells is increased.31 And for amprenavir, a marketed antiviral drug, TPGS has been used clinically to enhance the bioavailability of the drug.32

In the present work, QT loaded P123/TPGS mixed micelles were prepared by thin-film hydration method. The physicochemical properties and in vitro cytotoxicity of the drug-loaded micelles were investigated.

MATERIALS AND METHODS
Materials
QT was purchased from Xi’an Senmu Biological Technology Co. Ltd (Xi’an, China). TPGS was supplied by Wuhan Yuancheng Co. Ltd (Wuhan, China). P123, 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), trypsin and EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human breast carcinoma cell line MCF-7 and its adriamycin-resistant counterpart MCF-7/ADR were donated by Institute of Biochemical and Biotechnological Drug, School of Pharmaceutical Science, Shandong University. Penicillin streptomycin, RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersberg, MD, USA). All other chemicals were of analytical grade.

Preparation of QT Loaded Micelles
QT loaded micelles were prepared by thin-film hydration method.33-34 Briefly, 24mg of QT and 10 mM of copolymer carriers composed of P123 and TPGS with different
proportions were dissolved in alcohol. The solution was subsequently evaporated under reduced pressure by rotary vacuum evaporation to obtain a thin film of drug/polymer mixture, and the film was further dried over night at room temperature to remove any residual. After that, the obtained film was hydrated in 4mL of de-ionized water under magnetic stirring at room temperature to form a micellar suspension. Non-incorporated crystalline drug was separated by filtration through a 0.22 μm filter membrane, and a yellow clear solution of QT loaded mixed micelles was obtained.

QT loaded P123 micelles (QT-P123) and empty micelles were prepared according to the same procedure.

Characterization of Micelles

Particle Size Distribution

Particle size distributions and mean diameters of the prepared micelles were measured using the BI-200SM based on the light dynamic scattering method (DLS, Brookhaven Instruments Corporation, USA) at a scattering angle of 90° at room temperature. Each freshly prepared sample was placed into a quartz cuvette without additional treatment. The size distributions were extracted from the autocorrelation functions by the CONTIN program. For each sample, the size was measured in triplicate.

Surface Morphology

The morphology of the QT-P/T was observed under transmission electron microscope (TEM, JEM-1200EX, JEOL, Tokyo, Japan), and the accelerating voltage was 100 kv. To prepare the TEM samples, a drop of micellar solution was placed on a copper grid and stained with phosphotungstic acid solution (2%, w/v) about 15 s. Subsequently the sample was allowed to dry slowly in air and then examined under TEM.

Zeta-potential

Zeta-potential of different micelle solution was determined using Zeta Potential Analyzer Instrument (Brookhaven Instruments Corporation, USA). For each sample,
zeta-potential measurement was repeated eight times.

**Drug-loading and Entrapment Efficiency**

The concentration of QT in the micelles was determined with UV-Vis spectrophotometer (UV-2102, Shanghai Instrument Ltd, China) at a wavelength of 374 nm. The micellar solution was suitably diluted with alcohol prior to determination. The drug-loading (DL%) and entrapment efficiency (EE%) of QT in polymeric micelles were calculated from the following equations:\[^{24,37}\]

\[
DL\% = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding polymer and drug}} \times 100\% \quad (1)
\]

\[
EE\% = \frac{\text{weight of the drug in micelles}}{\text{weight of the feeding drug}} \times 100\% \quad (2)
\]

**Critical Micelle Concentration (CMC)**

In this study, CMC was analyzed by a fluorescence probe technique using pyrene as a hydrophobic probe.\[^{38-40}\] The concentration of encapsulated pyrene in micelle phase was determined using F-2500 fluorescence spectrometer (Hitachi, Japan). Pyrene dissolved in acetone was added to empty vials. After acetone evaporation, a series of micellar solutions were added to the vials. The final pyrene concentration was $6.0 \times 10^{-7}$ M, slightly below the saturation concentration of pyrene in water at 25 ºC, and the mixed solution was incubated overnight in the dark. All samples were excited at 334 nm, and fluorescence spectra were recorded between 350 nm and 500 nm. The excitation and emission slit widths were set at 5 nm. Upon formation of micelles, pyrene would move into the inside of the micelles from the aqueous phase, which could result in an alteration in the intensity ratio of I372/I383.

