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Grafting phenolics onto milk protein via conjugated polymerization for delivery of multiple functionalities: Synthesis and characterization

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ABSTRACT

A synthetic scenario for functionalization of β-lactoglobulin (βLg) with polymeric units containing caffeic acid (βLg-polyCA) was developed; and all intermediates and final products were structurally confirmed using nuclear magnetic resonance spectroscopy, matrix assisted laser desorption ionization time-of-flight mass spectrometry, and physico-chemically characterized using differential scanning calorimetry and circular dichroism. The antioxidant properties and emulsion stability of βLg, βLg-CA conjugate and βLg-polyCA based systems containing high percentage of fish oil (50%) were evaluated; and βLg-polyCA presented the highest antioxidant and free radical-scavenging activity based on DPPH, ABTS and HS scavenging assays (92.4, 87.92 and 67.35% respectively). Thiobarbituric acid (TBARS) test demonstrated that compared to native βLg, βLg-polyCA afford up 4–5 fold of inhibition of oxidative rancidity and displayed drastic secondary structure changes. Compared to native βLg based emulsions, βLg-polyCA had larger oil droplet sizes, stronger negative zeta potentials (−69.9 mv), narrower size distributions (PDI: 0.22) and less creaming index.

1. Introduction

Oil-in water emulsions are commonly used in agro-based industries for delivery of hydrophobic components including; vitamins, antioxidants, phytosterols, and essential fatty acids (n-3 PUFA) from fish oil (Wang, Li, Zeng, & Liu, 2008; Kargar, Spyropoulos, & Norton, 2011). Lipid oxidation in n-3 PUFA enriched o/w emulsions is a major hazard leading to developments of off-flavors, and potential toxic substances (Choe & Min, 2009; Eltweri et al., 2017). Synthetic antioxidants are widely used to inhibit lipid oxidation in n-3 PUFA emulsions. Natural antioxidants such as phenolic acids (Shan, Cai, Sun, & Corke, 2005) are now in consumer preferences to replace synthetic antioxidants. However, the efficacy of natural antioxidants depends on their chemical properties (Van Acker et al., 1996) and surrounding conditions in line with the polar paradox theory (Alvarado, Wang, & Moradi, 2011). It has been rationalized that, nonpolar antioxidants are more effective in emulsion systems while their polar counterparts are more effective in bulk oils (Lucas et al., 2010). Therefore, extending applications of natural antioxidants to complex oil-water systems, pharmaceuticals and cosmetics through chemical/enzymatic modification is desired (Ramadan-Hassanien, 2008; Laguerre et al., 2010).

The extension of the functionality of macromolecules for multipurpose applications has gained interest. Hence, the assembly of molecules bearing different functional components is the target of many studies. For instance, α-lactoalbumin was conjugated with 2 catechin (phenolic compound) units by Yi, Fan, Zhang, and Zhao (2016) for delivery of β-carotene in nanoemulsions. Another study grafted nine units of caffeic acid into β-lactoglobulin (βLg) for fish oil delivery in 30% fish oil emulsions (El-Maksoud et al., 2018). No study is available on the use of βLg-phenolic conjugates as antioxidant stabilizers in high load fish oil emulsions (≥50% fish oil). Enrichments of food products will be more effective using highly concentrated emulsions. However, the high percentage of fish oil and large o/w interface demands more protection from oxidation. Thus, incorporation of more antioxidant

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moieties into natural stabilizers or emulsifiers could deliver enhanced protection to sensitive ingredients in high load delivery systems. β-Lactoglobulin (βLg) is a globular protein with well-known emulsification tendencies (Liu, Chen, & Mao, 2007; Katsuda, McClements, Miglioranza, & Decker, 2008; Zimmerer & Jones, 2014; Oliveira, Ruiz-Henestrosa, von Staszewski, Pilosof, & Pintado, 2015). However, unmodified βLg is not a strong antioxidant to prevent peroxidation of sensitive oils.

