Antioxidant Coating of Micronsize Droplets for Prevention of Lipid Peroxidation in Oil-in-Water Emulsion

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ABSTRACT Fast lipid peroxidation in emulsified oils results in carcinogens formation and product rancidity. Prevention of oxidative degradation in oil-in-water emulsion has been achieved by encapsulating of each droplet of dispersed phase in antioxidant multilayer coating shell. The fabrication comprised placing a surface-active ionic emulsifier at the oil/water interface followed by stepwise alternate adsorption a biocompatible polyelectrolyte and antioxidant layers. Uncoupled polyelectrolyte macromolecules and antioxidant were thoroughly removed from formulation, thus the protection was entirely attributed to the droplets' shell. The experiments were performed using linseed oil, the richest source of highly unstable omega-3 alpha linolenic essential fatty acid. Bovine serum albumin (BSA) was exploited as an anionic emulsifier. The biodegradable coating shell was formed of poly-L-arginine (PARG) and dextran sulfate (DS) applied as a polycation and a polyanion respectively. Tannic acid (TA) known as a natural antioxidant and possessing antimicrobial properties was used as a protective remedy. Oil microdroplets coated with TA-containing shell displayed physical-chemical and mechanical stability in aqueous phase and over freeze-drying process as determined by ζ-potential measurements, dynamic light scattering (DLS), and confocal laser scanning microscopy (CLSM). Oxidation of emulsified oil was monitored by formation of malondialdehyde (MDA) in the samples quantified by Thiobarbituric Acid Reactive Substances (TBARS) assay. Coating shell with an incorporated layer of TA effectively suppressed oxidation in water-dispersed oil droplets and affected iron-catalyzed oxidation over 15 days of incubation at 37 °C in 0.3 mM $FeBr_2$ solution. Antioxidant activity of TA-containing shell assembled around each oil droplet was found to be higher than that of mixed tocopherols (MT) added to linseed oil in concentration of 10 000 ppm.

KEYWORDS: Layer-by-layer • Coating • Encapsulation • Tannic acid • Lipid peroxidation • Oxidative stability • Oil-in-water emulsion

INTRODUCTION

Oil-in-water emulsions are important ingredients of a wide range of drugs, functional foodstuffs, and consumer care products. Oils such as linseed oil, cod oil, and some marine oils are well-known for healing effect due to high content of polyunsaturated fatty acids. So-called essential fatty acids, such as alpha-linolenic acid and linoleic acid can't be synthesized by the human body but instead must be ingested with food to avoid many degenerative diseases including heart disease, cancer, incidences of stroke, and skin and autoimmune disorders (1). However, instability of polyunsaturated fatty acids to peroxidation often hampers practical use of oil-based products highly shortening their shelf life. Despite rancid smell, oxidized oils contain carcinogenic and mutagenic end products of lipid peroxidation (2). Therefore, they are strictly refused for bioapplications. Oxidative stability is an essential requirement for lipid-based drug delivery systems. Inflammatory centers and cancerous growth are the sources of free radicals, which can initiate the chain reaction of lipid peroxidation in drugs. Thus, therapeutic and healing properties of various products containing aqueous dispersed unsaturated lipids depend strongly on reliable protection against pro-oxidant damage.

Oxidative degradation in a diphasic system of emulsified oil proceeds even faster, if compared to a monophase oil in a flask. The main reasons are extensive oil/water interface in emulsion, relatively small volume of each oil droplet, and a broad range of free radicals and pro-oxidants that can face oil droplets on a way to their therapeutic targets. The surface of an emulsion droplet, being in closest contact with damaging agents of the surrounding water phase, is the place where the chain reaction of lipid peroxidation is usually initiated and prevalent. As suggested by Klinkesorn et al., endogenous transition metals that are naturally present in the oil, surfactant, and/or water are the important pro-oxidants in emulsion droplets (3). As such, utilization of cations' screening effect of positively charged emulsifiers at the oil/water interface and/or adding of chelates to the continuous phase became the key strategies to control oxidative degradation in oil-in water emulsion. For instance, protein emulsifiers like

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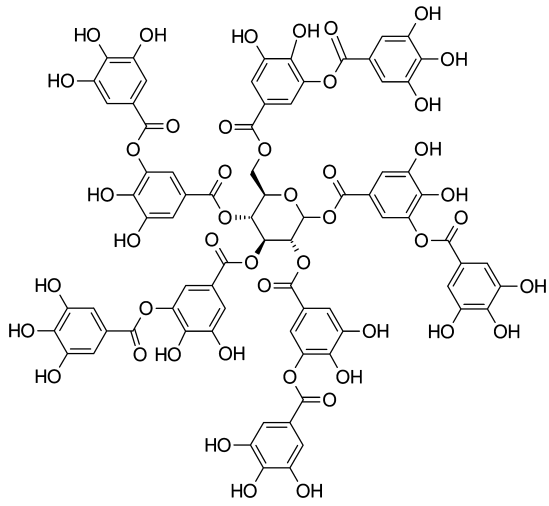

FIGURE 1. Chemical structure of tannic acid.

casein and whey proteins slowed down the oxidative degradation of incorporated oils at pH values well below their isoelectric point, i.e., when they are cationic, because of electrostatic repulsion of transition metals (4, 5). At the same time, adding of EDTA and free-radical scavengers (e.g. ferulic acid, coumaric acid, propyl gallate, gallic acid, and ascorbic acid) to the continuous phase also affected peroxidation in the emulsified oils (3, 6−8). The important disadvantage of the formulations described above is the dependency of their oxidative stability on the presence of external antioxidants or physical-chemical conditions the continuous phase limiting the range of their practical applications to a considerable extent. Therefore, a more versatile protection still has to be elaborated.

