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RESEARCH ARTICLE

Anticancer activities of (−)-epigallocatechin-3-gallate encapsulated nanoliposomes in MCF7 breast cancer cells

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Abstract

The chemopreventive actions exerted by green tea are thought to be due to its major polyphenol, (−)-epigallocatechin-3-gallate (EGCG). However, the low level of stability and bioavailability in the body makes administering EGCG at chemopreventive doses unrealistic. We synthesized EGCG encapsulated chitosan-coated nanoliposomes (CSLIPO-EGCG), and observed their antiproliferative and proapoptotic effect in MCF7 breast cancer cells. CSLIPO-EGCG significantly enhanced EGCG stability, improved sustained release, increased intracellular EGCG content in MCF7 cells, induced apoptosis of MCF7 cells, and inhibited MCF7 cell proliferation compared to native EGCG and void CSLIPO. The CSLIPO-EGCG retained its antiproliferative and proapoptotic effectiveness at 10 μM or lower, at which native EGCG does not have any beneficial effects. This study portends a potential breakthrough in the prevention or even treatment of breast cancer by using biocompatible and biodegradable CSLIPO-EGCG with enhanced chemopreventive efficacy and minimized immunogenicity and side-effects.

Keywords

Apoptosis, breast cancer, EGCG, nanoliposomes, viability

History

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Introduction

Breast cancer is the second most common cancer in women in the United States. Being second only to cancer of the skin, it accounts for one out of every three cancer cases diagnosed in the United States. The American Cancer Association expected about 226,870 new cases of invasive breast cancer and about 63,300 new cases of carcinoma *in situ* to be diagnosed in women in 2012 (American Cancer Society, 2011). Around one in eight women in the United States will develop breast cancer at some time in their lives (American Cancer Society, 2011).

Tea is one of the most popular beverages consumed worldwide. Green tea contains more catechins than red tea and oolong tea. Green tea catechins constitute about 33% of total dry tea weight (Wang et al., 2006). (−)-Epigallocatechin-3-Gallate (EGCG) is the most abundant catechin and comprises 48%–55% of total catechins (Basu & Lucas, 2007). EGCG has gained considerable attention due to its antioxidant, anti-tumorigenic, anti-inflammatory and antiangiogenic properties (Chyu et al., 2004; Khan & Mukhtar, 2008). One 2 g green tea bag contains about 330 mg of EGCG. Recent studies have shown that EGCG has potent chemopreventive activities effective in many different types of cancer, including breast cancer (Kim et al., 2006; Kushima et al., 2009). The chemopreventive effects of this polyphenol are attributed to EGCG's ability to inhibit cancer cell proliferation, induce apoptosis and cell cycle arrest, without causing damage in the normal/healthy cells (Chen et al., 1998). EGCG has positive effects on both estrogen-positive and estrogen-negative breast cancer cells at concentration range from 10 to 400 μM *in vitro* (Hsuuw & Chan, 2007; Kim et al., 2006). However, the evidence of human studies is inconclusive regarding its effectiveness for breast cancer prevention or treatment (Inoue et al., 2001; Nagano et al., 2001; Nakachi et al., 1998; Suzuki et al., 2004; Wu et al., 2003). The major problems are its low level of stability, bioavailability and target specificity in humans or research animals (Zhu et al., 2000). The absolute oral bioavailability of EGCG after drinking tea containing catechins at 10 mg/kg body weight is about 0.1% in humans and research animals (Lambert & Yang, 2003; Warden et al., 2001). The peak plasma EGCG concentration is 0.15 μM after drinking two cups of green tea (Lee et al., 2002). Moreover, EGCG is unstable in both water and physiological fluid (Barras et al., 2009; Lambert et al., 2003). EGCG stability is decreased by various metabolic transformations including methylation, glucuronidation, sulfation and oxidative degradation *in vivo* (Dou, 2009; Lu et al., 2003a,b; Vaidyanathan & Walle, 2002). The low concentrations and instability of EGCG cannot effectively inhibit tumor growth. Hence, there is a critical

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need to use biocompatible and biodegradable nanoparticles to increase EGCG stability and cellular bioavailability.

Nanotechnology involves the control of matter, generally in the range of 100 nm or smaller (Zhang et al., 2008). Studies have shown that nanocarriers smaller than 100 nm are extravasated effectively into tissues (Peer et al., 2007; Zhang et al., 2008) and cleared much slower than large carriers by the reticulo-endothelial system in the liver and spleen (Nishiyama, 2007). The application of nanotechnology to medicine, known as nanomedicine, has a bright future in developing novel diagnostic and therapeutic agents (Zhang et al., 2008). Nanomedicine has gained tremendous attention in cancer therapy, because nanocarriers can increase drug absorption, protect drugs from premature degradation, prolong drug circulation time, exhibit high differential uptake efficiency in the target cells (or tissue) over normal cells (or tissue), lower toxicity through preventing the drug from prematurely interacting with the biological environment, improve intracellular penetration and more (Peer et al., 2007). Chemoprevention through the use of natural phytochemicals is a novel and promising approach for cancer management. Since most biological processes, including breast cancer, occur at the nanoscale, nanoparticulate technology may be useful in the chemoprevention of breast cancer. Nanoliposomes have received considerable attention because they are composed of natural components and the synthesis processes are easily scaled-up. In this study, we synthesized biocompatible and biodegradable nanoliposomes, which were composed of hydrophobic cholesterol and amphipathic phosphatidylcholine, and EGCG was encapsulated into nanoliposomes. We also coated nanoliposomes by chitosan, a natural polysaccharide, acting as an absorption enhancer (Chen et al., 2009; Dube et al., 2010; Kushima et al., 2009; Lee et al., 2009). In this study, all substances are generally recognized as safe (GRAS) by U.S. Food and Drug Administration (FDA). We measured the effects of EGCG encapsulated chitosan -coated nanoliposomes (CSLIPO-EGCG) on EGCG stability, its uptake by MCF7 cells, and the viability and apoptosis of MCF7 breast cancer cells.

