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Preparation of PLGA Microparticles by a double Emulsion Solvent Technique for Sustained Release of (-)-epigallocatechin Gallate (EGCG) and Their Growth Inhibitory Effect on Rat Aortic Smooth Muscle Cells

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In this study, we fabricated porous PLGA microparticles loaded with EGCG by a modified water-in-oil-in-water (W/O/W) double emulsion solvent evaporation method. The physicochemical properties of the EGCG-loaded PLGA microparticles were studied using scanning electron microscopy (SEM), Fourier transformed-infrared (FT-IR) spectroscopy and *in vitro* release measurements. A remarkable burst effect was observed on first stage however the released amount was reduced largely with time. This result showed that the release of EGCG increased by its loading amount. Also, EGCG - loaded PLGA particles inhibited the growth of rat aortic smooth muslce cells (RASMC). This study demonstrates that PLGA microparticle system is potential as an efficient EGCG carrier in medical therapy.

Key words: (-)-epigallocatechin gallate, PLGA microparticles, sustained release, rat aortic smooth muscle cell, water-in-oil-in-water (W/O/W) double emulsion method

Introduction

Several epidemiological studies have suggested that green tea has pharmacological effects associated with a reduced risk of degenerative disease such as cancer and cardiovascular disease. Among the green tea polyphenols, epigallocatechin-3-gallate (EGCG) is the major component of green tea that mediates cardiovascular-related effects such as anti-proliferative,[1,2] anti-atherogenic[3] and anti-thrombotic.[4] Furthermore, recent studies have demonstrated that EGCG has ability of preventing angioplasty-induced restenosis, suppressing vascular smooth muscle cells (SMCs) proliferation and migration[4,5] and inhibiting the activities of some matrix metalloproteinases (MMPs).[6] On these bases, it can be assumed that EGCG has a protective effect on the vascular dysfunction and intimal hyperplasia after vascular damage. Local delivery of water-soluble EGCG may be needed for a successful vascularization from injured vessels.

For the purpose of a sustained drug release, a drug delivery system using biodegradable polymer microparticles has been focused with its potentials.[7,8] PLGA has been often used for drug delivery systems because of its proven safety and efficacy in sustained release. Drugs may be incorporated into PLGA microparticles by a variety of techniques including encapsulation method. Among the encapsulation techniques, the o/o solvent evaporation[9] and the w/o/w solvent extraction techniques[10-12] are two of the most convenient ways for the encapsulation of drug within PLGA microparticles. According to the used encapsulation methods, PLGA molecular weight, and stabilizers the drug encapsulation efficiency, diffusion of drug, morphological features of the PLGA microparticles and burst release can be determined.

In this study, the EGCG-loaded microparticles were prepared by a double emulsion solvent technique using a biodegradable copolymer of poly (lactic-co-glycolic acid) (PLGA) and *in vitro* EGCG release study from PLGA microparticles was performed.

Materials and Methods

Materials

PLGA (50:50) polymer (Mw 72.3 kDa) was purchased from Lakeshore Biomaterials, Inc (AL, USA). EGCG (MW 458.4) was supplied by Teavigo™ (DSM Nutritional Products Ltd., Basel, Switzerland) and its purity exceeded 93%. Dichloromethane (DCM), polyvinyl alcohol (PVA), DMSO and isopropyl alcohol were purchased from Sigma-Aldrich Co., (St. Louis., USA).

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Preparation of PLGA microparticles

EGCG-loaded PLGA microparticles were produced by modification of a water-in-oil-in-water double emulsion, solvent extraction technique. Briefly, 600 mg PLGA was dissolved in 5 mL of DCM and 0, 15, 30, 60 mg of EGCG were dissolved into 1 mL of distilled water, respectively. After mixing these two solutions vigorously for 10min by a vortex mixer, emulsified solution was re-emulsified in 50 mL of PVA (0.2%, w/v)., Solution was poured in 50 mL of aqueous isopropyl alcohol (2%, w/v) and stirred with a magnetic stirrer for overnight at room temperature to harden fabricated microparticles. Also, EGCG-free microparticles were prepared accordingly without EGCG. Fabricated PLGA microparticles were rinsed three times with pH 7.4 phosphate buffered saline (PBS) to remove the un-captured EGCG and lyophilized in a freeze-dryer. Dried microparticles were stored in -20 °C.

