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Theaflavins from Black Tea, Especially Theaflavin-3-gallate, Reduce the Incorporation of Cholesterol into Mixed Micelles

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Tea is one of the most widely consumed beverages in the world and may be associated with reduced heart disease rates. Theaflavins, which are formed in the production of black tea, have been suggested being responsible for the blood-cholesterol-lowering (BCL) effects of tea. We hypothesized that the effect of theaflavins on BCL could be through interference in the formation of dietary mixed micelles, which could result in reduced intestinal cholesterol absorption. Micelles were produced by mixing oleic acid, bile acids, lyso-phosphatidylcholine, and cholesterol. Theaflavin-treated micelles/particles were analyzed using electron microscopy (cryo-TEM), high-performance liquid chromatography (HPLC) analysis, and light-scattering particle size measurements. A dose-dependent inhibitory effect of theaflavins on the incorporation of ^{14}C-labeled cholesterol into micelles and a theaflavin-dependent increase in particle size was found. These particles consisted of insoluble large multilamellar vesicles with onion-like structures. Ultracentrifugation and HPLC analysis revealed that the pellets contained mainly theaflavin-3-gallate, while the remaining theaflavins were found to be present in the supernatant. Using purified theaflavin subtypes confirmed that mainly theaflavin-3-gallate is responsible for multilamellar vesicle formation. These results show that theaflavins can play a role in decreased intestinal cholesterol absorption via inhibition of micelle formation.

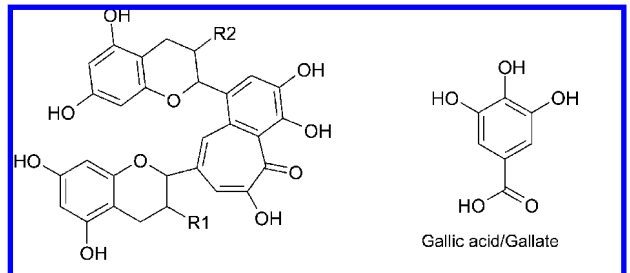
KEYWORDS: Black tea; theaflavins; polyphenols; cholesterol; micelles

1. INTRODUCTION

Tea is one of the most widely consumed beverages in the world. Tea comes in various kinds, but all true teas are produced from the same species of the plant *Camellia sinensis*. Teas are classified into three major categories according to the manufacturing process: unfermented green tea, fully fermented black tea, and partially fermented oolong tea.

Steaming or drying fresh tea leaves at elevated temperatures makes commercial green tea. Its chemical composition is similar to that of fresh tea leaves. Green tea contains polyphenols, which include flavanols, flavonol glycosides, and phenolic acids; these compounds may account for up to 30% of the dry weight. Most of the green tea polyphenols are flavanols, commonly known as catechins. Major green tea catechins are epigallocatechin-gallate (EGCg), epi-gallocatechin, epicatechin-gallate, and epicatechin.

In the manufacture of black tea, the monomeric flavan-3-ols undergo polyphenol oxidase-dependent oxidative polymerization, leading to the formation of bisflavanols, theaflavins, thearubigins, and other oligomers in an oxidation process commonly known as "fermentation". Theaflavins (about 1−2% of the total dry matter of black tea), including theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate, possess benzotropolone rings with dihydroxy- or trihydroxy-substitution systems (as shown in **Figure 1**), which give the characteristic color and taste of black tea. About 10−20% of the dry weight of black tea is made up of thearubigins, which are even more extensively oxidized and



R1 = OH	R2 = OH	Theaflavin	TF
R1 = OH	R2 = Gallate	Theaflavin-3-gallate	TF3g
R1 = Gallate	R2 = OH	Theaflavin-3'-gallate	TF3'g
R1 = Gallate	R2 = Gallate	Theaflavin-3,3'-digallate	TF3,3'dg

Figure 1. Molecular structures of the four major theaflavins present in black tea.

