Synthesis of gold and silver nanoparticles using flavonoid quercetin and their effects on lipopolysaccharide induced inflammatory response in microglial cells

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Abstract

Quercetin is a plant origin phytochemical with several pharmaceutical activities such as antioxidant, immunomodulatory, and anti-inflammatory effects. However, consumption of quercetin is limited due to its low aqueous solubility and poor bioavailability. The aim of the present study was to synthesize silver and gold nanoparticles of quercetin with a view to improve its aqueous phase solubility and investigate the effects on LPS-induced neuroinflammation in BV-2 microglial cells. The average size of silver and gold-quercetin nanoparticles was 53 and 27 nm, respectively. Absorption peaks in the UV–Vis spectra were observed at 555 and 405 nm for gold and silver-quercetin nanoparticles, respectively. The particle size and mapping of silver and gold-quercetin nanoparticles were also determined using a STEM detector. The inflammatory stimulation of the BV-2 cells with LPS caused an elevated release of proinflammatory prostaglandin, E2, nitric oxide (NO), upregulated cyclooxygenase-2, inducible NO synthase mRNA, and protein levels, which were markedly inhibited by the pretreatment with gold-quercetin nanoparticles (highly soluble in water) without causing any cytotoxic effects. The findings of the present study suggest that the potential of gold-quercetin nanoparticles are much better than quercetin and that gold-quercetin nanoparticles might provide protection against inflammatory neurodegenerative disease via suppression of acute microglial activation.

Keywords Quercetin · Microglia · Inflammation · Nanoparticle

Abbreviations

COX-2	Cyclooxygenase-2
iNOS	Inducible nitric oxide synthase
PGE2	Prostaglandin E2
NO	Nitric oxide
LPS	Lipopolysaccharide
EDX	Energy-dispersive X-ray

SEM	Scanning electron microscopy
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle medium
FBS	Fetal bovine serum
PAGE	Polyacrylamide gel electrophoresis
BCA	Bicinchoninic acid assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
MAPKs	Mitogen-activated protein kinases

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Introduction

Generally, microglial cells have been used as models to test the effects of herbal extracts and plant-derived compounds on neuro-inflammation induced by lipopolysaccharide, which activates toll-like receptors and upregulates proinflammatory genes and their active metabolites (Yu et al. 2015; Simonyi et al. 2015; Jeong et al. 2014). Activation of microglial cells in the inflammatory processes has increased phagocytosis and antigen presenting properties and this activation is involved in the immune response by secretion of cytokines, chemokines, reactive oxygen/nitrogen radicals, and arachidonic acid

metabolites (Kettenmann et al. 2011; Agostinho et al. 2010). The existing microglial activation damage of neurons through these proinflammatory products at minimum causes an increase in neuron death (Heneka et al. 2014; Marín-Teva et al. 2010). Therapeutic approaches with the use of bioflavonoids to alleviate LPS-induced proinflammatory gene activation underscore the important role of these compounds as potential therapeutic targets to ameliorate neuro-inflammatory neurodegenerative diseases (Choi et al. 2011; Baby et al. 2014).

Quercetin and sugar-containing derivatives, the most abundant flavonoids, are found widely in plants. Recent studies have demonstrated that bioflavonoid quercetin can suppress oxidative stress (Terao 2009; Olayinka et al. 2015; Gardi et al. 2015; Fraga et al. 2019; Abdel-Diam et al. 2019) and inflammatory responses (Gerin et al. 2016; Seo et al. 2015) in immune cells. However, the application of quercetin is limited due to its poor solubility, low bioavailability, and instability in a physiological medium, which restricts the use of this flavonoid for oral administration (Nathiya et al. 2014). Over the last decade, there have been an increasing number of newly developed drug molecules that exhibit poor water solubility, as well as limited availability. One of the biggest problems in drug development is to improve the solubility and oral bioavailability of these novel drugs (Savjani et al. 2012). Conjugation of poorly water-soluble quercetin with nanoparticles might be a good approach to overcome these limitations. The advantages of using nanoparticle carriers include an increase in the solubility and dissolution rate due to their high surface area and good adhesion to biological surfaces (Bhattacharjee et al. 2016). Gold nanoparticles are biologically stable materials and cause no severe side effects in biological systems, with demonstrated applications in drug delivery (Hornos Carneiro and Barbosa 2016). Silver nanoparticles are also widely considered for conjugation with biomolecules. There are several versatile methods to synthesize highly mono-dispersed nanoparticles (Jeevanandam et al. 2010).

