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Formulation, Characteristics and Anti-atherogenic Bioactivities of CD36-Targeted Epigallocatechin Gallate (EGCG)-Loaded Nanoparticles

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Abstract

Intimal macrophages are determinant cells for atherosclerotic lesion formation by releasing inflammatory factors and taking up oxidized low density lipoprotein (oxLDL) via scavenger receptors, primarily the CD36 receptor. (–)-Epigallocatechin-3-gallate (EGCG) has a potential to decrease cholesterol accumulation and inflammatory responses in macrophages. We made EGCG-loaded nanoparticles (Enano) using phosphatidylcholine, kolliphor HS15, alpha-tocopherol acetate and EGCG. 1-(Palmitoyl)-2-(5-keto-6-octene-diyl) phosphatidylcholine (KODiA-PC), a CD36-targeted ligand found on oxLDL, was incorporated on the surface of Enano to make ligand-Enano (L-Enano). The objectives of this study are to deliver EGCG to macrophages via CD36-targeted L-Enano and to determine its anti-atherogenic bioactivities. The optimized nanoparticles obtained in our study were spherical and around 108 nm in diameter, had about 10% of EGCG loading capacity and 96% of EGCG encapsulation efficiency. Compared to Enano, CD36-targeted L-Enano had significantly higher binding affinity to and uptake by macrophages at the same pattern as oxLDL. CD36-targeted L-Enano dramatically improved EGCG stability, increased macrophage EGCG content, delivered EGCG to macrophage cytosol and avoided lysosomes. L-Enano significantly decreased macrophage mRNA levels and protein secretion of monocyte chemoattractant protein 1, but did not significantly change macrophage cholesterol content. The innovative CD36-targeted nanoparticles may facilitate targeted delivery of diagnostic, preventive and therapeutic compounds to intimal macrophages for the diagnosis, prevention and treatment of atherosclerosis with enhanced efficacy and decreased side effects.

Keywords

Phytochemicals; (–)-Epigallocatechin-3-gallate; Nanoparticles; Targeted delivery; Biocompatible and biodegradable; Macrophages; Atherosclerosis

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1. Introduction

(-)-Epigallocatechin-3-gallate (EGCG) comprises 25 to 55% of the total green tea catechins [1, 2]. EGCG has a potential to decrease cholesterol accumulation and inflammatory responses in macrophages, which may in turn prevent atherosclerosis formation and development [3–6]. When apolipoprotein E null mice were given a daily dose of EGCG at 10 mg per kg body weight through intraperitoneal injections for twenty one days, cuff-induced evolving atherosclerotic lesion size was reduced by 55% [7]. Human studies have already shown that EGCG has beneficial effects for cardiovascular health, but the evidence is inconsistent [8–10]. The major reasons might be a low level of EGCG bioavailability and stability, a high level of metabolism by enzymes in the liver and other tissues, non-targeting effect on intimal macrophages [8–10]. Due to potential toxicity and side effects, human studies used low doses of EGCG [11]. The oral bioavailability of EGCG is about 0.1% in humans and research animals [12, 13]. EGCG is not stable in water and physiological fluids [1, 2]. Methylation, sulfation, glucuronidation, and other metabolic transformations dramatically decrease EGCG stability and bioactivities [8–10]. Nanotechnology could enhance EGCG stability, bioavailability, target specificity and bioactivities; reduce its toxicity through preventing EGCG from prematurely contacting with the biological environment, promoting intracellular penetration and sustained release of EGCG [14, 15].

Cardiovascular disease (CVD) is the No. 1 killer in the United States [16]. About 50% of the CVD death is caused by atherosclerosis [16]. Increased lipid, primarily cholesterol, accumulation and inflammatory responses in intimal macrophages are major contributors for atherosclerosis initiation, formation and progression [17, 18]. Monocyte chemoattractant protein 1 (MCP-1), a chemokine, directs blood inflammatory monocytes migration into the intimal layer of arteries [19, 20]. Monocytes in the intimal layer are activated and further differentiated into macrophages upon response to macrophage colony stimulating factors. Intimal macrophages express scavenger receptors for binding and taking up oxidized low density lipoprotein (oxLDL), primarily minimally oxLDL in humans. After macrophages accumulate cholesterol, they are transformed into foam cells, which are the hallmark of atherosclerosis. Macrophages and foam cells release inflammatory factors, especially MCP-1, which can recruit more inflammatory monocytes into the intimal layer of arteries, and amplify the local inflammatory response. Macrophage scavenger receptor AI, AII and CD36 are major membrane proteins involved in the uptake of cholesterol-rich oxLDL [21, 22]. Studies performed in mice lacking CD36 showed a significant reduction (76.5%) in aortic lesion size, and peritoneal macrophages isolated from those mice exhibited a 60–80% decrease in oxLDL binding and uptake [23]. This suggests that CD36-mediated oxLDL binding and uptake are required for foam cell formation and atherosclerotic lesion development. Oxidized phospholipids are enriched in atherosclerotic lesions in animals [24, 25]. They seem the most likely ligands for binding oxLDL to CD36. 1-(Palmitoyl)-2-(5-keto-6-octene-diyl) phosphatidylcholine (KODiA-PC), a type of oxidized phosphatidylcholines found on oxLDL, had a high binding affinity to CD36 and participated in CD36-mediated recognition and uptake of oxLDL and other particles by intimal macrophages [26, 27]. CD36 expression correlates well with lesion severity [26–28].