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Table 1. Composition of the Theaflavin Mix Used

	molecular weight (g/mol)	fraction in mix (w/w)	contribution to average molecular mass
theaflavin (TF)	569	0.10	57
theaflavin-3-gallate (TF-3-g)	721	0.27	195
theaflavin-3′-gallate (TF-3′-g)	721	0.14	101
theaflavin-3,3′-digallate (TF-3,3′-dg)	873	0.49	428
average molecular mass (g/mol)			781

polymerized, have a wide range of molecular weights, and are less well-characterized (*1*).

Oolong tea, a partially oxidized tea, contains monomeric catechins, theaflavins, and thearubigins at levels between green and black tea. Some characteristic components, such as epigallocatechin esters, theasinensins, and polymeric catechins (proanthocyanidins), are also found in oolong tea.

After theaflavin itself was first isolated in 1957 from black tea (*2*), this group of polyphenol pigments has been extended by including several new compounds. Theaflavins have been the subject of research with respect to properties, such as chemical structure, methods of purification and assay, formation mechanism, as well as synthesis methods. In recent years, attention has been paid to its pharmacological function, including properties as antioxidant, antipathogenic substance, and cancer suppressor (*3*). Furthermore, theaflavins have been tested for benefits to prevent coronary heart disease and hypertension and to treat diabetes (*4*−*7*).

A number of studies suggest that black tea can lower total and low-density lipoprotein (LDL) cholesterol as reviewed by Davies et al. (*8*). Maron et al. (*9*) showed that a theaflavin-enriched green tea extract lowered plasma cholesterol concentrations after 12 weeks of ingestion. In this double-blind, randomized, placebo-controlled, multicenter trial, 240 hypercholesterolemic subjects received 375 mg of total flavonoids (75 mg of theaflavins, 150 mg of catechins, and 150 mg of other tea phenols). The mean decrease in total and LDL cholesterol was 11 and 16%, respectively, while no significant adverse events were observed in this study.

The underlying mechanism behind this beneficial effect was not reported, but the first site of action might be interference in the formation of dietary mixed micelles in the intestine. Ikeda et al. (*10*) and Raederstorff et al. (*11*) studied the effects of catechins on micelle formation and concluded that relatively high concentrations of gallate-containing catechins are effective in inhibiting micelle formation. Because most theaflavins also contain gallate moieties, the same mechanism of action might also be applicable for the theaflavins.

The objective of the work described in this paper was to test the effect of black tea theaflavins on the *in vitro* incorporation of cholesterol in mixed micelles to provide a possible mechanism of action for the described cholesterol-lowering effect of theaflavins.

2. MATERIALS AND METHODS

2.1. Materials. The theaflavin mix was provided by colleagues from Unilever R&D Colworth, Sharnbrook, U.K., comprising the composition as shown in **Table 1**.

The individual pure theaflavins (theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate) were supplied by colleagues from Unilever R&D, Shanghai, China.

Lipton Yellow Label tea bags were used to make a black tea extract. Green tea extract was made using China Green Tea, Treasure Tea Company.