Methods

Nanoliposome preparation

Soy lecithin (Avanti Polar Lipids, Alabaster, AL) and free cholesterol (Sigma, St. Louis, MO) with a molar ratio of 4:1 were dissolved in chloroform, and the mixture was dried under a nitrogen evaporator and followed by a freeze-dry system for more than 24 h. We made EGCG encapsulated nanoliposomes (LIPO-EGCG) in 1× phosphate buffered saline (1 × PBS) solution containing 6 mM of EGCG and void nanoliposomes (V.LIPO) in 1 × PBS solution using a sonication method. Both LIPO-EGCG and V. LIPO were coated with 0.2% (w/v) of chitosan with medium molecular weight of 190,000–310,000. (Sigma, St. Louis, MO) using a magnetic stirrer for 1 h at 4 °C to form void CSLIPO (V. CSLIPO) and CSLIPO-EGCG, respectively. All steps during sonication and coating were performed under nitrogen to prevent EGCG and lipid degradation, oxidation and epimerization.

Measurement of nanoliposome characteristics

The size and morphology of nanoliposomes were analyzed using a scanning electron microscope (SEM) (Gemini Supra 35, LEO, Carl Zeiss, Oberkochen, Germany). The samples were diluted with deionized distilled water and dropped on conductive silicon substrate by pipette. After air dried, the samples were observed under SEM at the voltage of 7 kV. The size, size distribution and zeta potential were measured using Brookhaven BI-MAS and ZetaPALS analyzer, respectively. EGCG was detected using a high-performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA) with a C18 reverse-phase column and a UV detector (Agilent Technologies, Santa Clara, CA) as described previously in our paper (Chen et al., 2001). The mobile phase was composed of 83% water, 17% acetonitrile and 0.05% trifluoroacetic acid, and the flow rate was 1 mL/min. EGCG was detected at 280 nm as described previously (Chen et al., 2001). EGCG encapsulation efficiency and loading capacity are calculated thus:

$$\text{Encapsulation efficiency } (\%) = (\text{Weight of EGCG added} - \text{Weight of free EGCG})/\text{Weight of EGCG added} \times 100\%$$

$$\text{Loading capacity} = (\text{Weight of EGCG added} - \text{Weight of free EGCG})/\text{Weight of LIPO} - \text{EGCG} \times 100\%$$

Measurement of the stability of nanoliposomes, nanoencapsulated and native EGCG

The stability of LIPO-EGCG, CSLIPO-EGCG and native EGCG dissolved in 1 × PBS was measured at pH 7.2 at 4 °C and room temperature (RT) (22 °C). Nanoencapsulated EGCG was separated from nonencapsulated EGCG using a Sephadex™ G-25 column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Total and nanoencapsulated EGCG was measured using a HPLC system (Waters Corporation, Milford, MA) with a C18 reverse-phase column and a UV detector. Particle size, zeta potential, polydispersity index and nanoencapsulated EGCG concentrations of LIPO-EGCG and CSLIPO-EGCG were measured every 24 h for 7 d at 4 °C, and every 2 h for 12 h at 22 °C. The EGCG concentration changes of native EGCG dissolved in 1 × PBS were measured at the same time period and condition. The initial EGCG concentration was 500 μM in native EGCG, LIPO-EGCG and CSLIPO-EGCG.

Meanwhile, the stability of LIPO-EGCG, CSLIPO-EGCG and native EGCG was also measured in MCF7 cell culture medium at 4 °C, 22 °C and 37 °C. The cell culture medium was composed of Eagle's minimum essential cell culture medium supplemented with 10% fetal bovine serum, bovine insulin (0.01 mg/mL), penicillin (100 units/mL) and streptomycin (0.1 mg/mL). The initial EGCG concentration was 500 μM in native EGCG, LIPO-EGCG and CSLIPO-EGCG. The EGCG concentration changes in native EGCG, LIPO-EGCG and CSLIPO-EGCG at each time point were measured using a HPLC system.

In vitro release study

The *in vitro* release study of native EGCG, LIPO-EGCG and CSLIPO-EGCG were performed in the dissolution

medium of 1 × PBS (pH 5.0) using a dialysis method. Samples equivalent to 217 μg of EGCG were dispersed in 1 mL of 1 × PBS (pH 5.0) and then placed in the dialysis bags with MWCO 6000–8000. The dialysis bags were dipped with the help of a thread in a conical flask containing 15 mL of dissolution medium (37 °C) stirred at 250 rpm/min. In order to protect the released EGCG from degradation in the medium during a long-term release period, the dissolution medium was totally replaced by fresh pre-warmed medium every 2 h. The EGCG released into the medium at each time point was determined after appropriate dilution with mobile phase and submitted to the HPLC system.

Cell culture and treatments

Human estrogen receptor-positive MCF7 breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). MCF7 cells were cultured in Eagle's minimum essential cell culture medium supplemented with 10% fetal bovine serum, bovine insulin (0.01 mg/mL), penicillin (100 units/mL) and streptomycin (0.1 mg/mL) at 37 °C, 95% humidity and an atmosphere of 5% CO_2. Cells were treated with 1 × PBS (treatment 1), native EGCG (treatment 2), LIPO-EGCG (treatment 3), CSLIPO-EGCG (treatment 4), V. LIPO (treatment 5), V.CSLIPO (treatment 6) for 3 h.

Cellular uptake and distribution of fluorescent nanoliposome

MCF7 cells were seeded in a 96-well plate at a density of 5×10^4 cells per well in 100 μL of the culture medium and cultured for 24 h before the experiment. Cellular uptake and localization of 7-Nitro-2-1,3-benzoxadiazol-4-yl (NBD)-labeled LIPO or CSLIPO (liposome contained 1.0 mol% of NBD-phosphoethanolamine relative to the phospholipids) were measured using a fluorescence microscopy. Briefly, the attached cells were cultured in the cell culture medium and then treated with 1 × PBS (pH 7.4) (treatment 1), NBD-labeled LIPO dissolved in 1 × PBS (treatment 2) and NBD-labeled CSLIPO dissolved in 1 × PBS (treatment 3). Cells were incubated at 37 °C for 1, 2 and 4 h. After that, cells were washed three times with cold 1 × PBS (pH 7.4), fixed with 3.7% formaldehyde in 1 × PBS at room temperature for 10 min, followed by cell nuclei staining with DAPI for 5 min. Finally, the cells were imaged by an IX71® inverted microscope (Olympus America Inc, Center Valey, PA).