Layer-by-layer coating technique considerably advantageous for fabrication of complex and multifunctional coating films on plane surfaces and colloid particles (9−11) has been applied for encapsulation of micrometer- and submicrometersize oil-in water emulsions (12−16). Besides improved stability to coalescence and flocculation those double- or multilayer coated emulsions were characterized with better stability to oxidative stress. In this way, peroxidation rate of dispersed lipids of corn oil encapsulated into lecithin/chitosan bilayer was decreased (17). Katsuda et al. proposed to affect lipid peroxidation directly tailoring antioxidant molecules of beta-lactoglobulin (containing antioxidant ferulic acid) to bilayer coating shell. However, relatively high concentration of transition metals in the applied chemical speed-up the oxidative degradation in encapsulated fish oil (18). The examples mentioned above show a general need in the art for substantial improvement of the oxidative stability of aqueous dispersed lipids. Moreover, the protective tools must be universal and act effectively independent on the presence of external antioxidants or physical-chemical conditions of the surrounding medium.

This paper reports on LbL encapsulation of aqueous dispersed linseed-oil droplets into complex coating shell comprising a layer of antioxidant tannic acid (TA). TA, a polyphenol of natural origin (Figure 1), is known to possess high ability to scavenge free radicals and pro-oxidants (19). Among those, superoxide radical (20), mutagenic m-Chloroperoxybenzoic acid (21), and transition metals (22, 23). The use of TA as a building block in alternation with positively charged polymers in multilayer assemblies on a plane solid substrate and microcapsules was shown by Shutava et al. (24) Later the antioxidant properties of TA-containing multilayers have been demonstrated (25).

The aim of this study is to fabricate a formulation comprising oil-in-water emulsion, where each oil droplet is encapsulated in multilayer coating shell possessing high antioxidant activity independently on the physical-chemical conditions and antioxidants of the continuous phase, suitable for a broad range of practical applications. For this purpose we, first, construct a biocompatible polyelectrolyte/TA multilayer membrane at the interface of water dispersed droplets of linseed oil and examine the physical-chemical and mechanical stability of encapsulated emulsion. Second, we reveal the impact of the interfacial antioxidant presence to the oxidative stability of encapsulated oil relative to protective properties of antioxidant-free biodegradable multilayer coating shell assembled of poly-L-arginine and dextran sulfate (26). Third, the antioxidant activity of TA-based multilayers encapsulating the dispersed oil is evaluated in the pro-oxidant $FeBr_2$ containing environment. Fourth, we explore how the antioxidant location and mechanism of action affects the oxidative stability of emulsified linseed oil by the example of encapsulated emulsions protected by shell antioxidant (TA) and core antioxidant (mixed tocopherols, commonly known as vitamin E), respectively.

The obtained emulsions were characterized by ζ-potential measurements, dynamic light scattering (DLS), and confocal laser scanning microscopy (CLSM). UV−vis absorption spectroscopy was used to determine the amount of adsorbed TA. Oxidation of the emulsified oil was monitored by thiobarbituric acid reactive substances (TBARS) assay.

EXPERIMENTAL SECTION

Materials. Proteins (bovine serum albumin (BSA) and tetramethylrhodamine isothiocyanate labeled bovine serum albumin (TRITC-BSA)), polyelectrolytes (dextran sulfate (DS, MW. 20000), and poly-L-arginine hydrochloride (PARG, MW>70 000)), linseed oil, mixed tocopherols (MT) (FCC and FG grade reagent, mixture of D-alpha, D-beta, D-delta, and D-gamma-tocopherols), and tannic acid (TA) were purchased from Sigma-Aldrich. All chemicals were used as received without further purification. 3,4,9,10-Tetra-(hectoxy-carbonyl)-perylene (THCP) was synthesized as described elsewhere (27). TBARS Assay Kit was purchased from CELL BIOLABS, INC. and used according to enclosed protocol. Deionized water with specific resistivity higher than 18.2 $M\Omega\ m^{-1}$ from a three-stage Milli-Q Plus 185 purification system was used in the experiments.