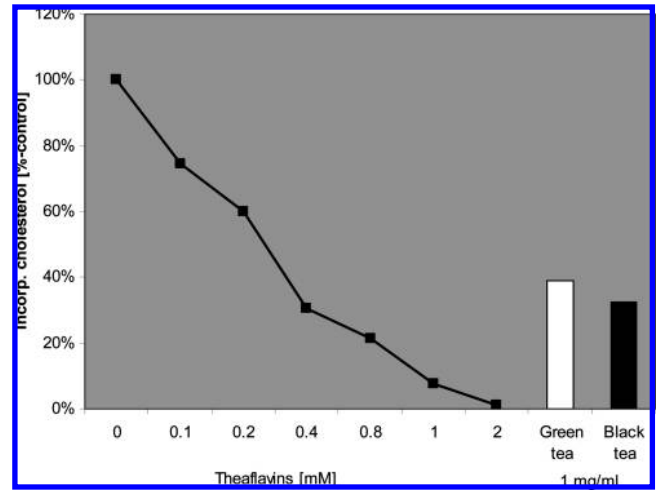

Figure 2. Theaflavins and black and green tea extracts (1 mg/mL) inhibit the incorporation of cholesterol into mixed micelles. Micelles were generated using mono-olein, oleic acid, lyso-phosphatidyl choline, cholesterol, and a bile acid mixture. A tracer amount of radiolabeled cholesterol was used to measure the incorporation of cholesterol into soluble micelles. Using increasing amounts of a theaflavin mix indicated the relation between the amount of theaflavins and the incorporation of cholesterol into micelles.

2.2. Micelle Formation Assay. Stock solutions of the micellar lipids mono-olein (Sigma), oleic acid (Sigma), lyso-phosphatidylcholine (Sigma), cholesterol (Sigma), and ^{14}C-labeled cholesterol (Amersham BioSciences) were pipetted in predefined ratios into tubes and mixed as previously described by Mel'nikov et al. (*12*). After drying with N_2, a 500 μL solution of theaflavin mix in a 2 mM bile acid mix (BA mix: glycocholic, taurocholic, glycodeoxycholic, taurodeoxycholic, taurochenodeoxycholic, and glycochenodeoxycholic acids, Sigma) in phosphate-buffered saline (PBS) at pH 6.5 was added to the above-mentioned dried lipids. This resulted in final lipid concentrations of 50 μM mono-olein, 100 μM oleic acid, 100 μM lyso-phosphatidylcholine, and a tracer amount of ~4000 dpm ^{14}C-labeled cholesterol. Unlabeled cholesterol was used at varying concentrations as indicated in the experimental methods. A concentration range of theaflavins was tested (0, 0.1, 0.2, 0.4, 0.8, 1, and 2 mM) on the incorporation of ^{14}C-labeled cholesterol in mixed micelles. Using PBS at an intestinal pH of 6.5, theaflavins are stable for a number of hours as shown by Jhoo et al. (*13*). Unlabeled cholesterol (80 μM) was added to the test compounds to saturate the formed micelles and thus create a more sensitive test system. After 30 min of sonification, the mixture was ultracentrifuged for 30 min at 50000*g* in a Beckmann Tabletop ultracentrifuge using 1.5 mL polycarbonate tubes, according to Eckhardt et al. (*14*). The amount of incorporated ^{14}C-labeled cholesterol in the supernatants, which represents micellar cholesterol, was measured by liquid scintillation counting. Comparable incorporation experiments were executed using ^{14}C-labeled micelle component cholesterol, glycocholic acid, and oleic acid to elucidate which micellar lipids are involved in the mechanism of action of theaflavins.

2.3. Light Scattering. Subsequent experiments were performed to assess the effect of theaflavins on the size of mixed micelles and vesicles as measured by light scattering. Micelles were produced using the same method as described above, except for ultracentrifugation and the use of ^{14}C-labeled cholesterol or other radiolabeled lipids. For this experiment, increasing theaflavin mix concentrations (0, 0.2, 0.4, and 0.8 mM) were used.

After the formation of the theaflavin-treated micelles, the whole mixture of lipids and test compounds was used for size measurements without any ultracentrifugation step. For each measurement, about 1 mL of micellar mixture was used in plastic cuvettes in a Zetasizer NanoSeries Nano ZS (Malvern, U.K.) particle characterization system and particle size distribution was measured using standard methods.

