Arch Pharm Res Vol 30, No 9, 1138-1143, 2007

Archives of Pharmacal Research
http://apr.psk.or.kr

Pharmaceutical Evaluation of Genistein-loaded Pluronic Micelles for Oral Delivery

Suk Hyung Kwon, Sun Young Kim, Kyoung Wook Ha, Myung Joo Kang, Jin Seo Huh, Tae Jong Im, Yong Min Kim, Young Mi Park, Kyoung Hoon Kang, Sangkil Lee, Jung Yun Chang[1], Jaehwi Lee, and Young Wook Choi

Division of Pharmaceutical Sciences, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea and [1]Korea Food and Drug Administration, Seoul 122-704, Korea

(Received September 13, 2006)

The purpose of the present study was to determine whether Pluronic F127 polymeric micelles could improve the oral bioavailability of a poor water-soluble drug, such as genistein. Genistein is a phytoestrogen that has estrogenic activity. F127 triblock copolymer consists of PEO_{100}-PPO_{65}-PEO_{100}. Genistein was incorporated in the Pluronic F127 polymeric micelles by a solid dispersion method. The genistein release of genistein-loaded polymeric micelles was studied *in vitro* (in pH 1.2 and pH 6.8). And the oral bioavailabilities of genistein powder and genistein-loaded micelles were estimated at a dose of 4.0 mg/kg as genistein in rats. Drug loading amount and drug loading efficiency were 11.18% and 97.41%, respectively. The average size of the genistein-loaded polymeric micelles was 27.76 nm. And genistein release of the genistein-loaded polymeric micelles *in vitro* was 58% (pH 1.2) and 82% (pH 6.8). The bioavailability of genistein-loaded polymeric micelles was better than genistein powder. Consequently, Pluronic F127 polymeric micelles are an effective delivery system for the oral administration of genistein.

Key words: Genistein, Polymeric micelles, Oral delivery, Pluronic, Bioavailability, Solubilization

INTRODUCTION

Genistein (4',5,7-trihydroxyisoflavone, Fig. 1) is a phytoestrogen that is found abundantly in soy products. Extensive epidemiological animal studies and *in vitro* experiments with genistein have indicated beneficial effects of this compound on a multitude of disorders, including cancer (Anderson *et al.*, 1998; Barnes, 1998), cardiovascular diseases (Honore *et al.*, 1997), osteoporosis and postmenopausal symptoms (Potter *et al.*, 1998).

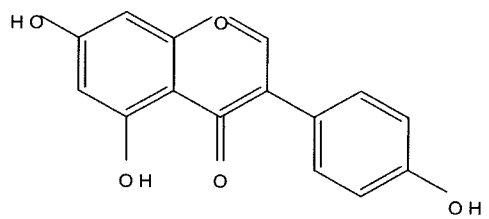

Fig. 1. Structure of 4',5,7-trihydroxyisoflavone (Genistein)

Correspondence to: Jaehwi Lee or Young Wook Choi, College of Pharmacy, Chung-Ang University, 221 Heuksuk-dong, Dongjak-gu, Seoul 156-756, Korea
Tel: 82-2-820-5606 (JL), 82-2-820-5609 (YWC)
Fax: 82-2-816-7338 (JL), 82-2-826-3781 (YWC)
E-mail: jaehwi@cau.ac.kr, ywchoi@cau.ac.kr

Polymeric micelles have been used in the pharmaceutics field as drug and gene delivery systems. Polymeric micelles can be used as efficient carriers for compounds having poor solubility, undesired pharmacokinetic characteristics and low stability in a physiological environment.

Pluronic F127 is one member of a family of tri-block copolymers composed of poly(ethylene oxide) (PEO)-poly(propylene oxide) (PPO)-poly(ethylene oxide) (PEO), generically called poloxamers (Schmolka, 1972). Preparation of polymeric micelles using Pluronics is simple, as they show good aqueous solubility and form micelles spontaneously with a diameter of typically 10-50 nm. The PPO segment of Pluronics is relatively hydrophobic compared to the PEO segments attached to it. Therefore, when several chains are placed into an aqueous solvent such as water, they aggregate to form self-assembled micellar structures above a given CMC (Lange, 1999). The hydrophobic core of such aggregates serves as a microenvironment for the incorporation of lipophilic compounds,

while the hydrophilic corona maintains the dispersion stability of the triblock copolymer aggregates. Thus, the noncovalent incorporation of drugs into the hydrophobic PPO core of the micelles results in an increased solubility, increased metabolic stability and increased systemic circulation time (Alakhov and Kabanov, 1998; Kataokaand Kabanov, 1999; Kwon and Okano, 1999; Kabanov and Alakhov, 2002).