Emulsion Preparation. A primary emulsion of linseed oil was obtained by dispersing 10 % v/v linseed oil in 90 % v/v emulsifier (BSA, 4 mg/mL) water solution. For this purpose an oil/emulsifier mixture was treated in Vibra-Cell (Sonics & Materials, Inc., USA) operating at a frequency of 20 kHz and power output of 300 W over 2 min. As no buffer was used to control the pH in BSA solution, ζ-potential of resulted colloids was measured prior deposition of a next layer of polyelectrolyte. ζ-potential measurements displayed the stabilized oil droplets were negatively charged, thus one part of this primary emulsion was mixed with two parts of PARG (2 mg/mL) water solution to

form a secondary emulsion. The mixture was transferred to a modified 50 mL stirred filtration cell (Millipore Corp., USA) and kept under vigorous agitation for 15 min followed by 3 washing cycles to remove uncoupled polyelectrolyte (28). In each washing cycle, the dispersion was placed into the cell, filled with water, and then 40 ml of aqueous phase were filtered through 0.22 μm hydrophilic surfactant free MF-Millipore membrane. To adsorb a next layer of either DS or TA, 10 mL of filtered emulsion was topped-up with 20 mL of DS (2 mg/mL) or TA (3 mg/mL) water solutions and vigorous stirred for 15 min followed by 3 washing cycles. The described routine was repeated alternating PARG with DS (or TA) to obtain the desired number of layers in the shell.

Oil-soluble antioxidant (MT) or fluorescent agent (THCP) were admixed to linseed oil before emulsification in amount of 10 000 ppm followed by multilayer shell assembly as described above.

ζ-Potential Measurements. Electrostatic charge of the droplets' surface in emulsion was measured by ZetaPlus system (Brookhaven Instrument Corporation, USA) placing 1 ml of 100 times diluted sample of 5 % v/v emulsion into 10 mm $\times$10 mm $\times$45 mm transparent polystyrene cells.

Particle Size Analysis. The hydrodynamic diameter of the water dispersed emulsion droplets was determined by Zetasizer Nano ZS (Malvern Instruments Ltd, UK) utilizing Dynamic Light Scattering (DLS) technique at 25 °C. Prior to measurements, 5 % v/v emulsion was 100 times diluted with water. For data analysis a model of aqueous dispersion of polystyrene latex beads was used.

Determination of the Amount of TA Coupled to Linseed Oil-in-Water Emulsion. The amount of adsorbed TA was quantified by means of UV−vis absorption spectroscopy. In details, the BSA/PARG coated oil droplets were immersed in the aqueous phase of known concentration and amount of TA. After 15 min of incubation, the aqueous phase with uncoupled TA was removed from the sample through a filter and absorption intensity in a 100 μl probe was detected at 283 nm (29) using the Infinite 200 PRO microplate reading spectrometer, Tecan Group Ltd., Switzerland. The concentration of the analyte was then determined using a calibration curve recorded from a series of standard solutions of known concentration of TA. The amount of TA coupled to oil droplets was calculated as a difference of the amount of TA initially added to the aqueous phase of the emulsion and detected amount of TA in the filtered supernatant. The determined values of the amount of TA associated with the dispersed phase and overall TA concentration in the samples with different content of emulsified oil are shown in Table 1. All data represent the mean of three measurements of different trials, and results are reported as the means and standard deviations of these measurements (Table 1).

Confocal Laser Scanning Microscopy (CLSM). Optical images were obtained on a Carl Zeiss Lsm510 META CLSM system (Carl Zeiss AG, Germany) equipped with a C-Apochromat 63X/1.2 Water Lens (Carl Zeiss AG, Germany) objective. The excitation (λ_{exc}) and emission (λ_{em}) wavelengths λ_{exc} = 529 nm, λ_{em} = 596 nm, and λ_{exc} =488 nm, λ_{em} =525 nm were used for scanning TRITC-BSA and visualization of encapsulated oil labeled with dissolved THCP respectively.

Freeze-Drying. The emulsion sample (25 ml) was transferred into a centrifuge tube, which was frozen by placing it overnight in a −80 °C freezer. A laboratory scale freeze-drying device (FreeZone 12, Labconco Corporation, USA) was used to dry the frozen emulsion at room temperature using a vacuum pressure of 0.006 mBar for 48 h.

Lipid Oxidation Measurement. Oxidative stability was evaluated by thiobarbituric acid reactive substances (TBARS) assay (30). TBARS is especially developed to quantify concentration of malondialdehyde (MDA), one of two natural biproducts of lipid peroxidation. TBARS assay kit was purchased from CELL BIOLABS, Inc. and used according to a protocol provided by supplier. To monitor lipid oxidation during storage, the samples containing 10 mL of of linseed oil-in-water emulsion were placed under air in tightly sealed microcentrifuge tubes and allowed to oxidize at 37 °C in the dark. Before MDA quantitation, each sample was diluted with water. The MDA-TBA adduct formed from the reaction of MDA in samples with TBA was measured colorimetrically. Absorbance was read at 532 nm, with background subtraction at 570 nm. TBARS levels were determined from a MDA equivalence standard. All data represent the mean of four measurements of two different trials, and results are reported as the means and standard deviations of these measurements. For direct comparison of samples with different initial content of oil microdroplets, measured concentration of MDA in a probe was recalculated per kg oil, assuming that the loss of oil microdroplets during the LbL coating did not exceed 1 %. Indeed, the use of a filtration cell for multilayer shell assembly limits the loss of colloids with the fraction of particles got stuck in pores of a filtering membrane. In our experiments, the filtering membrane remained unchanged until shell assembly in the sample was totally completed and its visual inspection did not reveal the evidence of considerable contamination of the pores.