2.4. Transmission Electron Microscopy (TEM). Micelles were generated using the same procedure as described above without using

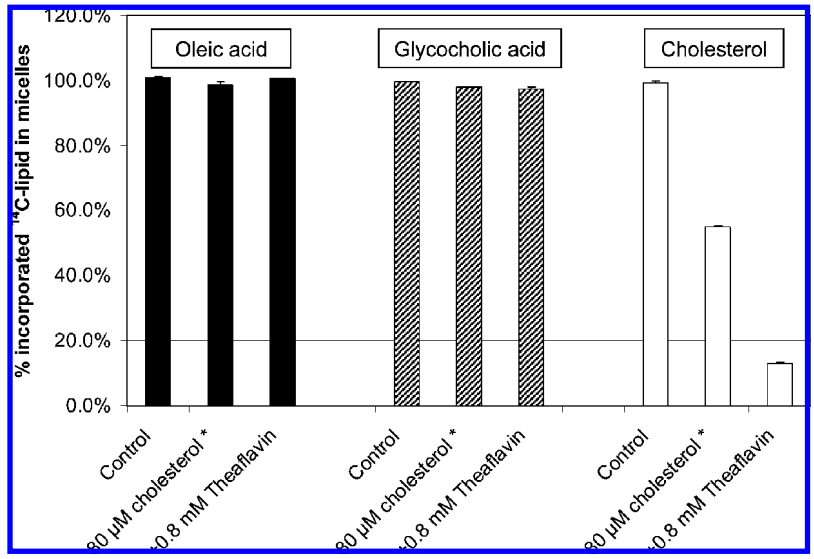

Figure 3. Incorporation of ^{14}C-labeled ingredients into mixed micelles. Tracer amounts of radiolabeled micellar lipids, glycocholic acid, oleic acid, and cholesterol, were used in the micelle formation assay. The effect of added theaflavins on lipid incorporation was determined by measuring the radiolabeled lipids in the formed soluble micelles. Dark bars, ^{14}C-labeled oleic acid; hatched bars, ^{14}C-labeled glycocholic acid; and open bars, ^{14}C-labeled cholesterol.

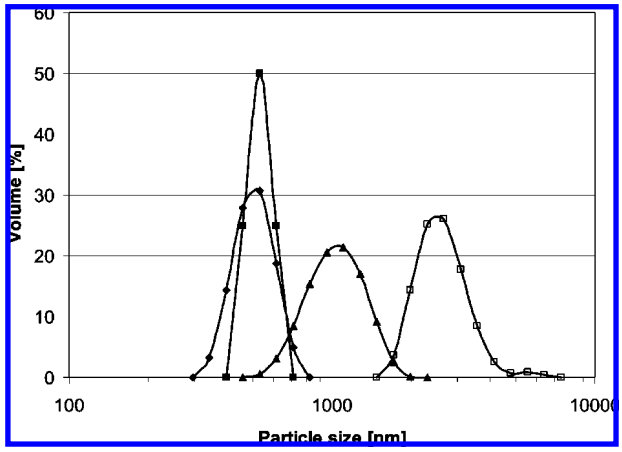

Figure 4. Theaflavins increased particle size of mixed micelles. The dose−response effect of added theaflavins on the particle size of micellar particles was measured by light scattering [◆, control micelles (80 μM cholesterol); ■, 0.2 mM theaflavin mix; ▲, 0.4 mM theaflavin mix; □, 0.8 mM theaflavin mix]. Micelles were generated using the standard micelle formation assay, and the size of the formed particles was measured without centrifugation on a Malvern Zetasizer particle characterization system.

^{14}C-labeled cholesterol. To visualize all particles formed, the mixture of lipids and theaflavins was not further treated by ultracentrifugation. For high-resolution imaging of the samples, 2 μL of a noncentrifuged mixed micelle suspension was applied to a 200 mesh holey carbon support grid (Ted Pella, Inc.). To obtain a thin liquid film, the excess fluid was blotted using Whatman No. 4 filter paper. To prevent evaporation artifacts, the blotting was performed in an environmental box (CEVS, ex Technion, Israel) saturated with water vapor. The thin film of the suspension was vitrified into liquid ethane and transferred under liquid nitrogen into a transmission electron microscope (Tecnai 20, FEI Company B.V., Hillsboro, OR). The thin vitrified specimen were imaged in the Tecnai 20 operated at 200 kV accelerating voltage using low (electron) dose routines.