Genistein has low aqueous solubility of approximately 0.81 μg/mL (Stancanelli *et al.*, 2007) so that genistein has demonstrated low oral bioavailability. The aim of the present study was therefore to develop and evaluate the genistein-loaded polymeric micellar formulations using Pluronic F127 to improve oral bioavailability. Pluronic F127 is biocompatible and after oral administration, the polymeric micelles containing genistein are expected to get through the intestinal barrier (Mathot *et al.*, 2006). *In vitro* characteristics of genistein-loaded Pluronic F127 micelles such as size, drug loading amount, drug loading efficiency and genistein release profiles were evaluated. *In vivo* bioavailability study was also carried out in rats after oral administration of genistein-loaded Pluronic micelles.

METERIALS AND METHODS

Materials

Genistein (purity > 94%) was obtained from Rex Gen Biotech Co., Ltd. (Korea). Pluronic F127 was supplied by BASF (Korea). Ammonium formate, sulfatase and 4-hydroxybenzophenone were purchased from Sigma Chemical Co. (U.S.A.). Acetonitrile, ethanol and methanol were of HPLC grade and purchased from Merck Co. (Germany). Potassium dihydrogen phosphate, sodium hydroxide, sodium chloride and hydrochloric acid were purchased from Wako Purechemical Co. (Japan). All other chemicals were of reagent grade and used as received. Deionized water was prepared by the NANOpure Infinity™ from Barnstead International (U.S.A.).

Preparation of genistein-loaded polymeric micelles using Pluronic F127

Genistein was incorporated into Pluronic micelles by a solid dispersion method (Burt *et al.*, 1999). Pluronic F127 (135 mg) and genistein (10-20 mg) were dissolved in 3 mL of ethanol. After 30 min of stirring at 37°C, ethanol was evaporated under reduced pressure at 45°C until a clear gel-like matrix was formed. Then, 50 mL of water at 60°C was added and the mixture was stirred until polymeric micelles containing genistein were formed. The resulting solutions was filtered with a 0.45 μm pore size membrane filter (Millex®, Millipore Co., U.S.A.) to remove undissolved genistein. A clear solution of polymeric micelles containing genistein was obtained. The resulting solutions were frozen at -80°C and lyophilized by a freeze dry system (Labconco Co., U.S.A.) for 2 days to give a solid freeze-dried product.

Measurement of drug loading amount and drug loading efficiency

Drug loading amount and drug loading efficiency were measured by reverse-phase HPLC after dissolving the genistein-loaded Pluronic micelles with ethanol (Liu *et al.*, 2005; Huh *et al.*, 2005). HPLC analysis was performed in Alliance 2690 chromatographic instruments (Waters Co., U.S.A.). Genistein was analyzed with XTerra C18 column (4.6×250 mm, 5 μm, Waters Co., U.S.A.) at 40°C and isocratic elution with the mobile phase of a mixture of acetonitrile:50 mM ammonium formate buffer (5:5, v/v) delivered at a flow rate of 1.0 mL/min. Samples (30 μL) were injected onto the HPLC system. The eluent was monitored at 260 nm with a UV/VIS detector. The drug loading amount of genistein in polymeric micelles was calculated from the amount of genistein incorporated into the polymeric micelles and the weight of the genistein-loaded polymeric micelles. The drug loading efficiency was calculated based on the amount of the genistein encapsulated into the polymeric micelles versus the amount of the genistein initially added to the polymeric micelles using the following equations:

$$\text{Drug loading amount (\%)} = \left(\frac{\text{amount of genistein in polymeric micelles}}{\text{amount of polymeric micelles containing genistein}}\right) \times 100$$

$$\text{Drug loading efficiency (\%)} = \left(\frac{\text{amount of genistein in polymeric micelles}}{\text{amount of genistein initially added to the formulation}}\right) \times 100$$