Table 1. Amount and Concentration of Antioxidants in Encapsulated Linseed Oil-in-Water Emulsions

	no. of TA layers in coating shell		
	0	1	2
$m_{[TA]}$ coupled to 2.5 % v/v emulsion[a] (mg)	0	31 ± 4	84 ± 11
$C_{[TA]}$ associated with dispersed phase[a] (mmol/L oil)	0	73 ± 9	197 ± 25
$C_{[TA]}$ in 10 mL of 0.625 % v/v emulsion[a] (mM)	0	0.46 ± 0.06	1.23 ± 0.16
$C_{[MT]}$ in dispersed phase[b] (mmol/L oil)	22	0	0
$C_{[MT]}$ in 10 mL of 0.125 % v/v emulsion[b] (mM)	0.275	0	0

[a] Core content: linseed oil, shell composition: BSA/PARG/[TA/PARG]$_n$. [b] Core content: linseed oil with MT, shell composition: BSA/PARG/DS/PARG. Standard deviation is given for the data derived from results of UV−vis absorption spectroscopy measurements. Molar concentration of 10 000 ppm MT admixed to linseed oil ($C_{[MT]}$) was calculated taking into account MW = 416.68 (average molecular weight of α-, δ-, and γ-tocopherols), $\rho_{[MT]} = \rho_{[linseed\ oil]} = 0.93$ g/mL.

RESULTS AND DISCUSSION

Layer-by-Layer Assembly on Dispersed Oil Droplets. PARG/TA/PARG or PARG/DS/PARG multilayer shell assembly was performed on negatively charged soft cores represented by aqueous dispersed oil droplets preliminary stabilized with BSA. Figure 2 shows changes of the surface charge of the particles after deposition of each layer. Initially negatively charged linseed oil/BSA cores became positively charged after deposition of a PARG layer. Deposition of a following DS layer shifted the surface charge of the particles again to a negative value, in agreement with LbL assembly principles (31−33). TA was expected to adsorb on BSA/PARG coated oil microdroplets as it is known form chemical bonds with arginine at pH 3.0 in concentrations between 0.025% and 2% (34−36). The conditions men-

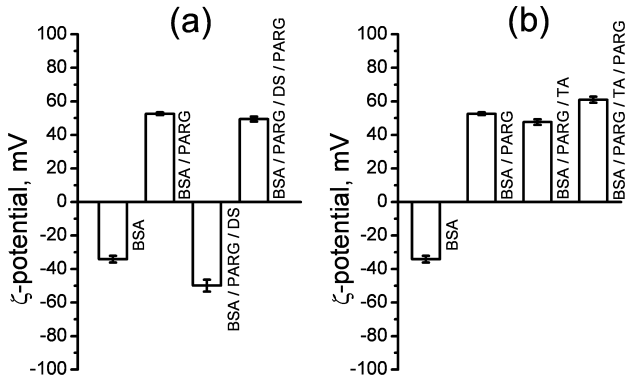

FIGURE 2. ζ-Potential of encapsulated droplets of linseed oil-in-water emulsion as a function of deposited layer: (a) BSA/PARG/DS/PARG; (b) BSA/PARG/TA/PARG.

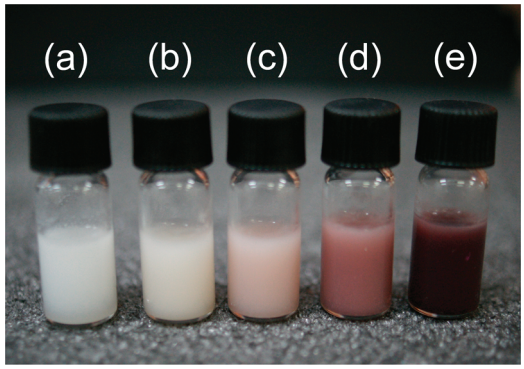

FIGURE 3. Color changes in encapsulated linseed oil-in-water emulsion in dependence on shell composition and water phase content: (a) BSA/PARG in H_2O; BSA/PARG/TA/PARG in (b) H_2O, (c) 0.03 mM $FeBr_2$, (d) 0.3 mM $FeBr_2$, and (e) 3 mM $FeBr_2$.

tioned above are a good match with the conditions of TA deposition in our routine. Indeed, the TA content in emulsion sample was 0.2 %, and the pH of the aqueous phase was measured to be ~3.5. However, adsorption of a TA layer was not reflected by an alternating change of the surface charge of the particles. ζ-potential of the BSA/PARG/TA shell still remained positive, although having lower absolute value than that of the BSA/PARG shell, possibly due to the lowering of charge of PARG on formation of bonds between amine groups of arginine and TA and a slight shift of slip plane on the covered emulsion droplets on adsorption of branched TA molecules.