2.5. High-Performance Liquid Chromatography (HPLC). Micellar samples were produced using the same procedure as for the cholesterol incorporation, except that no ^{14}C-labeled cholesterol was used in the samples. After formation of micelles with theaflavins, the samples were separated by ultracentrifugation for 30 min at 50000*g* in a Beckmann tabletop ultracentrifuge using 1.5 mL polycarbonate tubes. The supernatants were analyzed without pretreatment, while the precipitated material was solubilized in acetonitril in the same volume as the original volume of the sample. The theaflavin samples were separated using a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with an Inertsil 5 ODS-2 column (250 × 4.6 mm, 5 μm), (Varian, Inc., Palo Alto, CA). The column was kept at 30 °C and run isocratic at 1 mL/min using 1% acetic acid in acetonitril and 1% acetic acid in water in a ratio of 22.5/77.5% (v/v). After a run of 40 min, the column was washed with pure acetonitril/acetic acid. A Shimadzu diode array detector was used in the range of 200−400 nm to detect the elution of the individual theaflavins. Identification of the individual theaflavins was performed by using individual purified theaflavins or literature data (*15, 16*).

3. RESULTS

For the cholesterol-incorporation experiments, a theaflavin mix was used to assess its effect on the incorporation of cholesterol into mixed micelles. The described experiments resulted in a clear dose-dependent inhibitory effect of the theaflavins on cholesterol incorporation ($IC_{50} \sim 0.25$ mM) as shown in **Figure 2**. The highest concentrations resulted in an almost complete inhibition of cholesterol incorporation in the micelles. Also, two homemade tea extracts from green and black tea (1 mg/mL) reduced at a similar level the incorporation of cholesterol into the micelles.

To understand the role of each micellar lipid, three radiolabeled micelle components, oleic acid, glycocholic acid, and cholesterol, were separately used in micellar incorporation experiments in combination with the theaflavin mix. The results as depicted in **Figure 3** clearly show that only the incorporation of cholesterol is affected by theaflavins, as shown by the decrease of incorporated cholesterol. Oleic and glycocholic acids are incorporated into soluble lipid particles (micelles and vesicles) without any interference by the theaflavin mix.

Size-measurement experiments indicated that large particles are formed at increasing theaflavin mix concentrations. **Figure 4** shows that the addition of theaflavins increased the size of micellar particles compared to control micelles containing 80 μM cholesterol. The average size of the formed particles increased from 0.5 to 2.5 μm when incubated with the theaflavin mix.

To visualize the formed micelles, TEM analyses were performed using increasing amounts of theaflavins. The samples