Targeting intimal macrophages through the CD36 receptor is a promising avenue for diagnosis, prevention and treatment of atherosclerosis [29–32].

In our previously published study, we have successfully synthesized EGCG encapsulated nanostructured lipid carriers (NLCE) [33]. Nanoencapsulation significantly enhanced EGCG stability. NLCE compared to native EGCG significantly reduced MCP-1 expression and release from THP-1 derived macrophages. However, nanostructured lipid carriers (NLC) contained a large amount of triglyceride. If we further conduct *in vivo* studies by administering NLCE to animal or human bodies, NLC and NLCE have a potential to increase blood triglyceride concentrations, an independent risk factor for cardiovascular disease [34, 35]. To overcome this problem, we successfully develop a triglyceride free EGCG-loaded nanoparticles (Enano) in the present study by replacing triglyceride with (+)-alpha (α)-tocopherol acetate. Additionally, (+)- α -tocopherol acetate, as an anti-oxidant, can prevent EGCG and other components in nanoparticles from oxidation [36].

In this study, we made void nanoparticles (Vnano) using phosphatidylcholine, kolliphor HS15, (+)- α -tocopherol acetate and incorporated KODiA-PC on the surface of Vnano to form ligand-Vnano (L-Vnano). Kolliphor HS15 is a commonly used, nonionic solubilizer and emulsifying agent. We further encapsulated EGCG into Vnano and L-Vnano to synthesize Enano and L-Enano, respectively. In order to increase binding affinity of nanoparticles to macrophages, EGCG loading capacity and encapsulation efficiency we optimized composition of nanoparticles. We compared their binding affinity to and uptake by THP-1 derived macrophages in this study. We hypothesize that nanoencapsulation increases EGCG stability; L-Enano compared to Enano has higher binding affinity to THP-1 derived macrophages (Fig. 1), and further increases macrophage EGCG content, which might associate with decreased macrophage cholesterol content, and MCP-1 expression and secretion.

2. Materials and methods

2.1. Chemicals and reagents

EGCG (>95%), (+)- α -tocopherol acetate, phorbol 12- myristate 13-acetate (PMA), *Escherichia coli* lipopolysaccharide were purchased from Sigma-Aldrich Chemical, MO. KODiA-PC was purchased from Cayman Chemical, MI. Kolliphor HS15 was given as a gift from BASF Chemical, NJ. Soy phosphatidylcholine (PC) and 7-nitro-2-1, 3-benzoxadiazol-4-yl-phosphotidylcholine (NBD-PC) were purchased from Avanti Polar Lipids, AL. Trizol reagent, SuperScript™ III reverse transcriptase, and power SYBR green master mix were purchased from Life Technologies, CA.

2.2. Preparation of nanoparticles

Enano were synthesized using a lipid mixture containing 10% of EGCG, 36.2% of PC, 45% of kolliphor HS15, and 8.8% of (+)- α -tocopherol acetate in weight. After adding deionized water into the lipid mixture, the suspension was homogenized for 2 minutes followed by sonication for additional 1 to 2 minutes. L-Enano was made by using the above method via replacing 30 mol% of PC with KODiA-PC (Fig 1). Void nanoparticles (Vnano) and void

ligand-nanoparticles (L-Vnano) were prepared using the above materials and procedures without adding EGCG. For *in vitro* binding and uptake experiments, fluorescence dye NBD-PC (2 mol% of total PC) was used to make NBD-labeled nanoparticles.

2.3. Characteristics, encapsulation efficiency and loading capacity of nanoparticles

The particle size and polydispersity index were measured using a BI-MAS particle size analyzer, and zeta potential was measured using a ZetaPALS analyzer (Brookhaven Corporation, NY). The nanoparticle morphology was examined using a transmission electron microscope (TEM) instrument (200Kv Hitachi H-8100, Tokyo). The encapsulation efficiency and loading capacity of nanoparticles were measured as previously described [33]. Briefly, the total EGCG concentration (C_{total}) in the nanoparticle solution was measured using a high performance liquid chromatography (HPLC) system (Waters Co., Milford, MA) with a C18 reverse-phase column (150 mm×4.6 mm, 5 μ m size) and Waters 2489 UV/Visible detector. The mixture of water/acetonitrile/ethyl acetate/sulfuric acid (86:12:2:0.043, v:v:v:v) was used as a mobile phase with flow rate of 1 mL/min. The detection wavelength was selected as 254 nm. Native EGCG was separated from nanoencapsulated EGCG using an ultrafiltration method (Millipore Amicon Ultra-15), and measured by the HPLC system (C_{native}). In order to calculate the loading capacity, a certain volume of Enano or L-Enano (V) was dried using a vacuum freeze-drying system (FreeZone 4.5 plus, Labconco, Kansas City, MO). The weight of dried Enano or L-Enano was expressed as WNPS. The encapsulation efficiency and loading capacity of EGCG in the nanoparticles were calculated according to the following equations, respectively:

$$\text{Encapsulation efficiency} = (C_{total} - C_{native}) / C_{total} \times 100\%$$

$$\text{Loading capacity} = (C_{total}V - C_{native}V) / \text{WNPS} \times 100\%$$