Intracellular EGCG uptake by MCF7 cells

MCF7 cells were suspended in the culture medium (3 mL) at a density of 2×10^6 cells/well in 6-well flat-bottom tissue culture plates. After 24 h, cells were treated with 50 or 100 μM of native EGCG (treatment 1), LIPO-EGCG (treatment 2), CSLIPO-EGCG (treatment 3), which were dissolved into 1 mL of FBS-free culture media containing SOD (5 U/mL). After treating cells for 4 h at 37 °C, cells were washed with ice-cold 1 × PBS three times. Cells were then scraped from the plates and put into 200 μL of 2% ascorbic acid (pH 3.0). Each well was washed with 200 μL of methanol, which was then combined with previous 200 μL of 2% ascorbic acid. The volume of the mixture was measured and internal standard (epicatechin) was added into the mixture. After one cycle of freezing and thawing, mixtures were sonicated for 2 min in an ice-cold bath using a sonicator (Branson, Inc., Danbury, CT) followed by centrifugation at 10 000 × *g* for 20 min at 4 °C. The upper supernatant solution was collected and injected into the HPLC system. Precipitates were washed with 0.5 mL of deionized water to get rid of acid and dried in the hood overnight. The dried cells were digested by 0.5 N NaOH for 48 h. Total cellular protein concentrations were determined by using a bicinchoninic acid (BCA) assay kit (Pierce, Cramlington, UK). Total cellular EGCG uptake levels were expressed as μg/mg of protein.

Cell viability

The viability of MCF7 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well tissue culture plates in a concentration of 10 000 cells/well in a 96-well cell culture plate. After the 3-h treatment, cells were incubated with MTT reagent (5 mg/mL in 1 × PBS) for 2 hours at 37 °C followed by adding MTT solubilization solution and additional 1 hour incubation in the dark. The absorbance in each well was measured at 562 and 690 nm on the BioTek ELx800™ absorbance microplate reader (BioTek, Winooski, VT). The background absorbance (690 nm) was subtracted from the 562 nm measurements. Three EGCG concentrations (10, 2.5 and 0.625 μM) were tested among all treatments to observe dose-dependent changes in cell viability.

Detection of apoptosis

Apoptosis was quantified using a DeadEnd™ colorimetric terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick and labeling (TUNEL) kit (Promega Corporation, Madison, WI). After 3-h treatments, the cells were fixed by a formaldehyde-based method. The fixed cells were washed and incubated with equilibration buffer followed by incubation with biotinylated nucleotide mix and terminal deoxynucleotidyl transferase, recombinant, (rTdT) enzyme. After 1 h incubation at 37 °C, cells were washed using 1 × PBS. Endogenous peroxidases in cells were inactivated by adding 3% hydrogen peroxide. After washing three times with 1 × PBS, cells were incubated with horseradish peroxidase-labeled streptavidin for 30 min at room temperature. After washing cells with 1 × PBS three times, cells were incubated with diaminobenzidine (DAB). Using this procedure, apoptotic nuclei were stained dark brown. The stained cells were seen using an Olympus BX50 microscope (Olympus America Inc, Center Valey, PA) and photographed with the Nikon Digital Camera (model #Dxm1200) (Nikon, Melville, NY) attached to the microscope. NIH image J software (National Institutes of Health, Bethesda, MD) was used for data analysis.

Statistical analyses

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software (IBM corporation, Armonk, NY). One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was performed to compare multiple group means. Differences were considered significant at $p < 0.05$. Data are presented in text,

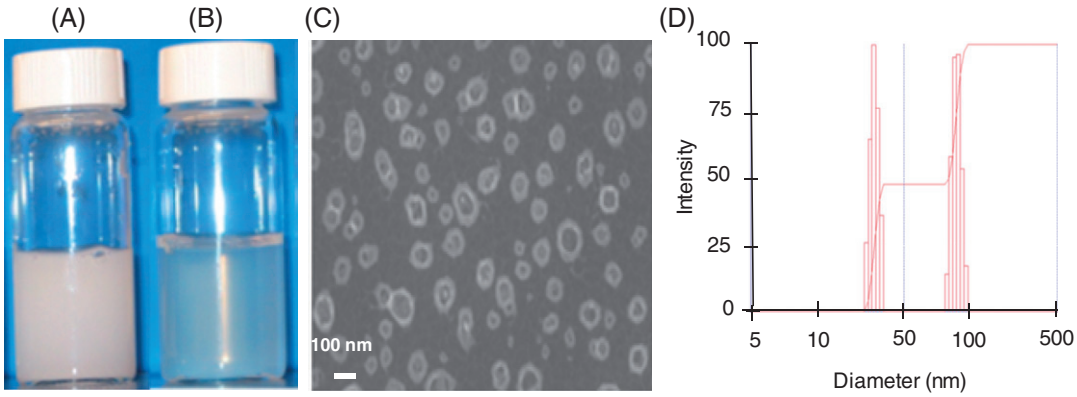


Figure 1. (A) A mixture of phosphatidylcholine, cholesterol, chitosan and EGCG in 1 × PBS; (B) CSLIPO-EGCG in 1 × PBS; (C) scanning electron microscope image of CSLIPO-EGCG; (D) the size of CSLIPO-EGCG measured using Brookhaven BI-MAS particle size analyzer.

Table 1. Characteristics of nanoliposomes.

Nanoliposomes	Effective diameter (nm)	Polydispersity	Zeta potential (mV)
LIPO-EGCG	56.0 ± 2.0	0.24 ± 0.01	−6.8 ± 1.8
V. LIPO	50.6 ± 3.0	0.22 ± 0.01	−9.6 ± 1.9
CSLIPO-EGCG	85.0 ± 6.6	0.35 ± 0.02	16.4 ± 2.8
V.CSLIPO	88.1 ± 8.2	0.38 ± 0.01	19.2 ± 2.6

figures and tables as means ± standard deviation (SD) from at least three independent measurements.