Measurement of micellar size

Size distribution of the micelles was obtained by photon correlation spectroscopy (PCS) using a Zetasizer Nano-ZS (Malvern Co., UK). The scattering angle was fixed at 173 and temperature was maintained at 25°C. For particle size analysis, each of the samples was taken and diluted with deionized water and filtered through a Millipore 0.45 μm pore size membrane (Millex®, Millipore Co., U.S.A.) before measurement (Liu *et al.*, 2005).

In vitro genistein release from Pluronic F127 micelles

The freeze-dried genistein-loaded polymeric micelles (2 mg as genistein) and 2 mL of water were introduced into dialysis tube (MWCO=12000-14000 Da, Spectra/Por, Spec-

trum Laboratories Ins., U.S.A.), immersed totally into one liter of hydrochloric acid-sodium chloride buffer (pH 1.2) or phosphate buffer (pH 6.8). The system was stirred at 100 rpm at 37°C. At predetermined time intervals, 3 mL of samples were withdrawn and replaced with an equal volume of fresh pH 1.2 or pH 6.8 buffer. Released amount of genistein was measured by HPLC as described above. For comparison, genistein release from bulk powder (dispersed in 2 mL of water by vortexing) placed in a dialysis tube was performed under the same conditions (Huh *et al.*, 2005; Cho *et al.*, 2004). All results from *in vitro* release studies were reported as means with standard deviations (n=3).

Pharmacokinetic evaluation

Male Sprague-Dawley rats, 8 weeks old and weighed about 250-310 g, were obtained from Orient Co. (Korea). Rats were fed an isoflavone-free diet from Orient Co. The animals had free access to food and water until 12 h before the experiments. Genistein aqueous suspension and genistein-loaded polymeric micelles were prepared for oral administration to rats. Six rats for each group were initially anesthetized with chloroform and the femoral artery was cannulated for blood sampling. The body temperature of the rats was maintained at 37°C with a heating pad. The oral dose of genistein in aqueous suspension and genistein-loaded polymeric micelles, dispersed in 2 mL of water, respectively was 4 mg/kg. Plasma samples were collected at predetermined time intervals after oral administration. Then, each blood sample was centrifuged at 12,000 rpm for 10 min. The plasma samples (about 200 μL) were stored at -20°C until analyzed by HPLC. (Supko and Phillips, 1995).

The plasma sample (50 μL) was incubated with 500 IU sulfatase and then 4-hydroxybenzophenone (20 μg/mL), an internal standard, and 5 mL ethyl acetate, extraction solvent, were added to the plasma samples. Extraction was processed by stirring vigorously for 50 min and centrifuged at 3000 rpm for 15 min at 4°C. The organic phase was transferred to a glass tube and evaporated under a stream of nitrogen at 40°C until dryness. Then, the residue was dissolved in 200 μL of 50% methanol for HPLC assay.

Genistein and 4-hydroxybenzophenone were analyzed with Capcell Pak column C18 (4.6×150 mm, Shiseido, Japan) at 40°C and gradient elution. The mobile phase used was acetonitrile: 50 mM ammonium formate (pH 3.5) buffer (6:4, v/v, A line) and acetonitrile: 50 mM ammonium formate (pH 3.5) buffer (2:8, v/v, B line). The elution conditions were as follow: 0-4 min 65% to 51% A line, 4-8 min 51% to 65% A line, 8-10 min 65% A line. Flow rate was 1.0 mL/min. Samples (50 μL) were injected onto the HPLC. The eluent was monitored at 268 nm with a UVdetector. The HPLC system used was Hitachi L-2400 chromatographic instruments (Hitachi, Japan).

The pharmacokinetic parameters were determined such as $AUC_{[0\text{-terminal}]}$, $AUC_{[0\text{-}\infty]}$, C_{max}, T_{max} and $t_{1/2}$ using BA-Calc 2002 (ver. 1.1.1, KFDA, Korea). The significance of difference was analyzed by ANOVA, and a significance level of less than 5% was considered significant.