This study was conducted to conjugate quercetin with gold and silver nanoparticles, which were synthesized in the presence of quercetin, by citrate reduction of chloroauric acid and silver nitrate. The potential effects of quercetin and its conjugates with nanoparticles on cytotoxicity, proinflammatory mRNA, and protein expression [cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS)] with prostaglandin E2 (PGE2) and NO production induced by lipopolysaccharide in microglial BV-2 cells were also investigated.

Materials and methods

Synthesis of gold and silver nanoparticles conjugated with quercetin

Gold nanoparticles were synthesized by reducing chloroauric acid (Sigma, St. Louis, MO, USA) with

trisodiumcitrate according to the Turkevich method (Turkevich et al. 1951). To synthesize gold nanoparticles, 0.5 mL of 1% trisodium citrate was added to 20 mL of 0.01% chloroauric acid under reflux. Quercetin (1 mg/100 μ L DMSO) was then added to the reaction mixture as soon as the mixture began to reflux. Silver nanoparticles were synthesized by reducing silver nitrate (Sigma, St. Louis, MO, USA) with trisodium citrate according to the Turkevich method (Turkevich et al. 1951). To synthesize silver nanoparticles, 5 mL of 1% trisodium citrate was added to 20 mL of 10^{-2} M silver nitrate under reflux. Quercetin (1 mg/100 μ L DMSO) was then added dropwise to the mixture to synthesize quercetin-silver nanoparticle conjugates. Gold and silver nanoparticles were then centrifuged at 10,000 rpm for 10 min, and after that, the obtained materials were washed three times with deionized water to remove unreacted quercetin and other undesired molecules.

Characterization of gold and silver-quercetin nanoparticles

The UV–Vis absorption spectra of free quercetin and its conjugates with nanoparticles were recorded on a Shimadzu UV-2450 spectrophotometer. The external morphology and the diameters of the particles were studied with a scanning electron microscopy (SEM) using a scanning transmission electron microscopy detector by EVO LS 10. The silver samples were coated with gold before taking SEM images. Dynamic light scattering (DLS) measurements were performed to obtain the size of particles using a Zetasizer ZEN3690 Malver. Energy-dispersive X-ray (EDX mapping) analysis was conducted using an EVO LS 10 high-resolution SEM to confirm the presence and distribution of gold and silver in the particles.

Cell culture

BV-2 mouse microglial cells were cultured in 75 cm² flasks containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 10 μ g/mL streptomycin, and grown in a 5% CO₂ atmosphere at 37 °C.

MTT assay

The cytotoxic effects of free quercetin, quercetin-gold, and quercetin-silver nanoparticles were evaluated by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reduction assay (Janjic and Wollheim 1992). BV-2 cells were treated with 0–40 μ g/mL free quercetin, 0–400 μ g/

mL gold-quercetin, and silver-quercetin nanoparticle conjugates in phenol-free red 1% FBS supplemented DMEM for 24 h and then rinsed three times with ice-cold PBS. MTT (Sigma, St. Louis, MO, USA) was added to make the final concentration of 0.5 mg/mL. After 4 h incubation, a solubilization buffer (10% sodium dodecyl sulfate in 0.01 mol/L HCl) was added and the colored formazan crystals were gently resuspended. The absorbance at 570 nm was recorded with a microplate reader (Bio-Tek ELX800, BioTek Instruments Inc., Winooski, VT, USA).

Quantitative real-time PCR

Total RNA isolation from cells was performed via phenol-guanidine thiocyanate extraction using RNeasy isolation reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. The total RNA (1 µg) was reverse-transcribed to cDNA using a Transcriptor High Fidelity cDNA synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) in a 20-µL reaction mixture. To quantify cDNA, qPCR was performed using FastStart Essential DNA probe master mix (Roche Diagnostics GmbH, Mannheim, Germany) and catalogue assay kits (kits consist a mixture of primers and probes for the determination of iNOS, COX-2, β -actin). Quantitative real-time PCR was conducted using a Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). For each sample, the level of the target gene transcripts was normalized to β -actin.