Results

Characteristics of nanoliposomes

Before making liposomes, all components were added into 1 × PBS solution. They were not soluble in 1 × PBS and formed a cloudy solution (Figure 1A). After sonication followed by coating, CSLIPO-EGCG were formed and the solution was clear (Figure 1B). Physical characteristics of nanoliposomes are summarized in Table 1. The mean size of LIPO-EGCG and CSLIPO-EGCG was less than 100 nm in diameter. CSLIPO-EGCG and V. CSLIPO had larger size (around 86 nm in diameter) than LIPO-EGCG and V. LIPO (around 55 nm in diameter). CSLIPO-EGCG and V. CSLIPO had positive zeta potentials, and LIPO-EGCG and V. LIPO had negative zeta potentials. EGCG encapsulation efficiency and loading capacity in CSLIPO were about 90% and 3%, respectively. CSLIPO-EGCG morphology was measured by using a scanning electron microscope (SEM) (Figure 1C). They are spherical and sizes are measured by SEM and Brookhaven BI-MAS analyzer (Brookhave, Holtsville, NY) (Figure 1D) are similar, around 86 nm in diameter.

The stability of nanoliposomes, nanoencapsulated and native EGCG

Particle size, zeta potential and polydispersity of LIPO-EGCG and CSLIPO-EGCG in 1 × PBS remain similar to the initial values after 7 d at 4 °C and after 12 h at 22 °C (Table 2). EGCG stability was significantly increased when it was encapsulated into CSLIPO. More than 95% of native EGCG in 1 × PBS (pH 7.2) was degraded after 12 h at 22 °C. However, only 10% of EGCG was degraded when the equivalent amount of EGCG was encapsulated into LIPO or CSLIPO at the same condition (Figure 2A). Native EGCG in 1 × PBS (pH 7.2) was completely degraded after 5 d at 4 °C. However, nanoencapsulated EGCG was degraded only 38% and 22% after 5 d at 4 °C in LIPO-EGCG and CSLIPO-EGCG, respectively (Figure 2B).

When we added native EGCG, LIPO-EGCG and CSLIPO-EGCG into cell culture mediums containing 10% FBS, the same stability trend and order were observed (Figure 3). The CSLIPO-EGCG showed the highest percentage remaining of EGCG in cell culture medium at 4 °C, 22 °C (RT) and 37 °C, whereas EGCG stability was significantly decreased when the temperature increased ($p < 0.01$). Native EGCG was completely degraded after 1 h at 37 °C. However, nanoencapsulated EGCG was degraded only 46% and 32% after 1 h at 37 °C in LIPO-EGCG and CSLIPO-EGCG, respectively.

In vitro release study

The EGCG release behavior of native EGCG, LIPO-EGCG and CSLIPO-EGCG was investigated using a dialysis method. Since EGCG is unstable in neutral and alkaline solutions, 1 × PBS (pH 5.0) was used as a receptor medium in order to provide stable condition. Native EGCG exhibited a significantly much faster dissolution rate with approximately 208.7 μg of the compound released within the initial 2-h period ($p < 0.01$) (Figure 4). In contrast, only 26.46 μg and 25.02 μg of EGCG were released from LIPO-EGCG and CSLIPO-EGCG within the first 2-h period, respectively. Both LIPO-EGCG and CSLIPO-EGCG exhibited controlled-release property after the minor burst EGCG release at the initial stage. The average release amount of EGCG at each 2-h period was about 6.54 μg and 6.32 μg in the later stage (from hour 6 to hour 34) for LIPO-EGCG and CSLIPO-EGCG, respectively.

Increased uptake of NBD-labeled CSLIPO

Intracellular uptake and distribution of NBD-labeled LIPO and NBD-labeled CSLIPO were observed by a fluorescent microscopy (Figure 5). MCF7 cells with no treatment showed undetectable background green fluorescence signals (Figure 5A). As the incubation time was increased from 1 h to 4 h, the uptake of both NBD-labeled LIPO (Figure 5B) and NBD-labeled CSLIPO (Figure 5C) was proportionally

Table 2. Particle size, zeta potential and polydispersity of LIPO-EGCG and CSLIPO-EGCG dissolved in 1 × PBS at pH 7.2 after storage at 4 °C and 22 °C.

Nanoliposome		Particle size (nm)		Zeta potential (mV)		Polydispersity	
Temperature		0 d	7 d	0 d	7 d	0 d	7 d
4 °C	LIPO-EGCG	52.0 ± 0.8	52.8 ± 1.5	−7.6 ± 2.9	−12.1 ± 2.8	0.24 ± 0.01	0.25 ± 0.02
	CSLIPO-EGCG	73.0 ± 0.3	83.2 ± 2.3	18.3 ± 2.4	18.6 ± 4.0	0.36 ± 0.01	0.36 ± 0.01
		0 h	12 h	0 h	12 h	0 h	12 h
22 °C	LIPO-EGCG	54.8 ± 0.3	61.5 ± 0.5	−19.1 ± 0.8	−17.5 ± 2.1	0.21 ± 0.01	0.25 ± 0.04
	CSLIPO-EGCG	78.5 ± 0.3	80.8 ± 1.1	14.3 ± 0.9	14.7 ± 1.5	0.31 ± 0.01	0.37 ± 0.02