RESULTS AND DISCUSSION

Evaluation of drug loading amount and drug loading efficiency

Genistein-loaded polymeric micelles were prepared by a solid dispersion method with various genistein/triblock copolymer weight ratios at three times. The incorporation profiles of genistein were examined for different genistein/triblock copolymer weight ratios (Fig. 2). The drug loading amount reflects the capability of the polymeric micelles to incorporate genistein into the core part of the micelles. As genistein weight ratio against Pluronic F127 was increased from 10/135 mg to 17.5/135 mg, drug loading amount was also increased from 6.51 to 11.18%. Genistein/Pluronic F127 copolymer below 17.5/135 mg produced clear or

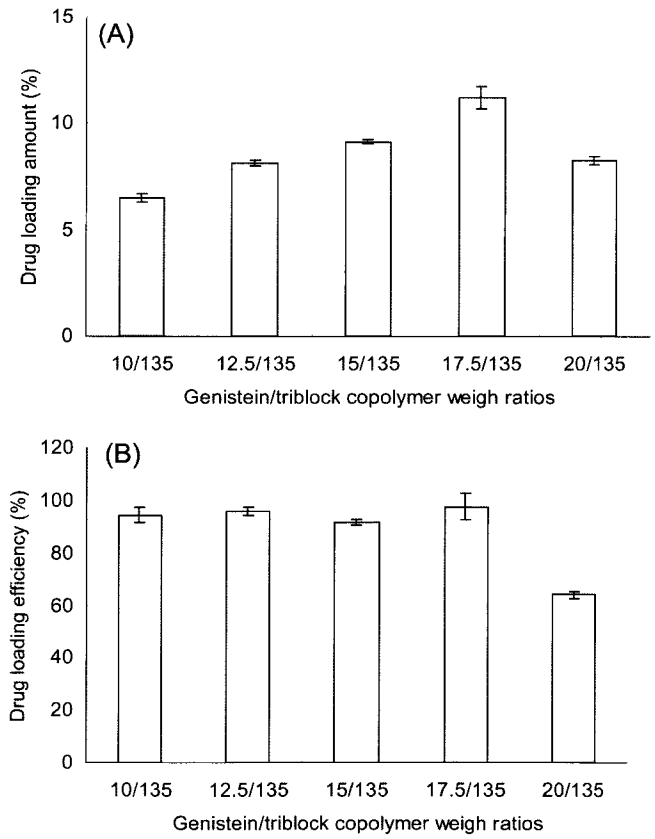

Fig. 2. The drug loading amount (A) and the drug loading efficiency (B) of Pluronic F127 micelles having various genistein/triblock copolymer weight ratios (Mean ± S.D., n=3)

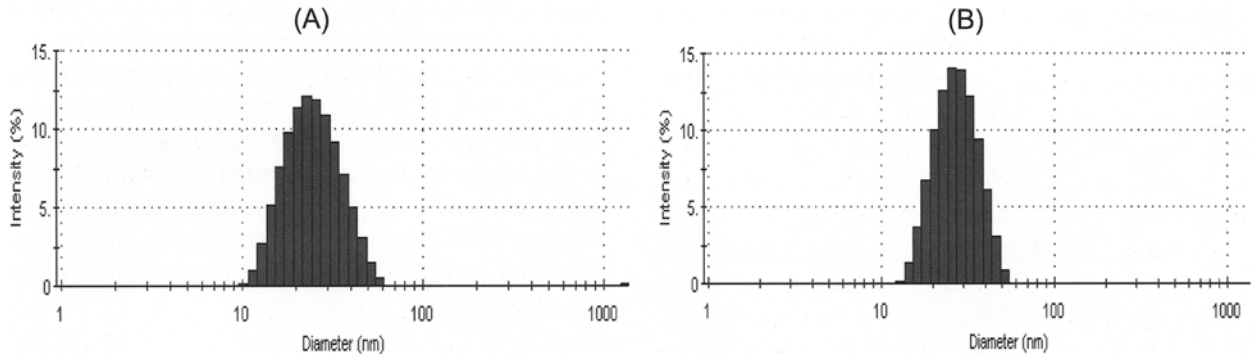

Fig. 3. Particle size distribution of genistein-loaded polymeric micelles (A) and drug free polymeric micelles (B)

light yellow solution but precipitation of genistein was observed with a weight ratio of 20.0/135 mg after 24 h of storage at room temperature. Therefore, the genistein/ Pluronic F127 copolymer ratio of 17.5/135 mg offered the best genistein encapsulation capability. At this ratio, the drug loading efficiency was measured to be 97.41%.