2.4. Cell culture and binding and uptake assay

Human monocytic THP-1 cells purchased from the American Type Tissue Culture Collection (ATCC, VA) were cultured in the RPMI1640 medium containing heat inactivated 10% fetal bovine serum (FBS) following ATCC instructions. THP-1 cells (2×10^5 /well) in a 24-well plate were differentiated into macrophages by incubating them with 75 ng/mL PMA for 72 hours. For measuring macrophage binding and uptake, THP-1 derived macrophages were treated with NBD-labeled void nanoparticles (Vnano or L-Vnano) at either 4°C or 37°C for 2 hours, or EGCG encapsulated nanoparticles (Enano or L-Enano) at 37°C for 2 hours. Cells were then washed at least three times with ice cold 1 X phosphate buffer saline (PBS, pH 7.4) and fixed with 3.7% formaldehyde in 1XPBS (pH 7.4) for 10 minutes at room temperature. After washing cells with ice cold 1XPBS (pH 7.4) at least three times, nuclei were stained with DAPI solution for 10 minutes at room temperature. Cells were washed again with ice cold 1XPBS (pH 7.4) and visualized under the EvoS Auto fluorescence microscope. Microscopy settings were identical for all measures to allow equal comparison of the images [33].

2.5. Nanoparticle formulation screening

Different ratios of ingredients including kolliphor HS15, PC, (+)- α -tocopherol acetate, KODiA-PC (ligand) and EGCG can give nanoparticles different binding affinity to and

uptake by macrophages, encapsulation efficiency and loading capacity. Total PC weight was considered as 1, kolliphor HS15, (+)- α -tocopherol acetate, target ligand KODiA-PC was compared to total PC. The tested weight ratios of kolliphor HS15 to PC were 0.5/1, 2/1, 4/1, 6/1, 12/1 and 18/1; the tested weight ratios of (+)- α -tocopherol acetate to PC were 0.25/1, 0.5/1, 1/1; the tested mole ratios of KODiA-PC to PC were 0/1, 0.3/1 and 0.5/1. When we changed one ratio, the ratios of other components remained the same as the optimized ones in nanoparticle formulas. The physical characteristics of nanoparticles remained at the similar level among all formulas by changing the sonication time. The nanoparticle size was in a range between 100 nm and 110 nm, zeta potential was in a range between -20 mV and -30 mV, and polydispersity index was less than 0.3. After determining the optimal ratios of ingredients, we added EGCG in a range of 3% to 14% (weight %) in nanoparticles to determine the optimal amount of EGCG for reaching a high loading capacity and a high encapsulation efficiency. The optimal formulation should also have a size less than 120 nm in diameter, a high level of macrophage binding affinity and uptake, low polydispersity. All experiments were conducted at 37°C.

2.6. Minimally oxLDL preparation, competitive binding and uptake assay of oxLDL and L-Vnano

LDL was isolated from human plasma by a sequential ultracentrifugation method [37]. Minimally oxLDL was prepared as previously described [37]. For the competitive binding and uptake experiment, THP-1 derived macrophages were treated with NBD-labeled Vnano or L-Vnano in combination with 40 μ g protein/mL of oxLDL for 2 hours at 37°C. After treatments, cells were washed with ice cold 1XPBS (pH 7.4), fixed with 3.7% formaldehyde in 1XPBS (pH 7.4), stained with DAPI and visualized under the EvoS fluorescence microscope as described in section 2.4.

2.7. Lysosome staining

In order to determine the intracellular location of Vnano and L-Vnano, lysosomes were stained with lyso-tracker (Life Technology, CA). Briefly, THP-1 cells were seeded on glass coverslips in a 6-well plate and differentiated to macrophages as described earlier in section 2.4. Macrophages were treated with NBD-labeled Vnano or L-Vnano for 2 hours at 37°C, followed by washing with cell suspension buffer (CBS, pH 7.4) three times, and incubated with lyso-tracker Red DND-99 (1 μ M) for an additional 30 minutes at 37°C. CBS contained 0.44 mM KH_2PO_4 , 110 mM NaCl, 0.35 mM Na_2HPO_4 , 1.3 mM CaCl_2 , 1 mM MgSO_4 , 5.4 mM KCl, 25 mM HEPES, 5 mM glucose and 2mM glutamine. Cells were washed and visualized under a confocal laser scanning microscope (Nikon T1-E microscope with A1 confocal system) to observe the intracellular distribution of the Vnano and L-Vnano.

2.8. EGCG stability and in vitro release study

To determine the EGCG stability at different temperatures, 45 μ g/mL of native EGCG dissolved or nanoencapsulated EGCG (Enano and L-Enano) suspended in 1XPBS (pH 7.4) were incubated at 4°C for 8 days, at 23°C for 12 hours and at 37°C for 4.5 hours, and EGCG concentrations were measured at each sample collection time point by the HPLC system. The *in vitro* release behavior was measured in 1XPBS (pH 5.0) using a dialysis method.