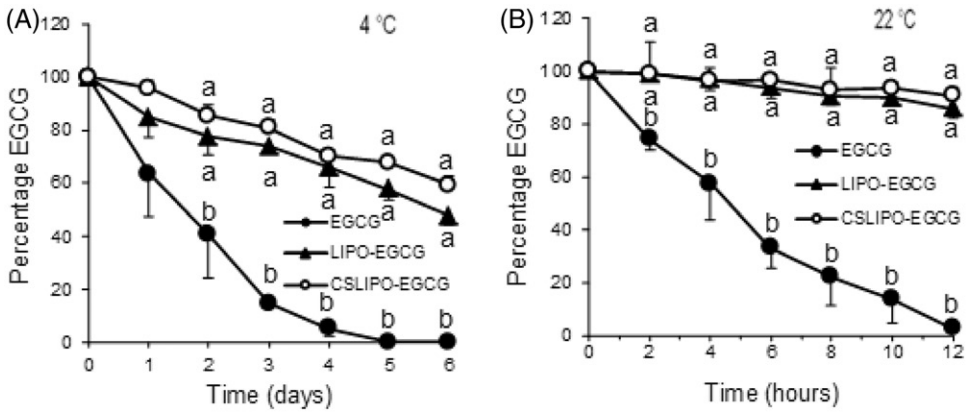


Figure 2. Stability of 0.5 mM of native EGCG and equivalent amounts of EGCG encapsulated into LIPO and CSLIPO in 1 × PBS (pH 7.2) at 4 °C (A) and 22 °C (B). Means at a time without a common superscript differ, $p < 0.05$.

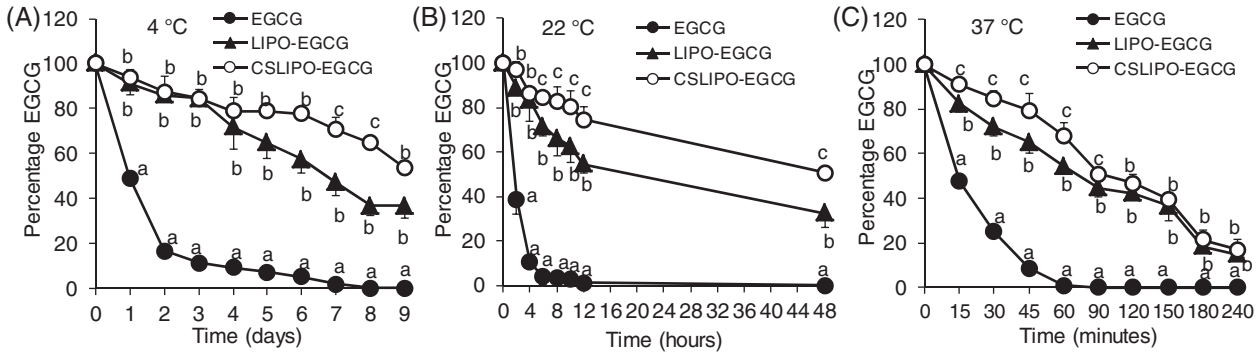


Figure 3. Stability of 0.5 mM of native EGCG and equivalent amounts of EGCG encapsulated into LIPO and CSLIPO in cell culture medium containing 10% FBS at 4 °C (A), 22 °C (B) and 37 °C (C). Means at a time without a common superscript differ, $p < 0.05$.

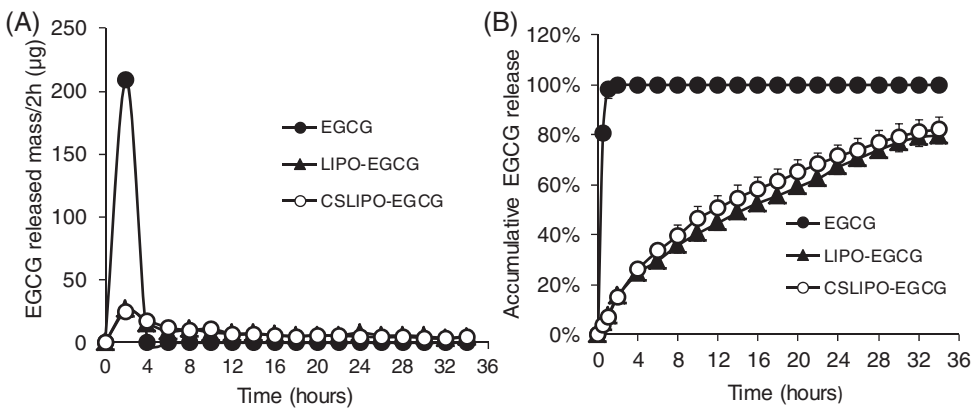


Figure 4. *In vitro* release profile of EGCG in 1 × PBS (pH 5.0) using a dialysis method at 37 °C.

elevated. However, cells treated with NBD-labeled CSLIPO had higher intracellular fluorescence intensity than cells treated with NBD-labeled LIPO. After MCF7 cells were exposed to NBD-labeled LIPO and NBD-labeled CSLIPO, the intact liposomes were found within the cells. Meanwhile, some diffused fluorescence was clearly observed in the entire intracellular matrix, but not located in the nuclei.