Characterization of PLGA microparticles

Determination of EGCG loading amount and efficiency

To determine EGCG amount loaded in the PLGA microparticles, 10 mg of microparticles were dissolved in 5 mL DMSO and analyzed at 312 nm by UV-Vis spectrophotometer. The concentration of EGCG released from particle was calculated from the standard calibration curve of EGCG solution. The relationship between the input amount of EGCG and the EGCG loading content of particle was investigated:

The yield of the produced microparticles and EGCG loading efficiency were calculated as followings;

$$\text{Yield} = \text{Weight of EGCG-loaded PLGA in production} / \text{Total weights of input EGCG and input PLGA} \times 100 \quad (1)$$

$$\text{EGCG loading efficiency} = \text{Actual loading amount of EGCG} / \text{Input amount of EGCG} \times 100 \quad (2)$$

PLGA microparticles morphology

Prepared PLGA microparticles were coated by gold and their surfaces and sizes were observed by a scanning electronic microscope (Hitachi S-800, Tokyo, Japan). The microparticles were mounted and sputter-coated with gold/platinum using an ion coater (E1010, Hitachi) and then observed at an accelerating voltage of 20 kV.

Fourier transformed-infrared (FT-IR) spectroscopy

Fourier transform infrared (FT-IR) spectra of EGCG, PLGA and EGCG- PLGA particle were obtained between 4000 and 650 cm^{-1} using an FT-IR spectrophotometer (Nicolet™ 380, Thermo Fisher Scientific, Inc., Waltham, MA). Samples were scanned 128 times at 8 cm^{-1} resolution.

***In vitro* release of EGCG from particle**

The 50 mg of EGCG-loaded PLGA microparticles were suspended in 5 mL of PBS (pH 7.4) and incubated at 37 °C with shaking at 224 rpm. Aliquots of supernatant were replaced with an equal amount of fresh PBS to maintain a constant volume of buffer at predetermined time intervals. The suspended solution was centrifuged at 25 °C and 1600 rpm for 10 min. The concentrations of EGCG from released buffer were determined at 280 nm by UV-Vis spectrophotometer from the standard calibration curve of EGCG solution. The accumulated release percentage of EGCG at each time point was detected from three replicate samples for 23 days.

Cell culture and growth inhibition assay of EGCG-PLGA particle

RASMCs (Rat Aortic smooth muscle cells) were purchased from BioBud (Seoul, Korea) and used between passages 7 and 15. The cells were routinely maintained in Dulbecco's modified Eagle's medium (Wellgene, Seoul, Korea) supplemented with 10% FBS (HyClone Laboratories Inc, UT, USA) and a 1% antibiotic antimycotic solution (including 10,000 U penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml, Wellgene) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

In order to examine the growth inhibitory effects of EGCG-loaded PLGA microparticles on RASMCs, the cells were seeded into 48well plates and incubated for 4hr at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the cells were attached, they were treated with EGCG-loaded PLGA particles (20 mg/ml) and incubated for 1d, 3d, and 5d, respectively. EGCG-loaded PLGA particles were sterilized by low temperature EO gas (3M™ Steri-Vac™ 5XL Gas Sterilizer/Aerators, 3M, St. Paul, MN) before the treatment to RASMC. Cell viability was determined by MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product].

Results and discussions

Preparation of biodegradable microparticles containing water-soluble bioactive agents has received much attention in recent years, due to their numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance and convenience. Various techniques are available to entrap water soluble bioactive agents into biodegradable microparticles. Among various methods, the W/O/W double emulsion technique is the most popular method.[13)]

EGCG-loaded PLGA microparticles were prepared by the W1/O/W2 emulsification-solvent evaporation method. The morphology of PLGA microparticles was observed by SEM (Figure 1). Although the size of microparticles was diverse, the average size was from 100 μm to 300 μm. Moreover, the PLGA microparticle were spherical with several pores distributed on their surface, irrespective of their dimension (Figure 1