Coating multilayers composed of BSA/PARG/TA/PARG and BSA/PARG/DS/PARG were both characterized with positive surface charge. Successful deposition of the TA layer was then verified by observing a slight change of emulsion color from a milky-white to a light brown, which did not disappear after sample washing (Figure 3a and b). Moreover, UV−vis Absorption Spectroscopy studies of the filtered supernatant displayed about 31 mg of TA coupled to 2.5 % v/v emulsion of linseed oil (Table 1).

Encapsulated emulsions were placed into 1.5 mL centrifuge tubes and monitored over 15 days of storage at 37 °C with the object to their stability to coalescence and shell integrity. Visual inspection revealed the dispersions remained homogeneous with no noticeable evidence of oil/water phase separation. After about 1 day of storage, fin

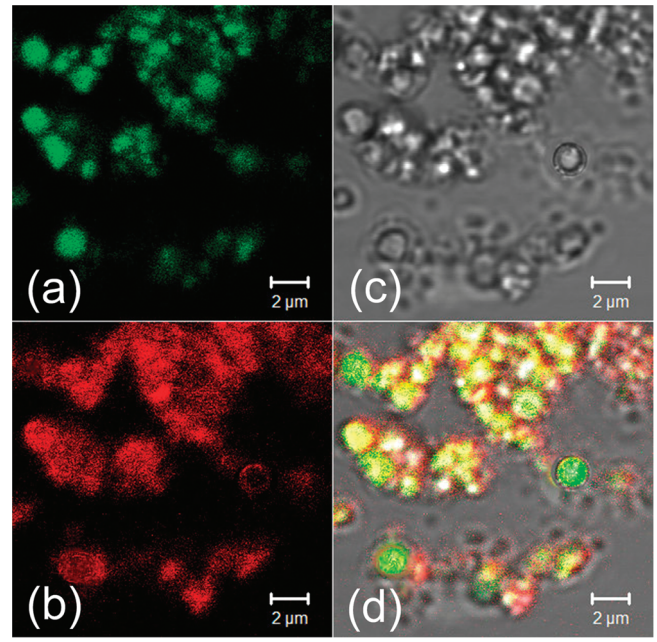

FIGURE 4. CLSM images of freeze-dried powder of BSA/PARG/TA/PARG encapsulated emulsion of linseed oil obtained at different excitation (λ_{exc}) and emission (λ_{em}) wavelengths. (a) λ_{exc} = 488 nm, λ_{em} = 525 nm; (b) λ_{exc} = 529 nm, λ_{em} = 596 nm; (c) transmission image; (d) overlay of images a, b, and c.

particle-enriched cream upper layer appeared as a result of gravitational separation of the emulsion samples (37). However, creaming of the encapsulated emulsion didn't lead to irreversible flocculation and completely disappeared after gentle shaking. Freeze-drying process also didn't affect the shell integrity. CLSM images of a tiny pinch of the dried emulsion are shown in Figure 4 where the green spots are attributed to oil cores with dissolved THCP (Figure 4a), and red color indicates the shells labeled with TRITC-BSA (Figure 4b). The overlay of the transmission (Figure 4c) and the fluorescence images (Figure 4d) displays the intact shells coating the polydisperse oil cores. Additionally, the stability of emulsion encapsulated into TA-containing multilayer shell to freeze-drying was examined analyzing the particle size distribution. For this purpose, the DLS measurements were performed in BSA/PARG/TA/PARG coated linseed-oil-in-water emulsion immediately after shell assembly and in the sample of its freeze-dried powder resuspended in water. The curves displaying the particle size distribution (PSD) in the corresponding samples are shown in Figure 5. Two maximum points on the PSD curve of the initial sample (Figure 5, curve a) indicate the presence of predominant fractions of coated microdroplets with the mean diameter of (308 ± 4) nm and (1622 ± 32) nm respectively determined by a Gaussian fit. The fraction of bigger particles appeared to be more polydisperse reflected by the full width at half maximum (FWHM). The freeze-drying process had negligible effect on the fraction of smaller particles. Indeed, no significant changes in position, intensity and FWHM of the corresponding peak can be mentioned (curve b in Figure 5). The mean diameter of the particles in the fraction obtained from a Gaussian fitting curve was (301 ± 8) nm. In contrast, the average size of bigger particles decreased after freeze-drying.

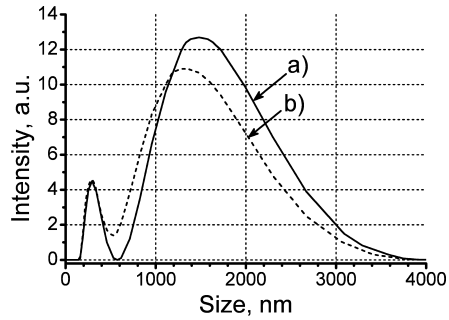

FIGURE 5. Particle size distribution in BSA/PARG/TA/PARG encapsulated emulsion of linseed oil recorded (a) immediately after shell assembly, and (b) in H_2O resuspended freeze-dried powder.