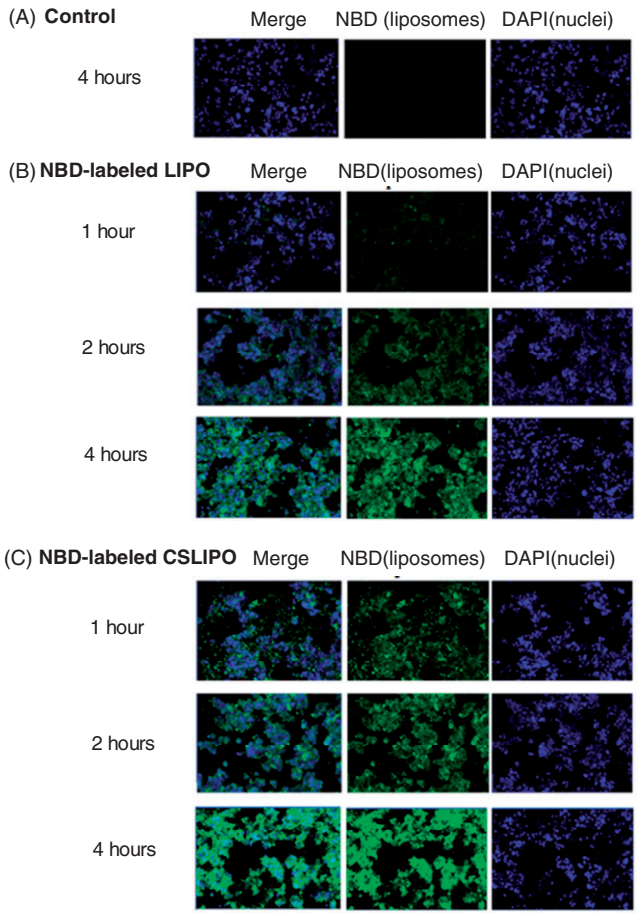


Figure 5. Fluorescent images of cellular uptake of $1 \times$ PBS as control (A), NBD-labeled LIPO (B), and NBD-labeled CSLIPO (C) by MCF7 human breast cancer cells. MCF7 cells were incubated with the above treatments for 1 h, 2 h and 4 h at 37 °C. Cell nuclei were stained blue by DAPI ($\lambda_{ex} = 358$nm, $\lambda_{em} = 461$ nm) and merged with green fluorescent signals from NBD-labeled liposomes ($\lambda_{ex} = 460$ nm, $\lambda_{em} = 535$ nm).

Increased cellular EGCG content

Cellular EGCG content was significantly increased by both LIPO-EGCG and CSLIPO-EGCG in MCF7 cells. Compared to 50 μM of EGCG, LIPO-EGCG and CSLIPO-EGCG at the equivalent concentration raised the cellular EGCG content by four folds and nine folds, respectively (Figure 6A). LIPO-EGCG and CSLIPO-EGCG at 100 μM increased the cellular EGCG content by six times and 34 times, respectively, compared to EGCG at the same concentration (Figure 6B).

Inhibitory effect of nanoencapsulated EGCG on cell viability

After treating MCF7 cells with 10 μM of native or nanoencapsulated EGCG for 3 h, CSLIPO-EGCG, but not LIPO-EGCG, significantly lower 40% of cell viability compared with $1 \times$ PBS and native EGCG. At the same concentration, native EGCG and V. CSLIPO did not significantly lower cell viability compared with $1 \times$ PBS (Figure 7A). The inhibitory effect of CSLIPO-EGCG on MCF7 cell viability was dose-dependent at the encapsulated EGCG concentration range of 0.625–10 μM. The reduction of cell viability was about 40%, 20% and 10% by 10, 2.5 and 0.625 μM of CSLIPO-EGCG, respectively (Figure 7B).

Proapoptotic effect of nanoencapsulated EGCG

Ten micromoles of CSLIPO-EGCG caused 27% of apoptosis in MCF7 cells. Ten micromoles of V. CSLIPO and native EGCG induced 4% and 6% of apoptosis, respectively. DNase is positive control of apoptosis, which induced 35% of apoptosis in MCF7 cells (Figure 8A and B). CSLIPO-EGCG significantly induced the apoptosis of MCF7 cells compared to native EGCG and V.CSLIPO.

Discussion

The study of EGCG as a chemopreventive agent has increased significantly in the past few years, due in part to EGCG's positive effects seen in *in vitro* studies and some research animal models. However, the evidence of human studies is inconclusive regarding its effectiveness for lowering breast cancer risk (Inoue et al., 2001; Nagano et al., 2001; Nakachi et al., 1998; Suzuki et al., 2004; Wu et al., 2003). The major problem is its low level of stability,

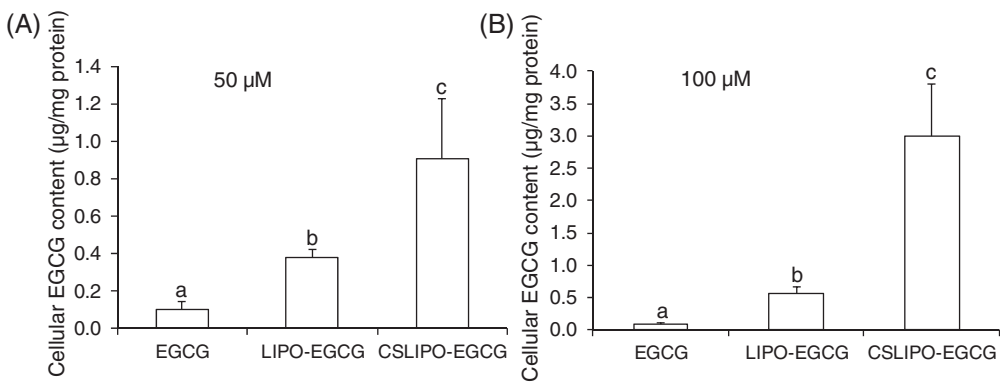


Figure 6. EGCG content in MCF7 cells after treating them with 50 μM and 100 μM of LIPO-EGCG and CSLIPO-EGCG for 4 h at 37 °C. Values are the means of four independent experiments, with standard deviations represented by vertical bars. Bars without a common superscript differ, $p < 0.01$.