Measurement of micellar size

The particle size and particle size distribution of genistein-loaded polymeric micelles were measured by photon correlation spectroscopy (PCS) using a Zetasizer Nano-ZS. Fig. 3 shows the typical size distribution of genistein-loaded polymeric micelles prepared with genistein/ Pluronic F127 copolymer weight ratio of 17.5/135 mg. For comparison, particle size of drug free polymeric micelles in water was also measured. The average sizes of the genistein-loaded polymeric micelles and drug free polymeric micelles were 27.76 ± 0.46 nm (polydispersity index = 0.26) and 24.89 ± 1.00 nm (polydispersity index = 0.21), respectively (n=3). The size distributions showed a narrow and monodisperse unimodal pattern.

In vitro drug release

The release rate of genistein from Pluronic F127 polymeric micelles in hydrochloric acid-sodium chloride buffer (pH 1.2) and phosphate buffer (pH 6.8) was faster than that from bulk powder (Fig. 4). The amount of genistein released from genistein-loaded polymeric micelles for 12 h was approximately 58% in pH 1.2 medium and 82% in pH 6.8 medium. On the other hand, genistein release from bulk powder was slower and resulted in released amounts of 48% in pH 1.2 medium and 44% in pH 6.8 medium for 12 h. Consequently, the increased genistein release from the polymeric micelles can be attributed to the solubilizing ability exhibited by the polymeric micelles. Additionally, genistein-loaded polymeric micelle is expected to release genistein more rapidly in the small intestine, compared to gastric environment.

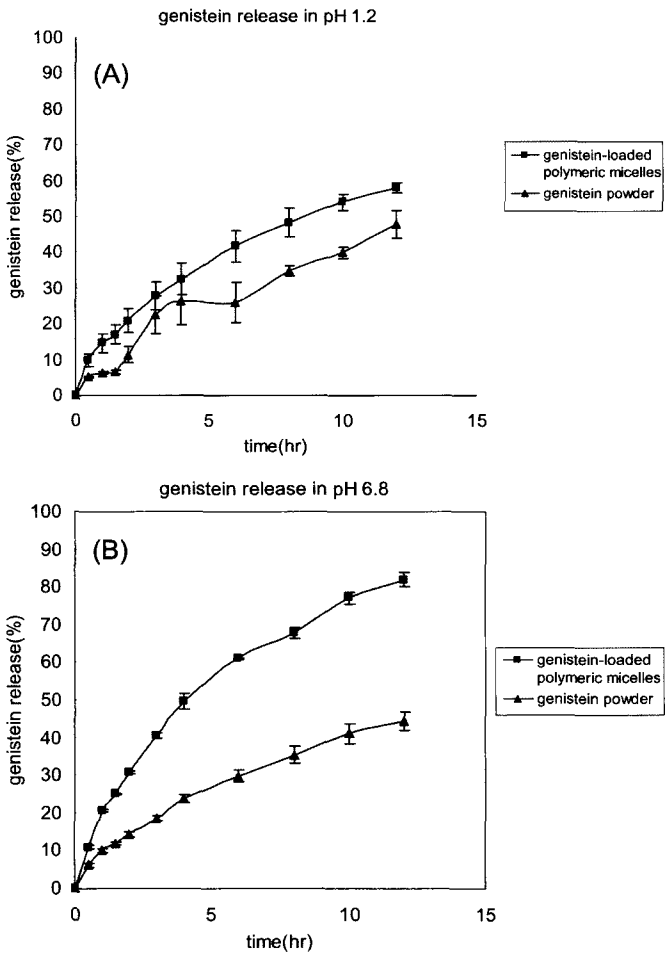

Fig. 4. Release profile of genistein from Pluronic F127 polymeric micelles and bulk powder in pH 1.2 buffer (A) and pH 6.8 buffer (B) (Mean ± S.D., *n*=3)

In vivo pharmacokinetic characteristics

In vivo study in rats was performed to evaluate the oral genistein bioavailability from genistein-loaded polymeric micelles in comparison with aqueous suspension of genistein after oral administration (Fig. 5).