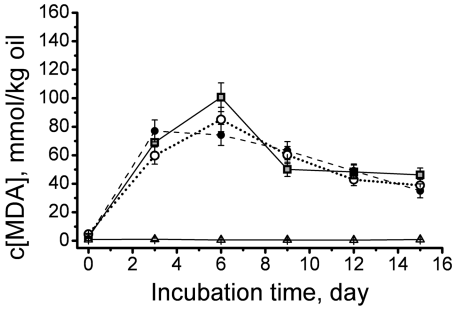

FIGURE 6. Formation of TBARS in encapsulated linseed-oil-in water emulsions over 15 days of storage at 37 °C in pure water in dependence on the thickness and composition of coating shell: BSA (—□—), BSA/PARG (--●--), BSA/PARG/DS/PARG (···○···), BSA/PARG/TA/PARG (—Δ—).

Thus, the mean particle diameter in the fraction was determined to be 1450 nm. However, the Gaussian fitted peaks at 1622 and 1450 nm were characterized with similar FWHM (1248 nm and 1270 nm respectively), that indicates no changes in particles' polydispersity caused by the freeze-drying process. The overall particles' loss over the freeze-drying process determined as a difference in the area under the PSD curves a and b in Figure 5 was about 12 %.

Influence of the Multilayer Shell Thickness, Composition, And Particle Surface Charge on Oxidative Stability of Encapsulated Linseed Oil-in-Water Emulsion. To clarify the influence of the thickness of the coating shell and the presence of TA on oxidative stability of encapsulated oil, we obtained the following samples: (i) linseed oil/BSA, (ii) linseed oil/BSA/PARG, (iii) linseed oil/BSA/PARG/DS/PARG, (iiii) linseed oil/BSA/PARG/TA/PARG. MDA was measured as a function of storage time in order to monitor the differences in oxidation kinetics. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage (30). Besides, MDA has been proved as a marker of lipid peroxidation to evaluate the oxidative stability of oil-in-water emulsions. Thus, the results of TBARS assay were in good agreement with the measured concentration of lipid hydroperoxide and headspace propanal (3, 5). Figure 6 displays a comparison of oxidation rates in encapsulated linseed-oil-in water emulsions stored over 15 days at 37 °C. The oxidative stability of linseed oil/BSA/PARG/TA/PARG emulsion comprising 2 double layers in the coating shell was not considerably improved if compared with emulsion coated with 1 double layer (linseed oil/BSA/PARG), and even with primary emulsion (linseed oil/BSA). Each of the above mention samples had a strong smell of rancidity already after 24 h of incubation at body temperature. Indeed, primary, secondary, and multilayer coated emulsions demonstrated almost the same oxidation rate with time as determined with TBARS assay. The difference between measured MDA concentrations in each mentioned sample after the same period of storage was within the standard deviation. Several papers have pointed out the thickness of the emulsion droplet interfacial membrane as a crucial tool to alter the rate of oxidative degradation in emulsified oil (38, 39). At first sight, those results are in contradiction with our observation. The contradiction is dissolved taking into account positive correlation between the thickness of polyelectrolyte multilayer film and the concentration of monovalent salt in the polyelectrolyte solutions used for film assembly (40). Thus, the thickness of PARG/DS/PARG shell composed in salt-free polymer solutions should not exceeding a few nm. At the same time, protein emulsifiers are known to form a relatively thick 10 nm layer at the oil/water interface (37). Accordingly, the main contribution into the thickness of BSA/polyelectrolyte(s) coating shell was made by a layer of the protein emulsifier (BSA). This fact explains the similarity in protective properties of polyelectrolyte multilayer coating compared to just a protein emulsifier placed at the oil/water interface.

Emulsions with positive surface charge have been found to be better stable to oxidation in comparison with negatively charged ones, presumably because of effective screening of transition metals with positively charged shell (5, 41). In our case, the emulsions coated with strongly positively charged BSA/PARG ($\zeta = (+52.6 \pm 0.7)$ mV) and BSA/PARG/DS/PARG ($\zeta = (+49.4 \pm 1.5)$ mV) shells did not have an advantage over negatively charged primary emulsion stabilized with BSA ($\zeta = (-34 \pm 2)$ mV) regarding the oxidative stability (Figure 2 and 6). The obtained result might indicate the presence of other damaging pro-oxidants in the emulsion samples not screened or scavenged by the interfacial membrane.

The situation changes drastically if 1 layer of TA was incorporated in the shell. No MDA were detected in the sample of TA coated emulsion over the whole period of observation (15 days) (Figure 6). Furthermore, the smell of rancidity did not appear as well. Thus, the polyelectrolyte multilayer coating shell comprising the antioxidant TA sandwiched between two layers of PARG prevented oxidative degradation in encapsulated linseed oil.