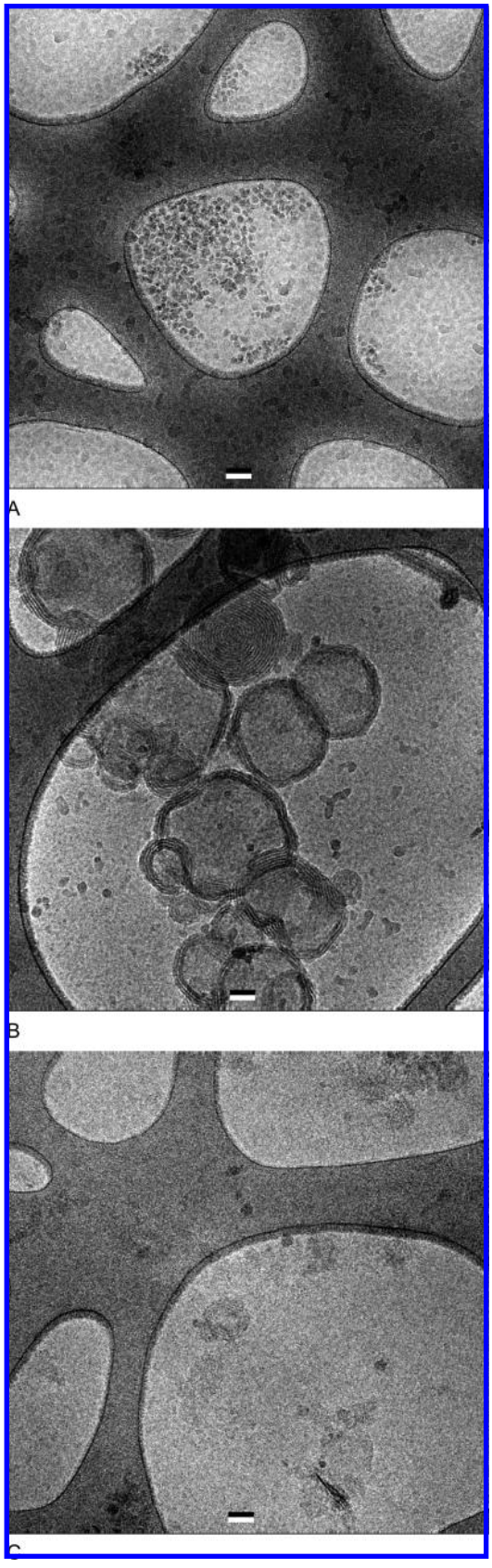

Figure 5. Multilamellar particles are formed from theaflavins and micellar lipids. The effect of a theaflavin mix on the micelle particle structure was measured by TEM. Micelles were generated by mixing mono-olein, oleic acid, lyso-phosphatidylcholine, and bile acids using the standard procedure without centrifugation. The formed particles were visualized by using cryo-TEM techniques. A, control micelles; B, micelles plus 0.4 mM theaflavin mix; C, bile acid mix plus 0.4 mM theaflavin mix. In A−C, bars = 50 nm.

Table 2. Distribution of Theaflavins in Supernatant and Pellet Fraction[a]

	before separation	after separation	
	starting mix (%)	supernatant (%)	pellet (%)
theaflavin	10	9.5	0.5
theaflavin-3-gallate	27	15.4	11.6
theaflavin-3′-gallate	14	10.5	3.5
theaflavin-3,3′-digallate	49	48.0	1.0
total	100%	100%	

[a] The relative composition of fractions of theaflavin-treated micelles based on HPLC analysis after centrifugation.

for electron microscopy analysis were not centrifuged; otherwise, the large particles would have been removed. **Figure 5** depicts the multilamellar vesicles formed in the presence of theaflavins, which are absent in the samples that do not contain theaflavins. As further shown in **Figure 5**, the bile acid mix plus theaflavin mix also did not show these large multilamellar particles. When lyso-phosphatidylcholine was omitted from the lipid mix, these multilamellar structures were not formed (results not shown).

To analyze the theaflavins present in the large onion-like structures, micelles were prepared with/without various concentrations of theaflavin mix. The micelle samples were centrifuged, and the supernatant and the pellet were applied to HPLC. Results showed that theaflavin-3-gallate was the major theaflavin present in the multilamellar vesicles in the pellet as shown in **Table 2**, while the other three theaflavins were mainly located in the supernatant.

The four individual theaflavins were also tested separately in the micelle formation assay by electron microscopy analysis. This method also confirmed that only micelles with theaflavin-3-gallate resulted in the formation of multilamellar structures. The other three theaflavins (theaflavin, theaflavin-3′-gallate, and theaflavin-3,3′-digallate) did not form such structures as shown in **Figure 6**.