**Solubility of QT in Water or Polymer Micelle Solution**

The solubility of QT in water was determined as follows, excessive crude QT powder was added to 10 mL of de-ionized water, and then the resulting mixture was stirred at 100 rpm at 25 ºC for 72 h and centrifuged at 10000 rpm for 15 min. The supernatant was taken and filtrated through a 0.22 μm filter membrane, and subsequently, the
content of QT in the obtained filtrate was analyzed with UV method at 374 nm.

1 mL of QT loaded micelle solution was suitably diluted with alcohol, and then the resulting solution was analyzed with UV method at 374 nm, the concentration of QT in micelle solution was calculated and the solubility of QT in polymer micelle solution was obtained.

In vitro Release of QT from Micelles

The release of QT from the micelles was investigated by dialysis method with 0.5% Tween 80 solution as release medium. The solution containing 3.0 mg of QT was introduced into a pre-swollen dialysis tube with a MWCO of 8KD-12KD (Xi’an Luosenbo Co. Ltd, Xi’an, China), and the dialysis tube was immersed into 200 mL release medium at 37 ± 0.5ºC with stirring speed at 100 rpm. At predetermined time intervals, 4 mL of the dissolution medium was withdrawn and the same volume of fresh medium was added. Then, the amount of released QT was measured by UV-Vis spectrophotometer at 374 nm, and the cumulative release percentage (Q%) was calculated. For comparison, the release of QT from propylene glycol solution was conducted under the same conditions.41

Cytotoxicity in vitro

The cytotoxicity in vitro of drug loaded micelles was evaluated on MCF-7 and MCF-7/ADR using the MTT method.30,34,42 The cells were cultured in RPMI-1640 medium, which was supplemented with 2 mM l-glutamine, 10% (v/v) FBS, 100 units/mL penicillin G, 0.25 µg/mL amphotericin B, and 100 µg/mL streptomycin at 37 ºC in a humidified 5% CO2 sterile incubator. The medium was changed once every two days.

MCF-7 cell lines were seeded at the density of 3×10³ cells per well in 96-well plates and 8×10³ cells per well for MCF-7/ADR cells. After 24 h incubation, 100 µL of medium containing the treatment agents such as QT DMSO solution (the final concentration of DMSO kept below 0.2%), empty micellar solutions and drug loaded micellar solutions of various concentrations was added. The concentration of QT
ranged from 0.5 to 60 μg/mL. After additional 48 h incubation, the cells were washed twice with phosphate buffer saline (PBS). Subsequently, the growth medium was refreshed and 20 μL of MTT solution (5 mg/mL) was added to each well. The plates were incubated at 37 °C for another 4 h and the medium was removed again. The intracellular metabolized product formazan crystals were dissolved by addition of 150 μL of DMSO to each well. The absorbance was measured using a multiwell scanning spectrophotometer Model 680 (Bio-Rad, USA) with the test wavelength at 570 nm and the reference wavelength at 630 nm. Cell viability was calculated by \[\frac{\text{absorbance of cells exposed to micelles or drug}}{\text{absorbance of cells cultured without micelles or drug}}\] in percentage.

**RESULTS AND DISCUSSION**

**Particle Size Distribution**

The particle size will directly affect the bio-distribution and circulation time *in vivo* of the carriers.\(^{43}\) The average size and size distribution for empty micelles and drug loaded micelles were presented in Fig.2. The average size of empty P123 micelles and mixed micelles composed of P123/TPGS was under 10 nm with rather narrow size distribution patterns. An increasing in the average size after QT loading was observed from 8.85 nm (Fig.2(C)) to 29.04 nm (Fig.2(A)) for P123 micelles and from 9.45 nm (Fig.2(D)) to 18.43 nm (Fig.2(B)) for mixed micelles composed of P123/TPGS with the molar ratio at 7:3, respectively.