Antioxidant Activity of TA-Containing Shell in $FeBr_2$ Solution. The ability of TA layer incorporated in multilayer shell to scavenge Fe^{2+} cations and inhibit lipid peroxidation was examined at different concentrations of $FeBr_2$ salt in the aqueous phase. Among those, 0.03 mM, 0.3 mM, and 3 mM were tested. $C[FeBr_2] = 0.03$ mM was chosen as corresponding to normal physiological conditions in blood serum of human adults, where the concentration of iron ranges from 10 to 35 μM (42). Immediately after

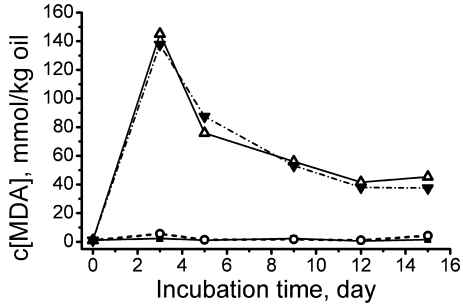

FIGURE 7. Formation of TBARS in encapsulated emulsion of linseed oil over 15 days of storage at 37 °C in $FeBr_2$ containing medium. The samples contained: BSA/PARG/TA/PARG encapsulated emulsion in 0.03 mM $FeBr_2$ (—■—), 0.3 mM $FeBr_2$ (· · ·○· · ·), and 3 mM $FeBr_2$ (—Δ—). BSA/PARG/TA/PARG/TA/PARG encapsulated emulsion in 3 mM $FeBr_2$ (- · -▼- · -). Error bars are overshadowed by the points' symbols.

adding $FeBr_2$ solution to the water phase of encapsulated emulsions with TA in the coating shell, the samples changed their color from light brown to pink attributed to formation of iron tannate at the oil/water interface. The intensity of pink color was in positive relationship with the added amount of iron salt (Figure 3b-e). The results of the oxidative stability study are shown in Figure 7. It can be seen that TBARS formation in emulsion of linseed oil encapsulated in multilayer shell with 1 sandwiched layer of TA was affected during 15 days of storage at 37 °C at the aqueous phase concentration of $FeBr_2$ not higher than 0.3 mM. In three millimolar $FeBr_2$ solution, oxidative degradation of the encapsulated oil became sufficient as determined by TBARS assay (Figure 7). At the same concentration of $FeBr_2$, a high level of MDA was detected in the BSA/PARG/TA/PARG/TA/PARG encapsulated emulsion comprising 2 layers of the antioxidant in its coating shell. Taking into account the data in Table 1 for 0.625 % v/v emulsion, which was used to test iron-catalyzed oxidation, we conclude that polyelectrolyte interfacial membrane with TA layer(s) sandwiched between PARG layers inhibited peroxidation of encapsulated lipids, if the total concentration of antioxidant in the sample was not below the concentration of Fe^{2+} cations in the aqueous phase. However, 1.5 times overall excess of TA in the sample ($C_{[TA]}$:$C_{[Fe^{2+}]}$ = 1.5:1) was already sufficient for effective elimination of Fe^{2+} cations by the coating shell, so that they could not initiate the oxidative process. The ratio of antioxidants to pro-oxidants $C_{[TA]}$:$C_{[Fe^{2+}]}$ = 1:2.4 achieved in the case of BSA/PARG/TA/PARG/TA/PARG encapsulated emulsion dispersed in 3 mM $FeBr_2$ possibly resulted in a certain amount of Fe^{2+} cations not entrapped by the TA layers. Hence, oxidizing species could catalyze oxidative degradation of oil core penetrating through the multilayers from the aqueous bulk. The obtained values suggest that TA and Fe^{2+} have to be taken in approximately 1:1 ratio for total scavenging of Fe^{2+} cations by sandwiched TA layer(s) and prevention of lipid peroxidation in emulsified oil.

Oxidative Stability of Encapsulated Linseed Oil Depending on Antioxidant Type and Location. Antioxidant activity of TA incorporated into the droplet's shell was compared with that of MT admixed into the oil phase prior to multilayer encapsulation. Actually, the

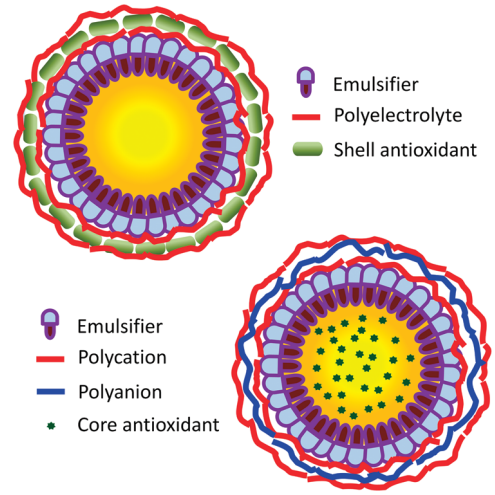

FIGURE 8. Schematic representation of encapsulated oil droplets protected by shell antioxidant (top) and core antioxidant (bottom).