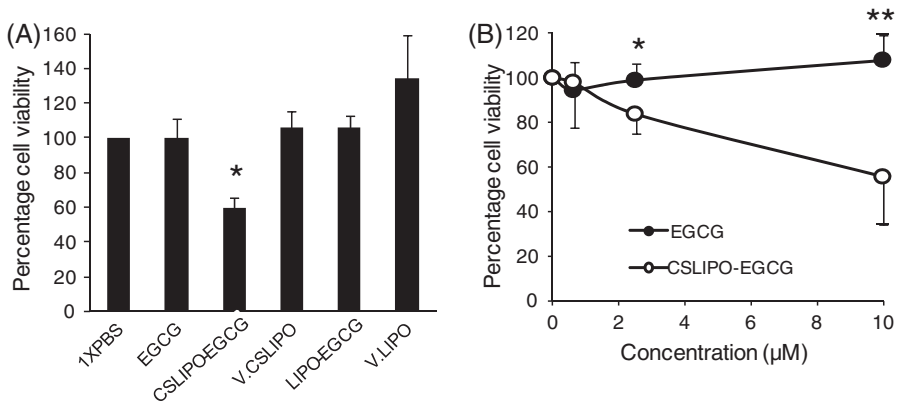
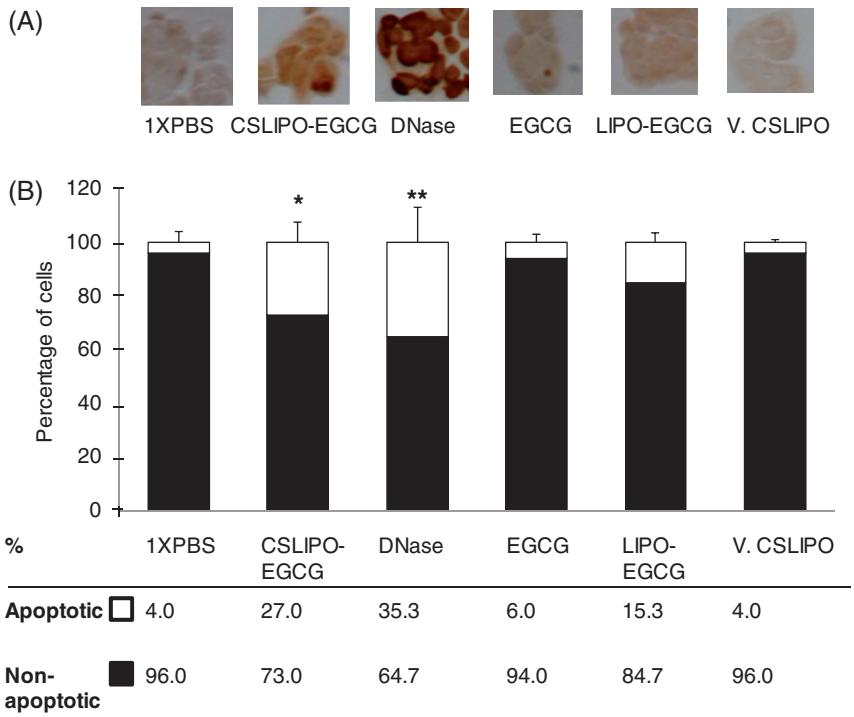


Figure 7. (A) Comparative effect of native EGCG and nanoencapsulated EGCG at 10 μM, and their void vehicles on MCF7 cell viability; (B) dose-dependent effect of CSLIPO-EGCG on MCF7 cell viability. *$p < 0.05$; **$p < 0.01$.



%	1XPBS	CSLIPO-EGCG	DNase	EGCG	LIPO-EGCG	V. CSLIPO
Apoptotic	4.0	27.0	35.3	6.0	15.3	4.0
Non-apoptotic	96.0	73.0	64.7	94.0	84.7	96.0

Figure 8. Apoptotic effect of EGCG encapsulated nanoparticles on MCF7 cells. (A) Representative apoptotic images from each treatment group, a darker tone denotes apoptosis; (B) percentage of apoptotic (white) and non-apoptotic cells (black) at treatment groups. Values are the means of three independent experiments, with standard deviations represented by vertical bars. *$p < 0.05$; **$p < 0.01$.

bioavailability and target specificity in humans (Zhu et al., 2000). In this study, we used biocompatible and biodegradable nanoliposomes to increase EGCG stability and enhance its antiproliferative and proapoptotic effects on MCF7 breast cancer cells.

LIPO-EGCG was composed of hydrophobic cholesterol and amphipathic phosphatidylcholine, and EGCG was encapsulated into the hydrophilic core. Both cholesterol and phosphatidylcholine are major cell membrane components. Therefore, the nanoliposomes are biocompatible and biodegradable. The average size of LIPO-EGCG was around 55 nm in diameter, and EGCG encapsulation efficiency was around 90%. Since the zeta potential of LIPO-EGCG was negative, we decided to coat LIPO-EGCG by chitosan, which can give LIPO-EGCG a positive charge. Chitosan coating amount on the stability of EGCG was investigated in our previous study at three concentrations (0.1%, 0.2% and 0.4%). No significant difference was found among them in degradation rate of EGCG in 1 × PBS (pH 7.2) at room temperature (data not shown). Higher concentration of chitosan can increase uptake of nanoparticles, but can also lower the cell culture medium and increase the nanoparticle size (Jaruszewski et al., 2011; Liu & Park, 2009). Based on these preliminary results, CSLIPO-EGCG were coated with 0.2% of chitosan in this study. CSLIPO-EGCG were spherical with size under 100 nm in diameter and positively charged. Nanoparticles with size between 10 and 100 nm in diameter can easily penetrate into tumor tissues by extravasation through increased permeability of the tumor vasculature and ineffective lymphatic drainage, and escaping renal elimination (Choi et al., 2011; Peer et al., 2007). Chitosan is a natural and biocompatible polysaccharide obtained by deacetylation

of chitin, which is the structural element in the exoskeleton of crustaceans, such as crabs and shrimps. Chitosan can also increase the stability and bioavailability of nanoparticles and nanoencapsulated compounds (Chen et al., 2009; Dube et al., 2010; Jaruszewski et al., 2011; Lee et al., 2009; Liu & Park, 2009). The size and zeta-potential of CSLIPO-EGCG remained stable after 6 days at 4 °C and 12 h at 22 °C. The stability of EGCG was significantly enhanced by nanoencapsulation, especially by CSLIPO. Native EGCG at a concentration of 0.5 mM was completely degraded after 5 days at 4 °C. However, we could detect more than 70% of EGCG, when it was encapsulated into CSLIPO at the same condition. The same stability trend and order were also found when they were added into cell culture medium containing 10%FBS. CSLIPO-EGCG had the significantly highest stability at three temperature levels (37 °C, 22 °C and 4 °C), and native EGCG had the lowest stability. *In vitro* release study demonstrated that LIPO-EGCG and CSLIPO-EGCG had sustained release feature. Native EGCG only showed a burst release at the initial 2-h period. This sustained release feature also means the lower leakage rate of EGCG from LIPO and CSLIPO. Moreover, the uptake of CSLIPO and nanoencapsulated EGCG was increased in MCF7 cells. Many studies have demonstrated that nanoparticles can increase the stability of encapsulated compounds and the cellular uptake of compounds in many cells (Dube et al., 2011; Shen et al., 2012). In this study, the highest cellular fluorescence intensity was obtained after 4 h of treating MCF7 cells with NBD-labeled liposomes. Relative lower intensity appeared at 1 h might be due to the time delay caused by endocytosis of transmembrane transport. However, a more significant finding was that a big visible difference of green color fluorescence intensity was observed between LIPO and CSLIPO. The data confirm that chitosan is an uptake enhancer. The difference may also result from LIPO degradation caused by the enzymes or environmental factors in cells during the incubation period, resulting in the loss of structural integrity and release of the fluorescent lipid molecule into the cytosol. Compared to free EGCG, both LIPO-EGCG and CSLIPO-EGCG significantly increase the cellular EGCG content in MCF7 cells at the same condition. Consistent to the uptake of NBD-labeled nanoliposome data, CSLIPO-EGCG increased cellular EGCG content much more than LIPO-EGCG (more than 2-fold changes). The improved stability, lower leakage rate and elevated cellular uptake of EGCG may partially contribute to the increased antiproliferative and proapoptotic effect of CSLIPO-EGCG on MCF7 breast cancer cells.