The micelle size obtained by DLS depends on both the block copolymer composition and the drug loading. After the micelle was formed, the micelle size was mainly influenced by the interaction of the hydrophobic fractions. The mean diameter of QT-P/T (18.43 nm) was smaller than that of QT-P123 (29.04 nm). This might be attributed to the influence of copolymer composition, in copolymer carrier kept constant at 10 mM, part of P123 molecules was replaced by TPGS with a smaller molecular weight and a smaller hydrophobic group than P123, so QT-P/T showed a significantly smaller hydrophobic volume than that of QT-P123, which might result in
smaller size.\textsuperscript{44}

Stable and small particle sizes (<200 nm) could reduce the uptake of the reticuloendothelial system (RES) and provide efficient passive tumor-targeting ability via the enhanced permeability and retention effects.\textsuperscript{33} Therefore, the size of prepared micelles was suitable for tumor-specific accumulation via the EPR effect.

\textbf{Surface Morphology and Zeta-potential}

As seen from Fig.3, the mixed micelles presented nanometric and homogeneous spheres, and a core-shell structure was also observed under TEM. The outer shell might be attributed to the hydrophilic PEG in TPGS and PEO in P123, and inner core should relate to hydrophobic PPO and Vitamin E. The possible organization of the mixed micelles made from copolymer P123 and TPGS in water through the hydrophobic interaction was depicted schematically in Fig.4. And the obtained QT loaded micellar solutions were clear and yellowish as shown in Fig.5 with the suspension of crude QT dispersed in water at the same drug concentration as control.

Both QT-P123 and QT-P/T were negatively charged with zeta-potential of about -4.08 mV and -10.18 mV, respectively. While the empty P123 micelles were almost neutral (0.78 mV) and the zeta-potential of the empty P/T micelles was -7.48 mV. The structure of P123, both the PPO and PEO segments, was nonionic, so the change of surface charge of the micelles should result from addition of drug and TPGS. As we know, the most acidic phenolic OH groups of QT can dissociate and result in the anionic property for QT.\textsuperscript{45} Therefore, the absolute value of zeta-potential was increased as addition of drug. Compared with QT-P123, an increase in the absolute value of zeta-potential for QT-P/T was observed, too. The increase of negative surface charge of QT-P/T might be attributed to TPGS which enhanced the solubility of QT and more anionic QT presented in solution.

\textbf{Incorporation of QT in Micelles}

The major factors which influenced the DL\% and EE\% of copolymer micelles were the nature and concentration of the solute, nature of the core forming block, core
block length, the nature and block length of the outer shell for micelle. Based on these reasons, copolymer carriers composed of P123 and TPGS with different proportions were studied. As shown in Table 1, the DL% of the QT-P/T with the molar ratio of P123 to TPGS at 7:3 (10.59 ± 0.38%) was higher than that of QT-P123 (8.25 ± 0.32%). This result might be related to the stable reaction among the aromatic ring in TPGS, PO groups in P123 and incorporated drug. However, when the proportion was at 5:5, the EE% (37.95 ± 3.27%) and DL% (5.84 ± 0.50%) were markedly decreased. The possible reason was that QT-P/T with the molar ratio of P123 to TPGS at 5:5 showed a significantly smaller hydrophobic volume than that of QT-P123 or QT-P/T with the molar ratio of P123 to TPGS at 7:3.

The solubility of QT in the obtained QT-P/T micelle solution was 5.56 ± 0.34 mg/mL, while only 2.03 ± 0.44 μg/mL for that of QT in water. That is to say, the solubility of QT in the polymeric micelles was about 2738-fold that of crude QT in water.