We hypothesize that grafting multiple units of caffeic acid onto βLg will offer superior protection of sensitive fish oils in high load emulsions. This concept was realized using simple conjugation techniques, which are well proven for enhancing the activity and functionality of biological molecules (Bontempo, Heredia, Fish, & Maynard, 2004). Specifically, atom transfer radical polymerization (ATRP) (Le Droumaguet & Velonia, 2008; Zdyrko & Luzinov, 2011) allowed coupling of caffeic acid homopolymers onto reactive sites of βLg. Tetra ethylene glycol (TEG) was used as a linker between caffeic acid units and βLg due to its low toxicity, biocompatibility and reduced immogenicity (Mantovani et al., 2005; Losso & Nakai, 2002; Krishnan & Srinivasan, 2003).

HO-TEG-Br [2(2(2(2-Hydroxyethoxy) ethoxy) ethoxy)-ethyl 2-bromo-2-methylpropanoate] was first synthesized by chemically coupling tetraethylene glycol (TEG) with α-Br-isobutyryl bromide. Succinimidyl-TEG-Br (1-(2,5-Dioxopyrrolidin-1-yloxy)-1-oxo-2,5,8,11-tetraoxatridecan-13-yl-α-bromo-2-isobutyrrate) was obtained by a further coupling of HO-TEG-Br with *N,N′*-Disuccinimidyl carbonate. The resulting Succinamide-TEG-Br was bio-conjugated onto βLg. Further co-polymerization with a synthetic phenolic-containing monomer [2-Hydroxyethyl methacrylate (HEMA) caffeic acid (CA), 2-((3-(3,4-dihydroxyphenyl)acryloyl) oxy)ethyl methacrylate)] via ATRP polymerization yielded the final product βLg-polyCA (Scheme 1). Thus, caffeic acid grafted as multi-copies in HEMA-polymers were attached to βLg protein scaffold using TEG as a linker.

Structural confirmation was by use of nuclear magnetic resonance (NMR), matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS), Fourier transform infrared (FTIR). Physico-chemical characterization was by using differential scanning calorimetry (DSC) and circular dichroism (CD). Antioxidant properties were assessed by 2,2-Diphenyl-1-picryl-hydrazil (DPPH) radical scavenging, hydroxyl radical scavenging and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) activities. Emulsification and prevention of lipid peroxidation in 20–50% fish oil delivery emulsions were determined by dynamic light scattering studies and thiobarbituric reactive substances (TBARS assay) respectively. This study has demonstrated that segments from nature can be grafted onto bio-macromolecules for improved functionality, by simple synthesis scenarios.

2. Experimental section

2.1. Materials

Chemicals used in this study were of analytical purity (> 99%) obtained from Sigma Aldrich (St Louis, Missouri). Water used for the polymerizations was of Milli-Q water grade. Milk protein (β-lactoglobulin) was obtained from Arla Foods, Denmark. Psyllium husk (*Plantago ovate*) was purchased from local market Denmark. Psyllium husk, (composed of hemicellulose (Fischer et al., 2004), was used as adjunct thickener and stabilizer (Verma & Mogra, 2013) in fish oil emulsions.