Native EGCG, Enano and L-Enano containing the same EGCG amount 0.5 mg were dispersed in 2 mL of 1XPBS (pH 5.0) and then placed in three different dialysis bags with MWCO 6,000–8,000. The dialysis bags were dipped with the help of a thread in a conical flask containing 25 mL of 1XPBS (pH 5.0) (dissolution medium) and stirred at 250 rpm/minute at 37°C for 20 hours. The dissolution medium was totally replaced by freshly pre-warmed dissolution medium every two hours in order to minimize the effect of EGCG degradation and imitate *in vivo* condition. EGCG released into the medium was determined every 2 hours using the HPLC system.

2.9. Cellular EGCG content

THP-1 derived macrophages were incubated with 45 µg/mL of native EGCG, Enano and L-Enano in RPMI1640 medium in combination with superoxide dismutases (SOD, 5U/mL) at either 4°C or 37°C for 4 hours. After washing cells, cellular EGCG was extracted and determined by the HPLC system as described previously [33]. Total cellular protein levels were determined using a bicinchoninic acid (BCA) kit (Pierce, IL). Cellular EGCG content was expressed as µg of EGCG per mg of protein.

2.10. Secretion of inflammatory factor MCP-1

THP-1 derived macrophages were pretreated with native EGCG, void nanoparticles (Vnano and L-Vnano) and EGCG nanoparticles (Enano and L-Enano) in 1XPBS (pH 7.4) at 37°C for 2 hours and then co-incubated with 50 ng/mL of *Escherichia coli* lipopolysaccharide (LPS) for additional 16 hours. EGCG concentrations were 5, 10, 20 and 40 µg/mL. MCP-1 protein concentrations in the culture medium were determined using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN) and cell protein levels were measured by a BCA kit. Released MCP-1 concentrations were expressed as ng per mg cell protein [37].

2.11. Real-time polymerase chain reaction

RNA was extracted from THP-1 derived macrophages, treated with the above treatments described in the section 2.10, at a concentration of 10 µg/mL using a Trizol reagent. cDNA was synthesized from RNA using SuperScript™ III reverse transcriptase according to the manufacturer's instructions. MCP-1 forward primer is TCGCTCAGCCAGATGCAAT and reverse primer is ATCTCCTTGGCCACAATGGTC. The amplification specificity and efficiency were tested in our previous study [37]. Beta-actin was used as an endogenous control. cDNA levels of MCP-1 were measured using a power SYBR green master mix on a Real-time PCR system (Eppendorf, NY). mRNA fold change was calculated using a 2(-Delta Delta C(T)) method [37].

2.12. Cellular cholesterol content measurement

THP-1 derived macrophages (1×10^6 /well) grown in 6-well plates were incubated with native EGCG, Vnano, L-Vnano, Enano and L-Enano in 1XPBS (pH 7.4) at 37°C at EGCG concentrations of 5, 10, 20 and 40 µg/mL in combination with 40 µg protein/mL of oxLDL for 18 hours. Non-esterified/free cholesterol (FC) and total cholesterol (TC) were measured using the HPLC system as described in our previous study [37]. Cholesteryl ester (CE) was

calculated as the difference between TC and FC and expressed as μmol of cholesterol per gram of protein [37].

2.13. Statistical analysis

Data analysis was conducted using Statistical Package for the Social Sciences (SPSS). One-way ANOVA followed by Tukey HSD or Dunnett's C Post Hoc test was performed to compare multiple groups. Differences were considered statistically significant at $p < 0.05$. Data in figures and tables are expressed as means $\pm$ standard deviation (SD).

3. Results

3.1. Characteristics of nanoparticles

Vnano, L-Vnano, Enano and L-Enano were successfully synthesized. They were spherical and around 108 nm in diameter (Fig. 2A, B, C, D). Their surface charges were between -20 to -30 mV. Polydispersity index of all nanoparticles was less than 0.3 (Table 1).

3.2. Nanoparticle formulation screening

The magnitude of macrophage binding and uptake of NBD-labeled Vnano was used to screen different formulae. Generally binding was at 4°C . However at 37°C , both binding and uptake were involved. As the ratios of kolliphor HS15 to PC increased, the binding and uptake of Vnano to macrophages was gradually decreased (Fig. 3A). Since decreased kolliphor HS15 amount reduces stability of nanoparticles [33], 1.25/1 of Kolliphor HS15 to PC was chosen and used in the final formulation. As the ratios of (+)- α -tocopherol acetate to PC were increased from 0.25/1 to 1/1, macrophage binding and uptake of NBD-labeled Vnano were gradually decreased (Fig. 3B). Vnano with 0.25/1 of (+)- α -tocopherol acetate to PC ratio had the highest binding and uptake to macrophages, we chose this ratio in the final formulation. In order to evaluate size effects on macrophage binding and uptake of nanoparticles, we changed the sonication time. As sonication time was increased, the size of nanoparticles decreased. The size of nanoparticles at 70 and 120 nm in diameter did not significantly affect macrophage binding and uptake of Vnano (Fig. 3C).