Critical Micelle Concentration (CMC)
Pluronic copolymers consist of ethylene oxide (EO) and propylene oxide (PO) blocks, and can undergo self-assembly into spherical micelles in aqueous solution. CMC was a parameter indicative of the micelle’s stability in vitro and in vivo. In this study, it was measured by fluorescence technique with the pyrene as a hydrophobic probe. The CMC value was obtained by plotting the ratio of I372/I383 of the emission spectra profile vs the concentration of copolymers as shown in Fig. 6. This ratio of I372/I383 was decreased with increasing the concentration of copolymer.

The CMC value for micellar solution made from P/T with the molar ratio of P123 to TPGS at 7:3 or P123 was as low as 1.93×10⁻⁵ M (Fig. 6 (A)) or 1.97×10⁻⁵ M (Fig. 6 (B)), respectively, which was in accordance with previous report. The addition of TPGS did not result in notable variation in the CMC. Because of the low CMC, the micelles had high stability and ability to maintain integrity even upon extreme dilution in body.
**In vitro Release of QT from Micelles**

The *in vitro* release of QT from micellar formulation under sink condition was investigated by dialysis method with 0.5% Tween 80 solution as release medium. As shown in Fig.7, only 15% of QT was released from QT-P/T and QT-P123 within the first 4 h, while almost all QT was released from the propylene glycol solution during the same time period. After 60 h, 20-30% of the initially incorporated drug still existed in the micelles. The result indicated that the micelles showed a sustained-release property for the incorporated QT, which was similar to the reported studies. The released mechanism of QT from micelles might be related to the drug diffusion and the polymer material erosions or swelling. It was noticed that the release of QT from mixed micelles was faster than that of P123 micelles. It could be explained that the addition of TPGS enlarged the ratio of hydrophilic part in the mixed micelles and facilitated water molecules into the core of the micelles, leading to more hydrophilic channels.

**Cytotoxicity in vitro**

The *in vitro* cytotoxicity of QT-P/T and QT-P123 was assessed on MCF-7 and MCF-7/ADR cells with QT DMSO solution as control. The empty micelles of P/T and P123 with the same copolymer concentrations as QT-P/T and QT-P123 were used as control, too. The cells were incubated for 48 h in the presence of the micelles or free QT, and then their survival was analyzed using the MTT assay. The viability of MCF-7 and MCF-7/ADR cells after incubation with various formulations of QT and empty micelles was presented in Fig.8. The IC\textsubscript{50} values on MCF-7 cells for free QT in DMSO solution, QT-P/T, and QT-P123 were 16.32 μg/mL, 7.13 μg/mL and 10.73 μg/mL, respectively, while 16.87 μg/mL, 7.23 μg/mL and 14.47 μg/mL on MCF-7/ADR cells (Fig.8 (A,C)). The results demonstrated that QT-P/T showed a higher cytotoxicity compared to the free drug and QT-P123 on both MCF-7 and MCF-7/ADR cells.

It was reported that P123 did not display obvious cytotoxicity to HDF fibroblast cells. However, in this study, it was obvious that the empty micelles of P/T and P123
displayed cytotoxicity on MCF-7 and MCF-7/ADR cell lines, and the cytotoxicity of P/T empty micelles was higher than that of P123 empty micelles (Fig. 8 (B,D)). This could be explained with addition of the TPGS. TPGS in the mixed micelles might act as P-glycoprotein inhibitor to reduce drug efflux. Moreover, animal studies of human cancer xenografts found that TPGS could effectively suppress tumor growth. The anticancer activity of TPGS was reported to be related to its unique apoptosis-inducing properties via the generation of reactive oxygen species (ROS). ROS could damage DNA, proteins, and fatty acids in cells, resulting in apoptotic cell death. Therefore, QT-P/T showed higher cytotoxicity and might be considered as an effective anticancer drug delivery system for cancer chemotherapy compared with QT-P123.