Western blot

After the treatment procedure, cells were washed in ice-cold PBS three times and lysed in RIPA buffer supplemented with 2 mM Na_3VO_4 and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany) at 4 °C. The lysate was clarified by centrifugation at 10,000 rpm for 10 min at 4 °C to remove insoluble components. Cell lysates were normalized for protein content using BCA reagent (Pierce, Rockford, IL, USA). Equal amounts (30 µg) of protein were loaded onto 10% PAGE gels and separated by a standard SDS-PAGE procedure. Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) and blocked with 5% non-fat dry milk in TBST. To detect protein expression, the blots were probed with the specific antibodies against COX-2, iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by the secondary antibodies coupled to horseradish peroxidase. The detection of GAPDH (Cell Signaling Technology INC. Beverly, MA, USA) with a specific antibody was used as an internal control. The immunoreactive proteins on the membrane were detected by chemiluminescence using SuperSignal® West Pico (Pierce, Rockford, IL, USA) on X-ray film.

Enzyme-linked immunosorbent assay (ELISA)

Mediums were collected after pretreatment with free quercetin and its nanoparticle conjugates for 24 h followed by LPS treatment (in phenol-free red, serum-free medium) for 6 h and assayed for cytokine PGE2 using SUNRED Biological Technology ELISA kits (Shanghai, China) following the manufacturer's protocol.

Total nitric oxide (NO_x) assay

The NO_x levels were determined by a vanadium chloride (VCl_3)/Griess assay (Miranda et al. 2001). Vanadium chloride (0.8% w/v in 1 M HCl) was used to reduce released nitrate to nitrite in the medium. Finally, Griess reagents (1:1, 2% w/v Sulphanilamide in 5% HCl: 0.1% w/v N (1 naphthyl) ethylenediamine dihydrochloride in H_2O) were added to convert nitrite into a deep purple azo compound. The absorbance of azochromophore at 540 nm was measured using a plate reader. Total NO_x levels in the medium were calculated from a standard curve of 10–1000 µmol/L of sodium nitrate.

Statistical analyses

Possible associations between the groups were analyzed with SigmaPlot 12 statistical software using the *t* test. *p* values < 0.05 were considered as statistically significant. An increase or decrease of mRNA levels was also calculated by REST (relative expression software tool) software developed for group-wise comparison and statistical analysis of relative expression results.

Results and discussion

Preparation and characterization of nanoparticles

One important observation in this study is that, dried nanoparticles resuspended in water formed a very homogeneous dispersion, unlike quercetin, which precipitates in water. Undissolved pieces of quercetin were clearly visible in the suspension. The formation of gold and silver nanoparticles following the addition of free quercetin to the reaction medium right after addition of chloroauric acid or silver nitrate solutions was observed visually by a change in the color of the solutions. The color of the gold nanoparticle solution changed from red wine to a pale violet color after drop-wise quercetin addition (Fig. 1). The color change in the silver nanoparticle solution appeared a pale orange color to a pale brown color after quercetin addition (Fig. 2). This is due to the excitation of the surface plasma vibrations, indicating the formation of nano-quercetins. The nanoparticles were primarily characterized

by UV–visible spectroscopy. Absorption peaks were observed at 555 and 405 nm for gold (Fig. 1) and silver (Fig. 2) nanoparticles (Fig. 1), respectively. Single peaks formed by the gold-quercetin and silver-quercetin nanoparticle conjugates observed in the UV–visible spectroscopic analyses confirmed the uniform size and shape of the nanoparticles. The average size of the particles and size distributions of the synthesized nano-quercetins were determined by the particle size analyzer and the results are shown in Fig. 1. The results show that the average particle diameter for gold-quercetin (Fig. 1) and silver-quercetin (Fig. 2) nanoparticles are 27 and 53 nm, respectively. An EDX technique can be used to determine the composition of a material. In this study, the X-ray mapping capability of the EDX technique was used to observe the elemental

distribution in the nanocomposite films. In the X-ray mapping technique, the positions of specific elements emitting characteristic X-rays within an inspection field can be indicated by a unique color. A STEM detector was used to measure the particle size and mapping of silver in the material. The standard EDX spectrum recorded on the examined sample is shown in Fig. 3a. In the right part of the spectrum, a peak located at 3 keV can be seen. This maximum is related to the silver characteristic line L. The maximum peak at 0.2 keV located on the left part of the spectrum comes from carbon and the maximum located at 0.5 keV relates to the oxygen characteristic line. The carbon and oxygen spots in the samples confirm the presence of stabilizers composed of quercetin chains and their oxygen. It can be understood that silver is homogeneously