As the ratios of KODiA-PC to total PC increased, macrophage binding and uptake of NBD-labeled L-Vnano was increased. Nanoparticles with 0.3/1 and 0.5/1 (mole ratios) of KODiA-PC to total PC had a high level of macrophage binding and uptake, but they did not have a significant difference (Fig. 3D). Due to the additional cost concern of KODiA-PC, we chose 0.3/1 as the final mole ratio of KODiA-PC to total PC in the final formulation of L-Vnano and L-Enano. During each component screening in nanoparticle formulation, the other components content and physical characteristics of nanoparticles were kept at the similar level for all the formulas, which included particle size (100 nm – 110 nm), zeta potential (-20 mV - -30 mV), polydispersity index (less than 0.3), excluding Fig.3C that the effect of “nanoparticle size” was investigated. Based on the above results, the optimum weight ratio of PC/kolliphor HS15/(+)- α -tocopherol acetate (w/w/w) was 1/1.25/0.25, which was used to make nanoparticles for the rest of experiments. The mole ratio of 0.3/1 (KODiA-PC/PC) was used for making L-Vnano and L-Enano.

When EGCG loading capacity was increased from 3% to 12% in the final formulation, EGCG encapsulation efficiency slightly decreased from 97% to 95%. When EGCG loading capacity was increased to 14%, the encapsulation efficiency decreased sharply to 88% (Table 2). We loaded the optimal EGCG amount to reach 10% of EGCG loading capacity and 96% of encapsulation efficiency in the final Enano and L-Enano formulations.

3.3. Target binding to and uptake by THP-1 macrophages

NBD-labeled L-Vnano had higher macrophage binding affinity at both 4°C (Fig. 4A) and 37°C (Fig. 4B) and macrophage uptake at 37°C (Fig. 4B) than NBD-labeled Vnano. More Vnano and L-Vnano were bound to and taken up by macrophages at 37°C than at 4°C (Fig. 4A, B). When co-incubation of NBD-labeled void nanoparticles (Vnano and L-Vnano) with human minimally oxLDL at 37°C, macrophage binding and uptake of L-Vnano was decreased by 3.8 folds. However, oxLDL only slightly decreased Vnano binding to and uptake by macrophages (Fig. 4B, C). After loading EGCG to the nanoparticles, NBD-labeled L-Enano also had higher macrophage binding and uptake than NBD-labeled Enano at 37°C (Fig. 4D).

3.4. Intracellular distribution

The intracellular distribution of Vnano and L-Vnano was imaged using a confocal microscopy (Nikon T1-E microscope with A1 confocal system). Most of the Vnano (green) were localized in lysosomal compartments (red), producing yellow fluorescence spots indicating that most of Vnano were entrapped within the lysosomes. In contrast, L-Vnano (green) was observed as more diffused spots in the cytosol, which was clearly distinguishable from lyso-tracker red fluorescence, implying successful and fast escape of L-Vnano from lysosomes and efficient delivery to the cytosol (Fig. 5).

3.5. Macrophage EGCG content

CD36-targeted L-Enano, not Enano, significantly increased macrophage EGCG content at 4°C because of its high binding affinity to macrophages. As compared to native EGCG, both Enano and L-Enano significantly increased macrophage EGCG content at 37°C by more than 7 and 6 folds, respectively (Fig. 6).

3.6. EGCG stability study

Nanoencapsulation significantly increased EGCG stability. In neutral pH 7.4, 45 µg/mL of native EGCG was degraded more than 90% after 2 days at 4°C, but more than 80% of EGCG was remained in Enano and L-Enano at the same initial concentration and conditions (Fig. 7A). The same enhanced EGCG stability trend by Enano and L-Enano was observed at 23°C and 37°C (Fig. 7B, C). However, there was no significant difference between Enano and L-Enano (Fig. 7).

3.7. In vitro release behavior of Enano, L-Enano and native EGCG

Enano and L-Enano had a sustained EGCG release property whereas native EGCG had a sudden/burst release pattern (Fig. 8). Native EGCG exhibited a complete release (100%) pattern in the initial 2-hour period and was not detectable in the dialysis bag after 2 hours.

Enano and L-Enano had a sustained release manner and the average release amount of EGCG was around 200 µg, 100 µg, 50 µg, 40 µg and 30 µg at the 1st, 2nd, 3rd, 4th and 5th 2-hour period, respectively.

3.8. Cytotoxicity

We also conducted the cell viability experiment using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The viability of THP-1 derived macrophages was more than 90% when treating them with either KODiA-PC, EGCG, Vnano, Enano, L-Vnano or L-Enano at concentrations of 5, 10, 20, 45 µg/mL (Supplementary Fig. 1).

3.9. Anti-atherogenic bioactivities

At 10 µg/mL of native or equivalent amount of nanoencapsulated EGCG concentrations or void nanoparticles, L-Enano significantly decreased macrophage MCP-1 mRNA levels compared to 1XPBS, native EGCG, Vnano and L-Vnano (Fig. 9A). As EGCG concentrations were increased from 5 to 40 µg/mL, MCP-1 protein secretion from macrophages was gradually decreased in Enano and L-Enano treatment groups (Fig. 9B). Compared with 1XPBS treatment, all nanoparticles (Vnano, Enano, L-Vnano, L-Enano) at EGCG concentrations of 10, 20 and 40 µg/mL significantly decreased MCP-1 secretion from THP-1 derived macrophages. Compared with native EGCG, only L-Enano significantly decreased MCP-1 secretion at 10 and 20 µg/mL of EGCG concentrations (Fig. 9B). For macrophage cholesterol accumulation, macrophage-targeted L-Vnano and L-Enano decreased macrophage CE levels compared with 1XPBS and EGCG group at 10 and 20 µg/mL of EGCG concentrations, but did not reach statistical significant difference (Fig. 10). At the highest concentration, L-Vnano decreased macrophage CE levels significantly compared with 1XPBS and L-Enano treatments (Fig. 10).