CONCLUSIONS

The mixed polymeric micelles, composed of P123 and TPGS with the proportion of 7:3, exhibited higher encapsulating efficiency and drug-loading for QT. The average size of QT loaded mixed micelles was 18.43 nm, and zeta potential was -10.18 mV. The solubility of QT in QT-P/T was 5.56 mg/mL, which was about 2738-fold that of crude QT in water. Compared with the free drug, QT-P/T showed a significantly enhanced cytotoxicity. Based on these results, it can be concluded that the polymeric micelles formulation developed in this study may be considered as a promising delivery system for QT.

Acknowledgements: This work is partly supported by a research grant (No.2008GG10002012) from Department of Shandong Science and technology, P. R. China.
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<th>Composition (molar ratio)</th>
<th>EE%</th>
<th>DL%</th>
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<tr>
<td>P123 (10)</td>
<td>87.06 ± 3.80</td>
<td>8.25 ± 0.32</td>
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<tr>
<td>P123:TPGS (5:5)</td>
<td>37.95 ± 3.27</td>
<td>5.84 ± 0.50</td>
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<tr>
<td>P123:TPGS (7:3)</td>
<td>88.94 ± 3.71</td>
<td>10.59 ± 0.38</td>
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Captions

Fig. 1 The structure of QT.

Fig. 2 DLS particle size distribution of QT-P123 (A), QT-P/T (B), empty P123 micelles (C), and empty P/T mixed micelles (D).

Fig. 3 TEM image of QT loaded P123/TPGS mixed micelles (×19,000).

Fig. 4 Schematic illustration of QT loaded micelle composed of P123 and TPGS.

Fig. 5 Photographic images of QT-P/T (A), QT-P123 (B) and QT suspension (C).

Fig. 6 Plot of I372/I383 vs concentrations of copolymers in deionized water. Copolymers of P123/TPGS (7:3) (A); P123 (B)

Fig. 7 Release profiles of QT from QT-P123 (▲), QT-P/T (■) and the propylene glycol solution (◆) in 0.5% Tween 80 solution at 37 ºC. Each point represents average ± SD (n = 3).

Fig. 8 Viability of MCF-7 cells after incubation with various formulations of QT (A), and empty micelles (B); Viability of MCF-7/ADR cells after incubation with various formulations of QT (C), empty micelles (D). Each point represents average ± SD (n = 3).
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Liyan Zhao, et al., Fig. 1
Fig. 2 DLS particle size distribution of QT-P123 (A), QT-P/T (B), empty P123 micelles (C), and empty P/T mixed micelles (D).

Liyan Zhao, et al., Fig. 2
Fig. 3 TEM image of QT loaded P123/TPGS mixed micelles (×19,000).

Liyan Zhao, et al., Fig. 3
Fig. 4 Schematic illustration of QT loaded micelle composed of P123 and TPGS.

Liyan Zhao, et al., Fig. 4
Fig. 5 Photographic images of QT-P/T (A), QT-P123 (B) and QT suspension (C).

Liyan Zhao, et al., Fig. 5
**Fig. 6** Plot of I372/I383 vs concentrations of copolymers in deionized water.

Copolymers of P123/TPGS (7:3) (A); P123 (B)

Liyan Zhao, et al., Fig. 6
**Fig. 7** Release profiles of QT from QT-P123 (▲), QT-P/T (■) and the propylene glycol solution (◆) in 0.5% Tween 80 solution at 37 °C. Each point represents average ± SD (n = 3).

Liyan Zhao, et al., Fig. 7
**Fig. 8** Viability of MCF-7 cells after incubation with various formulations of QT (A), and empty micelles (B); Viability of MCF-7/ADR cells after incubation with various formulations of QT (C), empty micelles (D). Each point represents average ± SD (n = 3).

Liyan Zhao, et al., Fig. 8