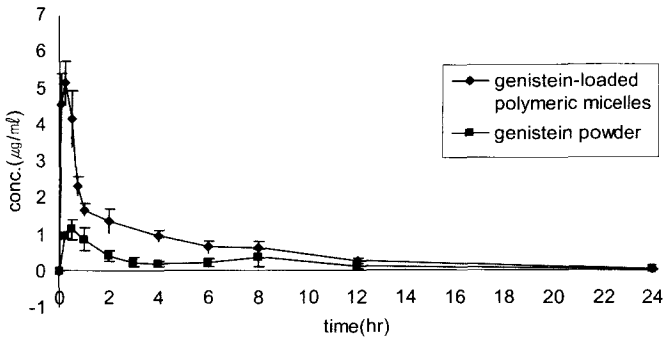

Fig. 5. Plasma concentration-time curve of genistein after oral administration at a dose of 4 mg/kg as genistein in rats (Mean ± S.E., *n*=5)

After oral administration of genistein-loaded in polymeric micelles (4 mg/kg as genistein), maximum plasma concentration (C_{max}) was observed at 5.68 μg/mL after 0.20 h (T_{max}, time to reach maximum plasma concentration) (Table I). On the other hand, C_{max} obtained with genistein powder was 1.22 μg/mL at 0.55 h (T_{max}). Thereafter plasma concentration declined gradually and the half life ($T_{1/2}$) was measured to be 3.63 h for genistein-loaded polymeric micelles and 4.54 h for genistein powder, respectively. Also AUC of genistein-loaded polymeric micelles was approximately 3-times greater than that of genistein powder.

As a result, genistein-loaded polymeric micelles were found to cause better bioavailability than genistein powder after oral administration. This increased bioavailability exhibited by the polymer micelles may be attributed to enhanced genistein solubility and release in the gastrointestinal tract (Sant *et al.*, 2005).

CONCLUSIONS

The oral delivery of poorly water-soluble drugs is a growing issue for the pharmaceutical industry as many drug candidates resulting from high throughput screening possess these physicochemical limitations. Polymeric micelles are promising drug carriers for these poorly soluble drugs but their potential use as oral drug delivery systems has not been widely demonstrated *in vivo*.

In this study, genistein-loaded Pluronic F127 polymeric micelles showed high solubilization capacity and nanoscopic particle size. The best drug loading amount and drug loading efficiency were 11.18% and 97.41%, respectively. And, the average size of the genistein-loaded polymeric micelles was 27.76 nm. The amount of genistein released for 12 h from genistein-loaded polymeric micelles *in vitro* was 58% (pH 1.2) and 82% (pH 6.8). The *in vivo* oral bioavailability of genistein loaded in polymeric micelles using rats was greater than genistein powder. Therefore, Pluronic F127 polymeric micelles are an effective solubilizing and delivering system for oral administration of poorly soluble genistein.

ACKNOWLEDGEMENTS

This work was supported by the Seoul R&BD program.

REFERENCES

Alakhov, V. Y. and Kabanov, A. V., Block copolymeric biotransport carriers as versatile vehicles for drug delivery. *Expert Opin. Invest. Drugs*, 7, 1453-1473 (1998).

Anderson, J. J., Ambrose, W. W. and Garner, S. C., Biphasic effects of genistein on bone tissue in the ovariectomized, lactating rat model. *Proc. Soc. Exp. Biol. Med.*, 217, 345-350 (1998).

Barnes, S., Evolution of the health benefits of soy isoflavones. *Proc. Soc. Exp. Biol. Med.*, 217, 386-392 (1998).