4. Discussion

Intimal macrophages play an important role in atherosclerotic lesion formation and progression by facilitating cholesterol accumulation and increasing inflammatory response in blood vessel walls [38]. Rupture of vulnerable lesions (plaques) followed by thrombi formation accounts for most coronary events and/or sudden deaths [40–42]. Vulnerable lesions are characterized by macrophage-dense inflammation, large lipid cores, thin fibrous caps and few smooth muscle cells [43]. Increased intimal macrophage accumulation enhances the development of lesions, especially vulnerable lesions [19, 44, 45]. One of the underlying mechanisms is that they secrete pro-inflammatory cytokines to amplify the local inflammatory reactions, activate endothelial cells and smooth muscle cells, activate and recruit more monocytes/macrophages and other immune cells into the lesions [47]. Targeted delivery of EGCG to intimal macrophages might decrease production of inflammatory factors and/or cholesterol accumulation, which is a promising avenue for atherosclerosis prevention and treatment. In this study, KODiA-PC as a macrophage-targeted ligand was incorporated on the surface of nanoparticles composing of phosphatidylcholine, kolliphor HS15, (+)-α-tocopherol acetate and EGCG. We optimized nanoparticle formulation and measured their binding affinity to and uptake by THP-1 derived macrophages. We also

encapsulated EGCG into the nanoparticles to measure and compare their characteristics and anti-atherogenic effects in macrophages.

In order to reach a high level of macrophage binding and uptake of nanoparticles, we optimized all components and physical characteristics in nanoparticles. The increased kolliphor HS15 content and nanoparticle size in the tested range had little effects on nanoparticle binding to and uptake by macrophages. However, the increased (+)- α -tocopherol acetate content plays a significant role in decreasing nanoparticle binding to and uptake by macrophage. Kolliphor HS15 as major surfactant was more likely to be on the surface of nanoparticles to facilitate nanoparticles formation and stabilization. The impact of (+) α -tocopherol acetate on nanoparticle binding and uptake by macrophage needs further investigation. In general, higher absolute surface charge leads to stronger repulsion interactions among nanoparticles in dispersion, and hence higher stability. The surface charges of all nanoparticles in this study were in the range of -20 to -30 mV, which can repel nanoparticles from aggregation for improving the physical stability of nanoparticles.

Macrophage-targeted nanoparticles have gained many attention in atherosclerosis research area. Iron oxide nanoparticles have been widely used to detect intimal macrophages, because of the phagocytotic function of macrophages on most foreigners including iron oxide particles in the body [48, 49]. However, safety concerns have arisen about accumulation of iron oxide nanoparticles in the body, which may lead to toxicity. Incorporation of antibodies on the surface of nanoparticles to target to macrophage scavenger receptors has been used and investigated, but they may increase immune reactions and responses in the body [50]. Synthetic high-density lipoproteins have also been used to target to intimal macrophages, but their target specificity is not high enough [51, 52]. Oxidized PC are isolated from oxLDL found in human and animal atherosclerotic lesions [32]. They are the major macrophage-targeted compounds on the surface of oxLDL via binding to scavenger receptors, primarily CD36 [25, 32]. Therefore, oxidized PC are the natural ligand for targeting to intimal macrophages. Studies showed that one type of the most potent oxidized PC targeting to the macrophage CD36 receptor was keto acid analogs, especially KODiA-PC [25]. In this study, L-Vnano carrying KODiA-PC as a target ligand had significantly higher binding affinity to and uptake by human THP-1 derived macrophages than Vnano. Our data in another study demonstrated that liposomes carrying KODiA-PC on their surface also had increased binding affinity to and uptake by both mouse and human macrophages via their CD36 receptors. When we co-incubated L-Vnano and oxLDL in this study, oxLDL significantly decreased macrophage binding and uptake of L-Vnano, which indicated that L-Vnano and oxLDL competitively bound to macrophages via the same scavenger receptor. On the surface of oxLDL, hydrophilic head/carboxylate group at the sn-2 position of KODiA-PC incorporated a terminal γ -hydroxy (or oxo)- α,β -unsaturated carbonyl, which was protruded from the phospholipid membrane into the aqueous phase for its high binding affinity to the macrophage CD36 receptor [25, 53, 54]. In current work, the KODiA-PC on the surface of L-Enano exhibited the similar structure. The hydrophilic head at the sn-2 position of KODiA-PC was protruded into the aqueous exterior for targeting to the CD36 receptor of intimal macrophages.