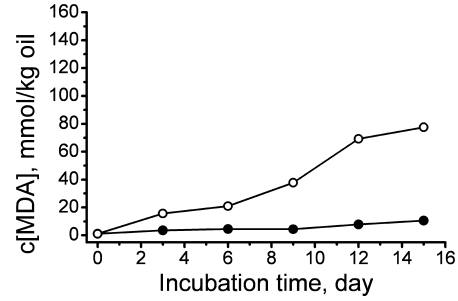

FIGURE 9. Antioxidant activity of mixed tocopherols dispersed in oil core of BSA/PARG/DS/PARG encapsulated emulsion in H_2O (—•—), and in 0.03 mM $FeBr_2$ (—○—). Error bars are overshadowed by the points' symbols.

comparison was made between two different pathways to affect oxidative process, i.e., preventive mechanism attributed to TA intercepting oxidizing species at the oil/water interface, and chain-breaking mechanism (41, 43) of MT targeting peroxyl radicals formed in oxidative process inside the oil core. (Schematic representation of encapsulated oil droplets comprising either shell or core antioxidant is shown in Figure 8) In contrast to TA, tochpherols don't react with Fe^{2+}. However, in a single oil droplet, the number of TA molecules at the interface required to scavenge iron from the water medium must not be considerably different from the number of MT molecules necessary to break the peroxidation chain reactions initiated by transition metal (at the same initial concentration of Fe^{2+} in each sample). Thus, the amount of each antioxidant per volume of dispersed phase was the most crucial parameter to consider the difference in the oxidative stability of emulsified oil.

To discover the impact of antioxidant type and location, MDA formation in BSA/PARG/DS/PARG coated emulsion with 10 000 ppm MT-containing oil cores was examined. The encapsulated oil droplets (1.25 % v/v) were dispersed in 10 mL of either pure water or 0.03 mM $FeBr_2$ solution and monitored over 15 days of storage at 37 °C. The results of the study are presented in Figure 9. Lipid peroxidation in water dispersed BSA/PARG/DS/PARG encapsulated oil droplets proceeded much slower in the presence of MT in the oil phase, although it was not totally suppressed like in case of

emulsion encapsulated in BSA/PARG/TA/PARG shell (Figures 6 and 9). Furthermore, MT-protected emulsion displayed high level of MDA concentration increasing with storage time already at 0.03 mM $FeBr_2$ in the aqueous phase. According to the data in Table 1, the amount of TA per volume of dispersed phase in BSA/PARG/TA/PARG encapsulated emulsion was of the same order of magnitude but slightly higher than that of MT. This fact might elucidate better antioxidant property of TA-protected oil droplets in water. However, the oxidative stability of TA-encapsulated emulsion in both 0.03 mM $FeBr_2$ and 0.3 mM $FeBr_2$ solutions (Figure 7) suggests antioxidant activity of TA-containing coating shell would still be sufficiently higher than that of MT dissolved in oil core of emulsion particles at comparable number of antioxidant molecules associated with dispersed phase. Hence, forming the pro-oxidants' scavenging coating shell at the oil/water interface appears to be an advantageous strategy to protect emulsified oil against oxidation rather than adding the chain-breaking antioxidant into the oil core.

CONCLUSION

In this paper, we demonstrated the effective protection of emulsified linseed oil against lipid peroxidation based on encapsulation of oil droplets inside antioxidant containing polyelectrolyte multilayer shell. Encapsulation was performed by means of LbL assembly of multilayer coating on aqueous dispersed oil cores preliminary stabilized with protein ionic emulsifier. Protection of encapsulated oil against oxidative degradation was attributed to antioxidant properties of tannic acid used as shell constituent in alternation with poly-L-arginine. The water dispersed emulsion encapsulated in multilayer shell comprising tannic acid did not oxidize over 15 days of storage at 37 °C and remained intact in solution of pro-oxidant Fe^{2+} added in concentration 10 times exceeding the physiological concentration of iron in human blood serum. The tannic acid-containing shell scavenged pro-oxidant from the surrounding medium, thus preventing initiation of the chain reaction of lipid peroxidation in the oil core. This mechanism of antioxidant activity was found to be advantageous to preserve the intact form of polyunsaturated fatty acids compare to action of chain-breaking antioxidant in the oil phase as shown by the example of mixed tocopherols. For instance, the emulsion of linseed oil having a layer of tannic acid at the oil/water interface demonstrated considerably better stability to oxidation in the presence of Fe^{2+} than emulsion encapsulated into antioxidant-free biocompatible shell with antioxidant mixed tocopherols located in the oil core.

The observed prominent improvement of the oxidative stability in the presence of a metal-scavenging compound supports the idea of transition metal cations being the most important pro-oxidants in oil-in-water emulsions. Similar effect can be expected of other chelates or metal-binding proteins capable to entrap metals at the interface preventing their further penetration inside oil core.

Together with better stability to oxidation, the emulsion droplets encapsulated in polyelectrolyte shell comprising a layer of tannic acid were stable to coalescence and flocculation in aqueous conditions. Shell integrity was not affected over freeze-drying of coated emulsion droplets.

The main benefit of the demonstrated approach for prevention of lipid peroxidation in emulsified oil comes from a direct tailoring of preventive antioxidant molecules to emulsion droplets' interface. So that, the antioxidant activity of the shell's compound is not affected by the physical and chemical factors of the surrounding medium. Furthermore, the protection provided by the antioxidant in the shell can be sufficient on its own and not requiring extra antioxidant compounds added either to the water or oil phase. It facilitates the development of formulation over a broader range of practical use that includes food, consumer care, and pharmaceutical related products.

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