4. DISCUSSION

The findings from this *in vitro* study suggest that theaflavins inhibit cholesterol incorporation in soluble micelles with a specific role for theaflavin-3-gallate as the most active theaflavin on micelle formation. Also, tea extracts from green and black tea reduced the incorporation of cholesterol into mixed micelles, which suggests that extracted compounds (e.g., catechins, theaflavins, flavonols, and thearubigins) from both teas reduce the incorporation of cholesterol into mixed micelles. The results also suggest that only the incorporation of cholesterol and not two other micellar lipids, glycocholic acid and oleic acid, was affected by theaflavins. Similar *in vitro* effects on decreased cholesterol incorporation in mixed micelles were reported by Ikeda et al. (*10, 17*), Raederstorff et al. (*11*), and others for green tea catechins, especially epigallocatechin-gallate (EGCg). In the production of black tea, EGCg is converted by the polyphenol oxidase (PPO)-induced enzymatic "fermentation" together with epicatechin into the most active theaflavin-3-gallate (*18*). The presence of a gallate moiety in tea polyphenols seems to play a major inhibitory role in the formation of mixed micelles.

Theaflavins, in the presence of cholesterol, form insoluble multilamellar membrane structures, which was also observed by Simon et al. (*19*) and Schrijvers et al. (*20*), when phospholipid micelles were incubated with tannic acid. Tannic acid contains one glucose molecule connected to five gallic acid polymers, suggesting again that gallated compounds might play a major role in the formation of the multilamellar vesicles.