Targeted L-Enano compared to untargeted Enano significantly increased macrophage EGCG content at 4°C, because more L-Enano than Enano were bound on macrophages at 4°C. Enano and L-Enano not only bind on macrophages, but also are taken up by macrophages at 37°C. Endocytosis is the major mechanism for cellular uptake of nanoparticles and other non-viral vectors. The process leads to the formation of intracellular vesicles, which can be fused into endosomes, and further into acidic lysosomes [55]. Similar to oxLDL, Enano and L-Enano might be taken up by macrophages via the receptor-mediated endocytosis. L-Enano compared to Enano did not increase macrophage EGCG content at 37°C. The reason may be that more L-Enano escape acidic lysosomes. Our previous study showed EGCG was very stable at 37°C when pH = 5.0 and not stable when pH = 7.0 [33]. Accordingly, we hypothesize that some L-Enano may escape lysosomes (pH 4.5~5.0) and go to the cytosol (pH > 7.0) where EGCG is easily degraded and metabolized. To prove this hypothesis, we used lysoTracker® Red DND-99, a red-fluorescent dye, to label lysosomes. Most of NBD-labeled (green) L-Vnano did not co-localize with the lysosomes, which suggests that L-Vnano escaped the lysosomes. However, most of NBD-labeled Vnano co-localized with the lysosomes. Even though macrophages take up more L-Enano than Enano, some L-Enano escaped lysosomes and were degraded and metabolized quickly, which explain similar EGCG content in macrophages treated with L-Enano and Enano at 37°C.

Cellular nanoparticle fluorescence intensity at 4°C was much weaker than it at 37°C, which confirms that uptake of nanoparticles was an energy-dependent and active transport process. Cellular EGCG content was higher at 4°C than it at 37°C. High EGCG stability at 4°C may be one major reason for high cellular EGCG content at 4°C. HT-29 human colon adenocarcinoma cells took up native EGCG at a concentration range from 5 to 640 µM through a passive diffusion process [9, 56]. The native EGCG was subsequently converted into methylated metabolites and glucuronides, which together with native EGCG were pumped out of the cells through multi-drug-resistance proteins or P-glycoproteins [9, 56]. This efflux rate was higher at 37°C than it at 4°C. This energy-dependent efflux process may partially explain lower cellular EGCG content at 37°C than at 4°C. In this study, nanoencapsulation also increased EGCG stability at 4°C, 23°C and 37°C. Many studies have demonstrated that nanoencapsulated compounds could be protected from premature degradation and oxidation, epimerization and polymerization contributed by many environmental factors such as temperature, pH of the system, oxygen levels and the presence of metal ions [1, 57]. Taken together, our nanoparticles increased EGCG stability, enhanced payload of EGCG, exhibited sustained release of EGCG, increased their binding affinity to and uptake by macrophages.

Inflammatory factors such as MCP-1 play a central role in atherosclerotic lesion initiation and development [18]. The binding of MCP-1 to its receptor CCR2 on monocytes promotes the recruitment and migration of monocytes into the artery intima layer [18]. When using mutant mice with MCP-1 or its receptor CCR2 deficiency, there was a striking decrease in arterial lipid deposition [58]. Studies also suggested that blood MCP-1 can be considered as a biomarker of atherosclerosis [59, 60]. Moreover, studies have demonstrated that EGCG decreased mRNA and protein levels of MCP-1 in other types of cells including tumor necrosis factor alpha (TNF-α)-stimulated human umbilical vein endothelial cells, LPS-stimulated L02 hepatocyte, PMA-stimulated endothelial cells and polychlorinated biphenyls

(PCB) 126-induced endothelial cells [4, 61–63]. Decreased MCP-1 expression by EGCG may be through down-regulated nuclear factor-kappaB (NF- κ B) and p38 mitogen-activated protein kinases (MAPK), and up-regulated peroxisome proliferator activated receptor gamma (PPAR γ) [4, 64]. Compared with native EGCG, only L-Enano at doses of 10 and 20 μ g/mL markedly inhibited MCP-1 protein release from THP-1 derived macrophages. Compared with 1XPBS, all nanoparticles (Vnano, Enano, L-Vnano, L-Enano) at doses of 10, 20 and 40 μ g/mL significantly decreased MCP-1 secretion, which might be due to bioactive (+)- α -tocopherol acetate presenting in all nanoparticles. Both EGCG and (+)- α -tocopherol acetate have therapeutic bioactivities [65]. A clinical study showed that vitamin E treatment, as α -tocopherol acetate, significantly decreased MCP-1 in patients with type 1 diabetes [66]. When male apolipoprotein E null mice fed with an atherogenic diet and given a green tea extract drink (0.8 g/L) or a vehicle drink, the green tea extract drink resulted in a 23 and 27% reduction in the atheromatous area and aortic cholesterol content, respectively [67]. A recent study also showed orally administered nanoencapsulated EGCG significantly decreased lipid deposition in aortic wall of rabbits [68]. However, Enano and L-Enano did not significantly decrease macrophage cholesterol levels compared with 1XPBS and EGCG in the current study. Additional experiments are required to investigate the effects of L-Enano on expression of other inflammatory factors in THP-1 derived macrophages, and measure anti-inflammatory responses in other types of macrophages. Atherosclerosis animal model such as pigs, apolipoprotein E null mice or LDL receptor null mice will be needed to confirm target specificity of L-Enano to intimal macrophages, and to measure their anti-atherogenic bioactivities and underlying mechanisms.