Cho, Y. W., Lee, J., Lee, S.C., Huh, K. M., and Park, K., Hydrotropic agents for study of in vitro paclitaxel release from polymeric micelles. *J. Control. Release,* 97, 249-257 (2004).

Honore, E. K., Williams, J. K., Anthony, M. S., and Clarkson, T. B., Soy isoflavones enhance coronary vascular reactivity in atherosclerotic female macaques. *Fertil. Steril.*, 67, 148-154 (1997).

Huh, K. M., Lee, S. C., Cho, Y. W., Lee, J., Jeong, J. H., and Park, K., Hydrotropic polymer micelle system for delivery of paclitaxel. *J. Control. Release,* 101, 59-68 (2005).

Kabanov, A. V. and Alakhov, V. Y., Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers. *Crit. Rev. Ther. Drug Carrier Syst.*, 19, 1-73 (2002).

Kataoka, K. and Kabanov, A. V., Special Issue: Polymeric Micelles in Biology and Pharmaceutics. *Coll. Surf. B: Biointerf.*, Elsevier, Amsterdam (1999).

Kwon, G. S. and Okano, T., Soluble self-assembled block copolymers for drug delivery. *Pharm. Res.*, 16, 597-600 (1999).

Table I. Summary result of pharmacokinetic parameters after oral administration of genistein bulk powder and genistein-loaded polymeric micelles (Mean ± S.E., *n*=5)

Formulation	T_{max} (h)	C_{max} (μg/mL)	$AUC_{(0\text{-terminal})}$ (μg·h/mL)	$AUC_{(0\text{-}\infty)}$ (μg·h/mL)	$T_{1/2}$ (h)
Genistein powder	0.55 ± 0.12	1.22 ± 0.24	4.00 ± 1.91	4.14 ± 2.01	4.54
Genistien-loaded micelles	0.20 ± 0.08*	5.68 ± 0.81*	12.12 ± 1.28*	12.31 ± 1.30*	3.63

*Significantly different from genistein powder at P<0.05

Lange, K. R., Surfactants: A Practical Handbook. Hanser Gardner, Cincinnati (1999).

Liu, S. Q., Tong, Y. W., and Yang, Y.-Y., Incorporation and in vitro release of doxorubicin in thermally sensitive micelles made from poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)-b-poly(d,l-lactide-co-glycolide) with varying compositions. *Biomaterials*, 24, 5064-5074 (2005).

Malmsten, M., Surfactants and Polymers in Drug Delivery. Marcel Dekker, New York (2002).

Mathot, F., Beijsterveldt, L., Préat, V., Brewster, M., and Ariën, A., Intestinal uptake and biodistribution of novel polymeric micelles after oral administration. *J. Control. Release*, 111, 47-55 (2006).

Potter, S. M., Baum, J. A., Teng, H., Stillman, R. J., Shay, N. F., and Erdman Jr, J. W., Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.*, 68, 1375S-1379S (1998).

Sant, V. P., Smith, D., and Leroux, J. C., Enhancement of oral bioavailability of poorly water-soluble drugs by poly(ethylene glycol)-block-poly(alkyl acrylate-co-methacrylic acid) self-assemblies, *J. Control. Release,* 104, 289-300 (2005).

Schmolka, I. R., Artificial skin 1. Preparation and properties of Pluronic F-127 gels for treatment of burns. *J. Biomed. Mater. Res.*, 6, 571-582 (1972).

Stancanelli, R., Mazzaglia, A., Tommasini, S., Calabrò, M. L., Villari, V., Guardo, M., Ficarra, P., and Ficarra, R., The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: A spectroscopic investigation with implications in the pharmaceutical analysis. *J. Pharm. Biomed. Anal.,* 44, 980-984 (2007).

Supko, J. G. and Phillips, L. R., High-performance liquid chromatographic assay for genistein in biological fluids. *J. Chromatogr. B*, 166, 157-167 (1995).

Wilhelm, M., Zhao, C., Wang, Y., Xu, R., Winnik, M., Mura, J., Riess, G., and Croucher, M., Poly(styrene-ethylene oxide) block copolymer micelle formation in water: a fluorescence probe study, *Macromolecules*, 24, 1033-1040 (1991).