2.2. Methods

2.2.1. Synthesis and chemical characterization of polymer

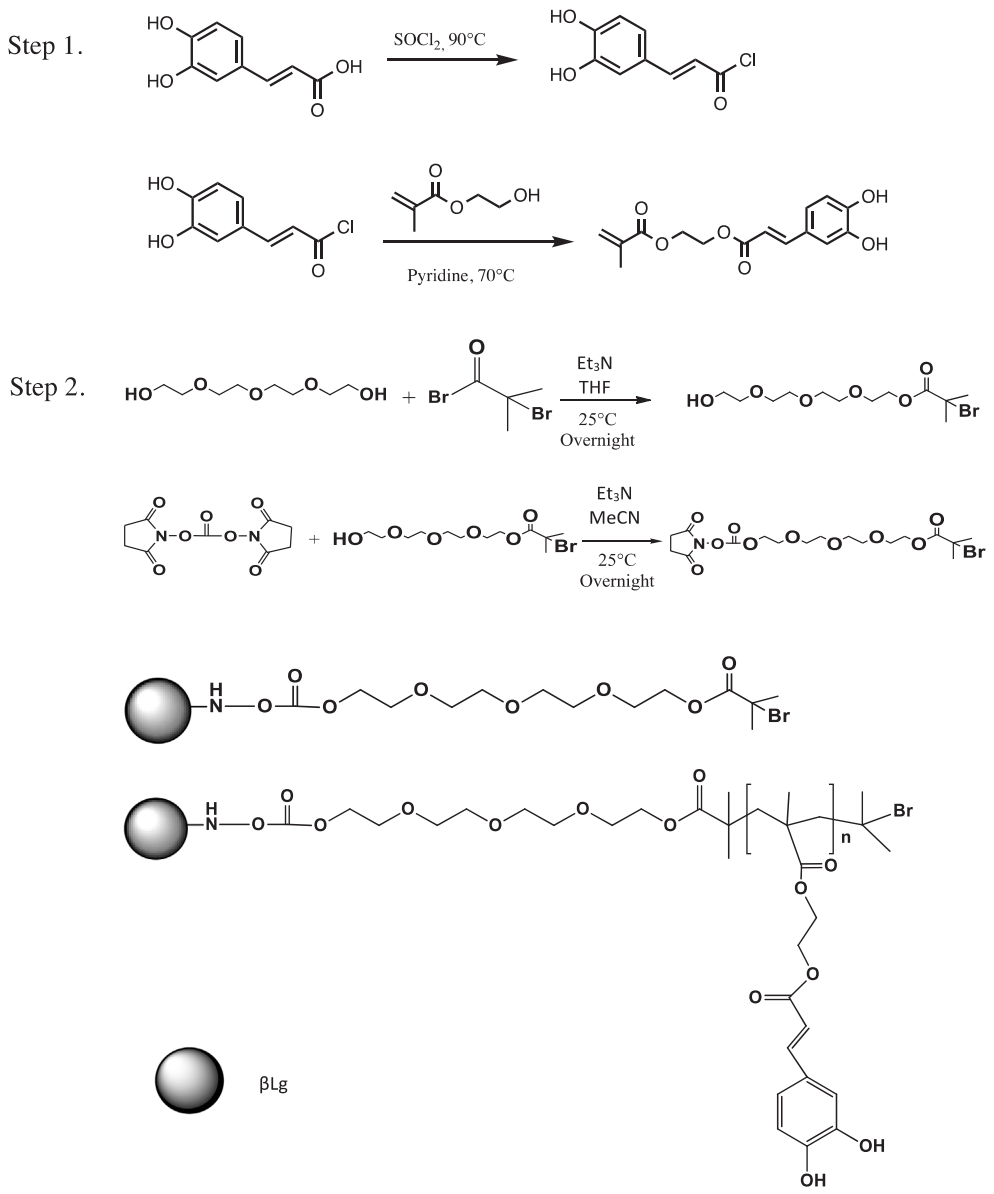
2.2.1.1. Synthesis of 2-((3-(3,4-dihydroxyphenyl)acryloyl)oxy)ethyl methacrylate, HEMA-CA ($C_{15}H_{16}O_6$). Caffeoyl chloride was synthesized via a two step reaction based on a previously reported method with some modification (Scheme 1) (Anankanbil, Pérez, Yang, Banerjee, & Guo, 2017). 2-Hydroxyethyl methacrylate (HEMA) (1:2 mol ratio, HEMA:caffeic chloride) was added to cooled caffeoyl chloride, the reaction mixture heated to 70 °C and kept at that temperature for 4 h while stirring at 360 rpm. Unreacted caffeoyl chloride was removed by washing with diethyl ether three times. The obtained yellow powder was characterized by electrospray ionization mass spectrometry (ESI-MS) and ^{1}H-nuclear magnetic resonance (NMR). The MS spectra of HEMA-CA gave a molecular ion at *m/z* (M + Na) requires 315.0792 *m/z*, found 315.0862 (Supporting information Fig. S1). ^{1}H NMR approved the presence of HEMA-CA after reaction (Supporting information Fig. S2).