Nanoliposomes have been used widely in cancer treatment. Liposomes can serve as delivery vesicles for both hydrophobic and hydrophilic molecules due to their structure of hydrophobic lipid bilayers and a hydrophilic core. Liposomes have been used to deliver drug, phytochemicals, vaccine, DNA/RNA and so on (Fang et al., 2005; Henriksen-Lacey et al., 2011; Langer, 1998; Xiong et al., 2011). Since nanoliposomes provide more surface area than liposomes, they have the potential to increase solubility, bioavailability and target specificity of encapsulated compounds, and improve controlled release (Mozafari, 2010). Tumor tissues have impaired vasculature and enhanced permeability and retention (EPR) by which they accumulate more nanoliposomes than normal tissues do (Mozafari et al., 2009). Cancer researchers have used nanoliposomes to deliver anticancer drug, siRNA and diagnostic agents. Myocet (liposomal doxorubicin) and Doxil/Caelyx (liposome- polyethylene glycol doxorubicin) are clinically approved anticancer drugs. Nanoencapsulation can increase drug solubility, stability, bioavailability, prolong drug circulation time, exhibit high levels of target specificity, lower toxicity through preventing the drug from prematurely interacting with the biological environment, improve intracellular penetration and more (Peer et al., 2007).

The use of bioactive food components to reduce the risk of cancer, also called chemoprevention, is gaining tremendous attention. EGCG is one of the most potent chemopreventive agents. Some studies using chitosan nanoparticles and lipid nanocapsules to encapsulate EGCG demonstrate that nanoencapsulation dramatically increases the EGCG stability (Barras et al., 2009; Dube et al., 2010), which are consistent with our current results. Furthermore, chitosan nanoparticles enhance the intestinal absorption of nanoencapsulated EGCG in rodent animal models (Dube et al., 2010), and the use of these nanoparticles could be a promising strategy for improving EGCG bioavailability in humans. Nanoencapsulated EGCG has demonstrated significantly increased proapoptotic, antiproliferative and antiangiogenic efficacy in *in vitro* and *in vivo* studies (Sanna et al., 2011; Siddiqui & Mukhtar, 2010; Siddiqui et al., 2009). Siddiqui et al (2009) have determined the effect of EGCG encapsulated polylactic acid (PLA)–polyethylene glycol (PEG) nanoparticles on human prostate cancer under both in cell culture and animal studies. Nanoencapsulated EGCG at 2.74 μM can significantly inhibit the proliferation of PCa prostate cancer cells and induce their apoptosis, which exhibits more than 10-fold dose advantage over native EGCG. Furthermore, nanoencapsulated EGCG retains EGCG's mechanistic signature. EGCG encapsulated into PLA-PEG nanoparticles can significantly decrease antiapoptotic Bcl-2 levels, increase proapoptotic Bax levels and the Bax/Bcl-2 ratio, induce p21 and p27 expression, which have been shown to be regulated exactly by native EGCG at high concentrations (Siddiqui & Mukhtar, 2010). Nanoencapsulated EGCG also dramatically reduces tumor volume in male athymic nude mice implanted with human prostate cancer cells (Siddiqui et al., 2009). Sanna et al (2011) enhance the therapeutic effectiveness by increasing the target specificity of EGCG encapsulated poly(lactic-coglycolic acid) (PLGA)-PEG nanoparticles through incorporating a target ligand on the surface of nanoparticles. The ligand has a high binding affinity to the prostate specific membrane antigen (PSMA) expressed on PCa prostate cancer cells, and further allows EGCG nanoparticles to selectively inhibit the growth of prostate-specific membrane antigen (PSMA) positive PCa cells. Our study is the first one to investigate the effect of nanoencapsulated EGCG on breast cancer. Numerous studies have demonstrated that native EGCG can lower cell viability and induce apoptosis of MCF7 cells at concentrations more than 50 μM (Bigelow & Cardelli, 2006; Hsu & Liou, 2011; Sen et al., 2009). The current study demonstrates that CSLIPO-EGCG can exhibit the same chemopreventive effects in MCF7 cells even at a

concentration of 10 μM or lower, further confirming its remarkable dose advantage. More studies are required to determine the oral and cellular bioavailability of CSLIPO-EGCG, and their tumor suppressive capacity and safety in research animals and humans.

Conclusions

In conclusion, EGCG stability was significantly increased through encapsulation into LIPO and CSLIPO. CSLIPO-EGCG significantly elevated the uptake of nanoliposomes and cellular EGCG content in MCF7 cells. CSLIPO-EGCG significantly lowered the viability of MCF7 cells and induced their apoptosis at 10 μM or lower. This study could serve as a basis for the use of biodegradable and biocompatible nanoparticle-mediated delivery systems to increase bioavailability, solubility, stability and payload of chemopreventive agents.

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Declaration of interest

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