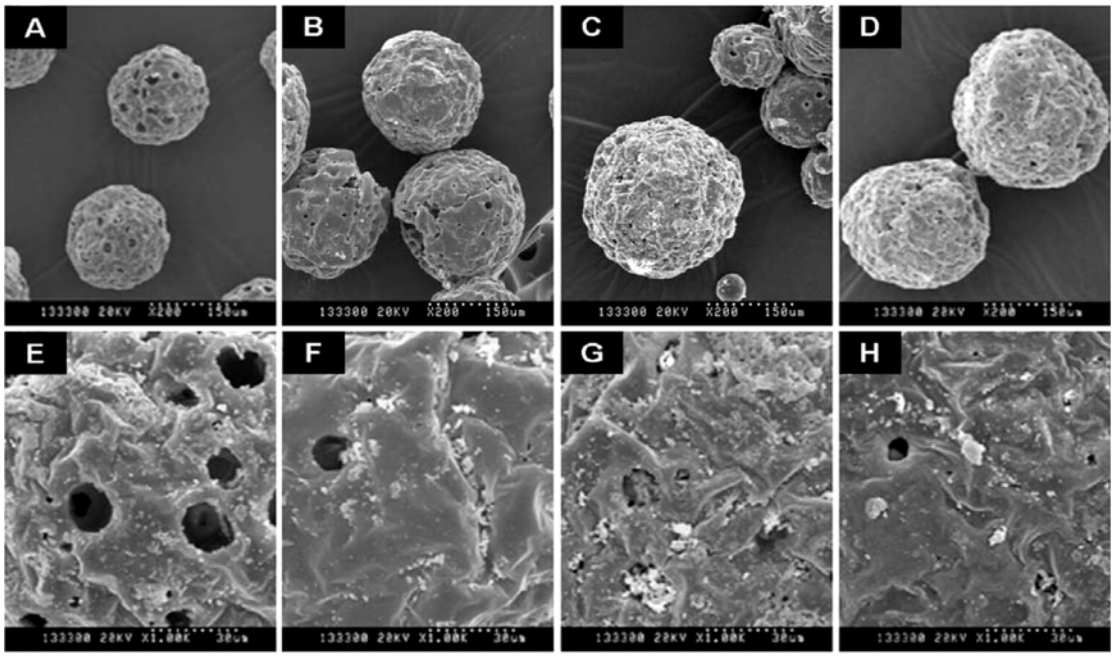

Figure 1. Scanning electron micrographs images (SEM) of PLGA microparticles. (A, E) 0 mg ; (B, F) 15 mg ; (C, G) 30 mg ; (D, H) 60 mg of EGCG was loaded microparticles. Upper panel: 200 magnification, down panel: 1.0 K magnification.

Table 1. Encapsulation efficiency and burst effect of EGCG-loaded PLGA microparticles.

EGCG(mg)/PLGA(mg)	Yield	Theoretical loaded EGCG (mg)	Actual loaded-EGCG (mg)	EGCG loading efficiency (%)
0/600	94.58±1.58	-	-	-
15/600	90.09±0.98	15	1.59±0.39	10.61±2.58
30/600	84.21±0.34	30	3.11±0.53	10.36±1.78
60/600	76.67±0.37	600	5.50±0.63	9.17±1.04

A, B, C, D). However, the pore number decreased with increasing EGCG concentration (Figure 1 E, F, G, H). Porosity of microparticles is known to vary with numerous factors such as polymer molecular mass, co-solvent concentration, dispersed phase to continuous phase ratio, drug concentration, rate and method of solvent removal.[15] Pores are formed because of the inner aqueous phase during microparticle formation in the water-in-oil-in-water (W/O/W) method, in comparsion with the oil-in-water method. In the organic phase, the polymer concentration continuously increases during organic solvent extraction/evaporation. The PLGA begins to precipitate and encapsulate the drug as well as inner water droplets. By elimination of the water during drying, empty holes remain and are uniformly distributed throughout the microparticles, irrespective of their size.[14]

However, once the microparticles are dried, in EGCG-loaded particles the empty holes are filled by EGCG that was included in water. We observed that the inside of pore is filled with loaded-EGCG compared with non-treated microparticles (Figure 1 E, F, G, H). Moreover, it has been observed that the size of pores decreased with the increasing EGCG concentration in EGCG-loaded microparticles.

The actual load of EGCG in PLGA microparticles was calculated by spectrophotometric analysis. The actual loading amount and efficiency of EGCG are presented Table 1. The production yield of PLGA microparticles gradually was decreased by addition of EGCG. The actual EGCG loading was almost identical in all cases. The EGCG loading efficiencies were found to be 8~13 % with no significant difference with increasing EGCG concentration. Although the loading efficiency has been reported to be related to surfactant concentration and viscosity in oil phase, stirring time period and stirring rate in the preparation procedure of primary emulsion, stirring rate in secondary emulsion preparation, polymer concentration, and osmotic pressure, drug loading efficiency and particle size of microparticle can not be completely controlled.[15,16]

The FT-IR spectra of the representative formulations confirmed that the EGCG was dissolved in the microparticle (Figure 2). FT-IR spectra of EGCG-loaded particles were dominated by

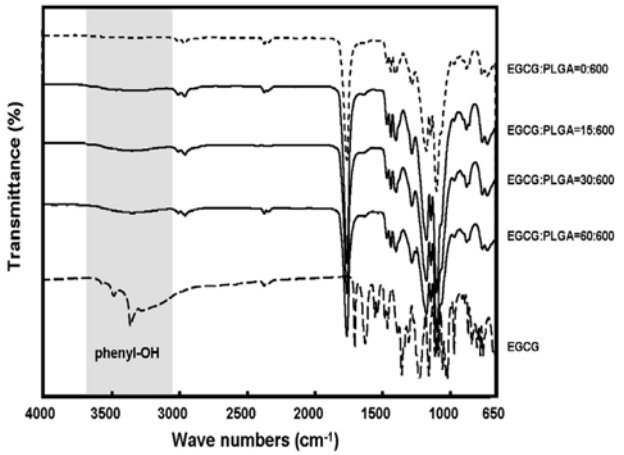

Figure 2. FT-IR spectrums of EGCG-loaded PLGA microparticles.