Fig. 1 Characteristic features of gold-quercetin nano particles. Color changes (yellow color solution: free quercetin in DMSO, red wine color solution: gold nanoparticle, pale violet color solution: gold-quercetin nanoparticle conjugates), size distributions and UV–Vis spectra of the synthesized gold-quercetin nanoparticle conjugates

Fig. 2 Characteristic features of silver-quercetin nano particles. Color changes (yellow color solution: free quercetin in DMSO, pale orange color solution: silver nanoparticle, pale brown color solution: silver-quercetin nanoparticle conjugates), size distributions and UV–Vis spectra of the synthesized silver-quercetin nanoparticle conjugates

Fig. 3 Characterization of silver-quercetin nanoparticle conjugates. **a** EDX spectrum, **b** EDX mapping, **c** EDX size distribution of silver-quercetin nanoparticle conjugates

Fig. 4 Characterization of gold-quercetin nanoparticle conjugates. **a** EDX spectrum, **b** EDX mapping, **c** EDX size distribution of gold-quercetin nanoparticle conjugates

distributed in the material investigated in the mapping (Fig. 3b). The size of particles was also measured using STEM. According to the STEM results in Fig. 3c, the silver particles have an average size of 10 nm. EDX mapping results (Fig. 4b) were obtained using a STEM detector and observed that the gold nanoparticles did not show homogeneous distribution in Fig. 4c. The EDX spectrum in Fig. 4a shows the existence of a characteristic gold peak at 2 keV. Particle size was measured with two different methods for both silver and gold nanoparticles. Results show that the average particle sizes of nanoparticles range from 10 nm to 40 nm for both silver and gold according to the particle size analyzer and STEM studies.

Anti-neuroinflammatory activities of quercetin and quercetin-nanoparticles

First, the effects of quercetin and quercetin-nanoparticles on BV-2 cell viability were investigated (Fig. 5). Quercetin dose triggered microglial cell death. After 24 h incubation, quercetin significantly reduced the viability of cultured

Fig. 5 Cytotoxicity of quercetin and nano-materials. Effects of quercetin, gold-quercetin, and silver-quercetin nanoparticle conjugates on BV-2 cell viability. $n=4$, $*p < 0.05$ vs. control cells

microglial cells at the concentration (conc.) > 2.5 μg/mL. While 2.5 μg/mL or lower concentrations of quercetin decreased cultured cell viability, the differences did not reach any statistical significance. 24-h treatment with gold-quercetin and silver-quercetin significantly reduced the

viability of cultured microglial cells at the conc. $> 200 \mu\text{g/mL}$. The non-toxic concentration of 1 and $2.5 \mu\text{g/mL}$ quercetin, 100 and $200 \mu\text{g/mL}$ gold and silver-quercetin nanoparticles were used for further experiments to test their protective effects against LPS-induced neuro-inflammation.

Microglial cells play a significant role in neuro-inflammation and host defense mechanisms that have been extensively studied in LPS-stimulated BV-2 macrophage cells, because the cells are very susceptible to LPS stimulation by activation of multiple inflammatory genes (Svensson et al. 2010; Petrova et al. 1999). The effects of LPS doses on activation of proinflammatory mediators COX-2 and iNOS at the levels of mRNA (Fig. 6a) and protein (Fig. 6b) after 6-h incubation time were investigated. Following LPS stimulation, up-regulation of the COX-2 and iNOS mRNA levels accompanied by an increase in their protein levels were observed in a LPS dose of 1.50 and $2.00 \mu\text{g/mL}$. Consequently, a $2.00 \mu\text{g/mL}$ LPS dose during 6 h incubation was used for further assays of stimulation of neuroinflammatory response in microglial cells.