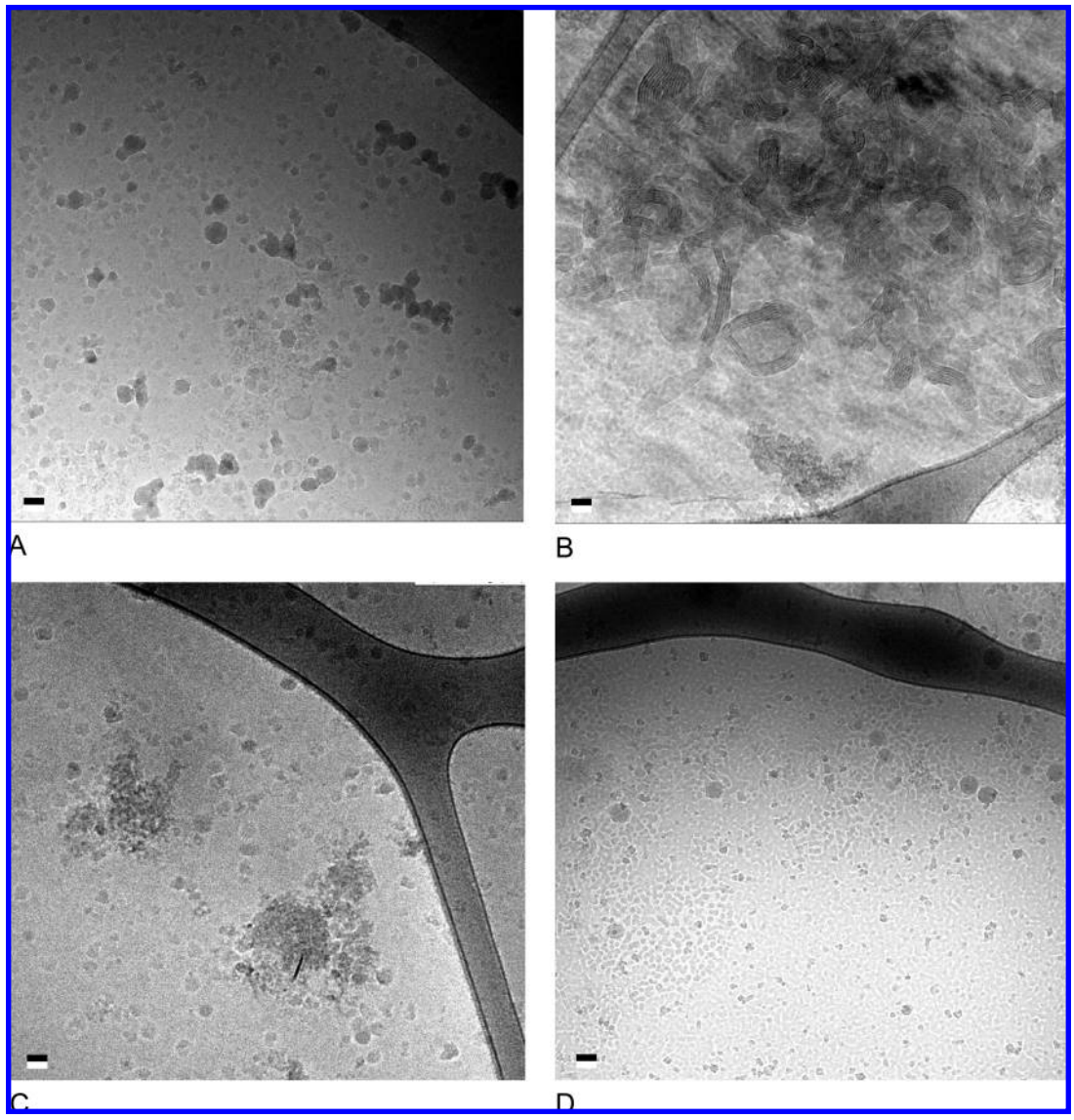

Figure 6. Visual appearance of micellar structures formed using individual theaflavins. The effect of four individual theaflavins (0.4 mM) on the visual appearance of formed micellar structures was visualized using TEM techniques. Micelles were generated by mixing mono-olein, oleic acid, lyso-phosphatidylcholine, and bile acids using the standard procedure without centrifugation using four purified theaflavins. A, theaflavin; B, theaflavin-3-gallate; C, theaflavin-3′-gallate; D, theaflavin-3,3′-digallate. In A−D, bars = 50 nm.

Theaflavins, especially theaflavin-3-gallate, might form insoluble complexes with lyso-phosphatidylcholine and "entrap" cholesterol, which is shown by the large particles seen in size measurements and electron microscopy imaging.

On the basis of earlier publications by Ikeda et al. (*10, 17*) and Stangl et al. (*21−23*), gallated polyphenols in tea play a major role in the potential health benefits of green and black tea consumption. Several human studies have shown that green and black tea consumption reduced total cholesterol and LDL cholesterol concentrations (*8, 9, 24*). The results described in this paper suggest that the main mechanism of action of theaflavins on cholesterol metabolism is on the inhibition of cholesterol incorporation in micelles, which results in a reduced intestinal cholesterol uptake. The gut is most likely the main site of action of the theaflavins, because the bioavailability of theaflavins is very low, as shown by human plasma levels after black tea consumption by Mulder et al. (*25*). A regular cup of black tea contains ∼16−24 mg of theaflavins per serving of 200−230 mL (*26−28*), resulting in an average theaflavin concentration of 100−150 μM. The experiments described in this paper used concentrations at a higher range (200−800 μM) of what is present in a regular cup of black tea. The reported experimental data suggest that concentrated black tea or black tea supplements, both containing theaflavins, might have even stronger inhibitory effects on micellar cholesterol incorporation than a regular cup of black tea.

In summary, the experiments described in this paper demonstrate that theaflavins exhibit an inhibitory effect on the incorporation of cholesterol into mixed micelles in this *in vitro* test system, while the incorporation of other micellar components (oleic acid and glycocholic acid) was not affected. Adding a theaflavin mix to mixed micelles induced the formation of large particles as shown by particle size analysis and TEM. HPLC analysis in combination with electron microscopy showed that theaflavin-3-gallate was the largest peak in the precipitated material, while the other three theaflavins were mainly present in the supernatant. The described findings could offer a mechanistic explanation for the cholesterol-lowering effect of theaflavins reported by Maron et al. (*9*) when giving high amounts of theaflavins to human volunteers. It remains to be established to what extent the *in vitro* findings reported here are also relevant *in vivo*. Therefore, animal or human studies

are required to confirm a possible cholesterol-lowering effect of theaflavins.

5. ABBREVIATIONS USED

BA, bile acids; BCL, blood cholesterol lowering; EGCg, epigallocatechin-gallate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PPO, polyphenol oxidase; TEM, transmission electron microscopy; TF, theaflavin.

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