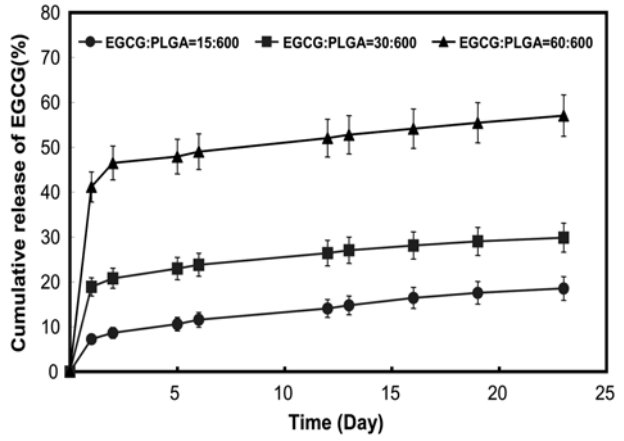

Figure 3. *In vitro* release behavior of EGCG from PLGA microparticles. Released EGCG was observed at 280 nm with control for 23 days (PBS buffer, pH 7.4, 37 °C).

a broad notable band at phenyl-OH group (3600–3150 cm^{-1}), which is the characteristic peak of EGCG. Therefore, the characteristic phenyl-OH peak was stretched with increasing EGCG concentration. The change in this peak was similar with EGCG releasing PLCL film prepared by solvent casting.[17] It seems that EGCG was encapsulated in the PLGA microparticle, probably by dispersion of EGCG in polymer.

The release experiments *in vitro* were carried out by spectrophotometrical method for 23 days. The EGCG release profiles from the PLGA microparticles in PBS are illustrated in Figure 3 for different concentration of EGCG. The EGCG release profiles were biphasic, remarkable burst effect was observed on the first and second day. However, the released concentration of EGCG was largely reduced from the third day and kept constant for 3 weeks. The highest burst effect and release rate was observed in microparticles with the highest concentration of EGCG. Burst release from PLGA microparticles has been attributed to either the surface or uniform distribution of the associated drug. Therefore, to reach the sustained release the drug should be distributed exclusively inside the microparticle. Porosity increases the surface area of the microparticle, which itself can contribute to the burst.[18] A successful microparticle should yield high encapsulation efficiency, and desired particle size.

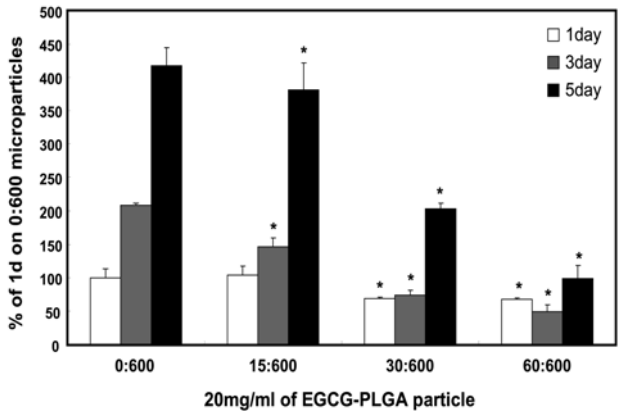

Figure 4. Effect of EGCG-loaded PLGA microparticles on RASMC proliferation. The results are reported as means ± SD (n = 4). The data are analyzed by a *t*-test. The values marked with asterisks are significantly different from the unloaded PLGA microparticle (0:600) (*$p < 0.05$).

Other studies have already shown that EGCG has pharmacological activites, including antioxidant, anti-proliferative, antithrombotic and anti-migrative effect on smooth muscle cells.[1,4,19] In this study, we examined the growth inhibitory effect on RASMC by EGCG-loaded PLGA microparticles (Figure 4). When the cells were treated with EGCG-loaded microparticles, cell proliferation significantly decreased with increasing EGCG concentration dose dependently in comparison with the unloaded PLGA microparticles. Our previous results had shown that proliferation and migration in serum-stimulated RASMC was inhibited in more than 200 μM EGCG.[5] By calculation, PLGA microparticles manufactured by different concentration of EGCG (15:600, 30:600 and 60:600) loaded 1.5 mg, 30 mg and 60 mg, respectively. Our results showed that RASMC proliferation was inhibited by EGCG released from PLGA microparticles for the first 5 days.

Microparticles should provide effective and sustainable release to control the drug release kinetics over periods of days to months. Thus, PLGA microparticles for local delivery of EGCG can be very helpful to optimize the therapeutic efficiency and to reduce possible side effects in medical application of EGCG.

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