The in vitro and in vivo anti-inflammatory potential of quercetin can be elucidated on different cell lines and in animal models. Quercetin was able to inhibit LPS-induced tumor necrosis factor α and nitric oxide (NO) production in Raw 264.7 macrophages (Manjeet and Ghosh 1999). Furthermore, in N9 microglial cells, pretreatment with quercetin caused a reduction of LPS-induced proinflammatory cytokines TNF- α and interleukin (IL)- 1α mRNA levels. This effect of quercetin resulted in a decreased apoptotic neuronal cell death induced by microglial activation (Bureau et al. 2008). Treatment with quercetin regulated inflammatory response in BV-2 microglial cells upregulate hemeoxygenase-1 levels against endotoxemic stress through the involvement of MAPKs (Sun et al. 2015). Quercetin, but not quercetin-3-glucuronide, ameliorated systemic inflammation by enhancing anti-inflammatory IL-10 levels in a LPS-injected mice sepsis model (Liao and Lin 2015). Quercetin

Fig. 7 Anti-inflammatory effects of nano-materials. Effects of quercetin, gold-quercetin, and silver-quercetin (indicated concentrations) on iNOS and COX-2 mRNA levels in LPS ($2 \mu\text{g/mL}$) activated BV-2 cells. $n = 3$, $*p < 0.05$ vs. LPS treated cells

inhibits the activation of the proinflammatory enzymes cyclooxygenase, lipoxygenase, and iNOS accompanied by production of their inflammation enhancing products including prostaglandin E2 and NO (Kim et al. 1998). In this study, we provided evidence that the transcriptional over activation of COX-2 and iNOS with protein levels by LPS-stimulated microglial cells can be inhibited markedly by low-dose quercetin ($1 \mu\text{g/mL}$) pretreatment. An aqueous solution of gold-quercetin nanoparticles demonstrated markedly better anti-inflammatory effects by decreasing inflammation-producing enzymes (COX-2 and iNOS) expression at both transcriptional (Fig. 7) and translational (Fig. 8) levels compared to a DMSO solution of free quercetin. As expected, LPS-stimulated NO (Fig. 9) and PGE2 (Fig. 10) biosynthesis in microglial cells. These findings emphasize that aqueous solutions of both gold and silver nanoparticles demonstrated better results by inhibiting NO and PGE2 production compared to DMSO solutions of free quercetin.

Fig. 6 Proinflammatory markers activated by LPS. **a** Effects of lipopolysaccharide on iNOS and COX-2 mRNA levels, **b** effects of lipopolysaccharide on iNOS and COX-2 protein levels $n = 3$, $*p < 0.05$ vs. control cells

Fig. 8 Anti-inflammatory effects of nano-materials. Effects of quercetin, gold-quercetin, and silver-quercetin (indicated concentrations) on iNOS and COX-2 protein levels in LPS activated BV-2 cells. $n=3$, $*p<0.05$ vs. LPS-treated cells

Fig. 9 Anti-inflammatory effects of nano-materials. Effects of quercetin (Q), gold-quercetin (Au-Q), and silver-quercetin (Ag-Q) (indicated concentrations) on nitric oxide (NO_x) production in LPS-activated BV-2 cells. $n=4$, $*p<0.05$ vs. Control cells, $#p<0.05$ vs. LPS-treated cells

Fig. 10 Anti-inflammatory effects of nano-materials. Effects of quercetin (Q), gold-quercetin (Au-Q), and silver-quercetin (Ag-Q) (indicated concentrations) on prostaglandin E2 (PGE2) production in LPS-activated BV-2 cells. $n=4$, $*p<0.05$ vs. control cells, $#p<0.05$ vs. LPS-treated cells

Conclusion

Conjugation of quercetin with gold and silver nanoparticles can overcome the solubility problem of quercetin in non-toxic aqueous mediums. The findings of the present study suggest that the potential of gold-quercetin nanoparticle conjugates is much better than pure quercetin treatment. Gold-quercetin nanoparticle conjugates might provide protection against inflammatory neurodegenerative disease via suppression of acute microglial activation.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest to declare.

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