In conclusion, we have successfully synthesized L-Enano, which are composed of PC, kolliphor HS15, (+)- α -tocopherol acetate, EGCG and KODiA-PC. L-Enano increased EGCG stability, exhibited sustained release of EGCG, had a high binding affinity to and uptake by macrophages, increased macrophage EGCG content, decreased macrophage MCP-1 mRNA levels and secretion. The CD36-targeted nanoparticles might also be used as a carrier for targeted delivery of diagnostic agents and other anti-atherogenic compounds to intimal macrophages for the diagnosis, prevention and treatment of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Illustration of targeted L-Enano composition, structure and targeting mechanisms to macrophages.

Fig. 2. Transmission electron microscope (TEM) images of Enano (A) and L-Enano (B) stained by 2% of uranyl acetate. The size and distribution of Enano (C) and L-Enano (D) were measured using a Brookhaven BI-90 particle size analyzer.

Fig. 3.

Formulation screening based on the binding affinity to and uptake of NBD-labeled void nanoparticles (Vnano) by THP-1 derived macrophages *in vitro*. The following parameters were changed: weight ratios of kolliphor HS15 to PC from 0.5/1 to 18/1 (A); weight ratios of (+)- α -tocopherol acetate to PC from 0.25/1 to 1/1 (B); the size of nanoparticles (C); mole ratios of KODiA-PC to PC from 0/1 to 0.5/1 (D). NBD-labeled void nanoparticles are shown in green (λ of excitation is 460 nm, λ of emission is 535 nm). Cell nuclei were stained by DAPI and shown in blue (λ of excitation is 358 nm, λ of emission is 461 nm).

Fig. 4.

Macrophage binding and uptake of NBD-labeled nanoparticles *in vitro*. THP-1 derived macrophages were treated with NBD-labeled Vnano and L-Vnano at either 4°C (A) or 37°C (B); the macrophages were treated with NBD-labeled Vnano or L-Vnano in combination with oxLDL at 37°C (C); Macrophages were treated with NBD-labeled Enano and L-Enano at 37°C (D). Images are representatives of three independent experiments.

Fig. 5. Representative Intracellular distribution images of NBD-labeled Vnano or L-Vnano (green) taken by a confocal laser scanning microscope. Lysosomes were labeled by the lyso-tracker red fluorescence dye; Yellow dots in merged picture indicate that the nanoparticles are trapped within acidic lysosomes.

Fig. 6. Macrophage EGCG contents. THP-1 derived macrophages were treated with 45 µg/mL of native EGCG, Enano and L-Enano at either 4°C (A) or 37°C (B). Bars in A or B without a common superscript differ, $p < 0.05$. More than three independent experiments were conducted.

Fig. 7. EGCG stability. Native EGCG, Enano and L-Enano containing 45 $\mu\text{g/mL}$ of EGCG in 1XPBS (pH 7.4) at either 4°C (A), 23°C (B) or 37°C (C). Each time point without a common superscript differs, $p < 0.05$. Three independent experiments were conducted.

Fig. 8.

In vitro EGCG release profiles of native EGCG, Enano and L-Enano. Three independent experiments were conducted.

Fig. 9.

MCP-1 mRNA levels in THP-1 derived macrophages treated with 1XPBS, native EGCG, Vnano, Enano, L-Vnano or L-Enano at EGCG dose of 10 µg/mL (A). MCP-1 protein secretion from THP-1 derived macrophages. Macrophages were pretreated with 1XPBS, native EGCG, void nanoparticles (Vnano and L-Vnano) and EGCG nanoparticles (Enano and L-Enano) containing EGCG at doses of 5, 10, 20 and 40 µg/mL at 37°C for 2 hours and then co-incubated with LPS for additional 16 hours (B). Bars without a common superscript differ, $p < 0.05$. Three independent experiments were conducted.

Fig. 10.

Cholesterol content in THP-1 derived macrophage. Macrophages were treated with 1XPBS, native EGCG, void nanoparticles (Vnano and L-Vnano) and EGCG nanoparticles (Enano and L-Enano) containing EGCG at doses of 5, 10, 20, 40 µg/mL at 37°C for 18 hours. Three independent experiments were conducted. TC: total cholesterol; FC: free cholesterol; CE: cholesteryl ester. Bars at the same EGCG concentration without a common superscript differ, $p < 0.05$. Three independent experiments were conducted.

Table 1

Particle size, zeta potential, and polydispersity index (PI) of Nanoparticles.

Name	Vnano	Enano	L-Vnano	L-Enano
Particle size (nm)	110.8 ± 1.5	109.6 ± 1.9	107.7±1.7	104.8±0.4
Zeta potential (mV)	-27.02 ± 0.82	-26.18 ± 8.30	-21.81 ± 1.18	-20.30 ± 1.57
Polydispersity	0.23 ± 0.004	0.19 ± 0.010	0.21 ± 0.007	0.18 ± 0.020

Table 2

Formulation screening based on EGCG's loading capacity and encapsulation efficiency in Enano.

% Loading capacity	% Encapsulation efficiency
3 ± 0.12	97.42 ± 0.53
6 ± 0.27	98.26 ± 0.95
9 ± 0.71	95.76 ± 3.18
12 ± 0.16	95.07 ± 2.05
14 ± 1.05	87.66 ± 3.29 *

Values are means ± SD, n = 3.

* denotes the encapsulation efficiency significantly differs at $p < 0.05$.