2.2.1.2. Synthesis of 2(2(2(2-Hydroxyethoxy) ethoxy) ethoxy)-ethyl 2-bromo-2-methylpropanoate, HO-TEG-Br. TEG-Br was prepared as described before (Magnusson, Bersani, Salmaso, Alexander, & Caliceti, 2010). Triethylamine (5.7 g, 0.05 mol) and tetra ethylene glycol (TEG, 100 g, 0.05 mol) in 100 ml of tetrahydrofuran (THF) were dissolved at −2 °C. α-Br-isobutyryl was added slowly and the reaction continued for 1 h under constant stirring and cooling. The reaction temperature was raised to room temperature and continued under nitrogen atmosphere overnight. Solvents evaporated after filtration of the reaction mixture. The crude product was dissolved in water and extracted with dicholomethane (150 ml). Anhydrous sodium sulfate was added to the dicloromethane extract. After filtration, the solvent was concentrated and the crude product purified by column chromatography. Diethyl ether, methanol (90:10, v/v) was used as developing solvent. The final product was an oily liquid (denoted as TEG-Br) ($C_{12}H_{23}BrO_6$, Mw, 342.07). HR-MS (ESI-TOF). $C_{12}H_{23}BrO_6$ (M + NH_4) requires 360.1038 *m/z* and found 360.1035 *m/z* (Fig. S3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.07–1.10 (t, J_H = 4 MHz), 1.77 (m), 1.87 (m), 3.43–3.59 (m), 3.93–3.97 (m), (D, J = 8, 1.31H), 4.14–4.17 (m) (Fig. S4).

2.2.1.3. Synthesis of succinimidyl-TEG-Br (1-(2,5-dioxopyrrolidin-1-yloxy)-1-oxo-2,5,8,11-tetraoxatridecan-13-yl-α-bromo-2-isobutyrrate). HO-TEG-Br (2.45 g, 7.1 mmol) was added to 5 ml acetonitrile followed by additions of *N,N′*-Disuccinimidyl carbonate (2.16 g, 8.5 mmol) and Et_3N (0.87 g, 8.5 mmol). The reaction was stirred overnight at room temperature (Scheme 1). After the reaction was deemed complete, the solvent was evaporated and the residue re-dissolved in chloroform (150 ml). The concentrated crude product was purified by column chromatography using diethyl ether:methanol (90:10, v/v) as developing solvent (Magnusson et al., 2010). The product (Sc-TEG-Br) resulted as a yellow oil. HR-MS (ESI-TOF). $C_{17}H_{26}BrNO_{10}$ (M + NH_4) requires 561.1030 *m/z* and found 516.2327 (Fig. S5). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81–0.84 (t, J_{HZ} = 4 MHz), 1.17–1.26 (m), 1.88 (s), 2.78 (s), 3.53–3.69 (m), 3.92 (m), 4.03–4.08 (m), 4.26–4.28 (m) (Fig. S6).

2.2.1.4. Conjugation of succinamide-TEG-Br to βLg. About 100 mg of β-lactoglobulin (βLg, 0.0054 mmol) was dissolved in 50 ml of 0.1 M phosphate buffer (pH 7.0). Sc-TEG-Br (39.45 mg, 0.081 mmol) in 1 ml of DMSO was added stepwise and the mixture stirred for 90 min at 20 °C. Dialysis was performed on the mixture for 72 h at 4 °C. Sodium azide (0.02%) was used to prevent microbial growth. Lysophilization of the resulting dialyzed product gave βLg-TEG-Br.

2.2.1.5. Synthesis of βLg-polyCA via ATRP and βLg-CA. The obtained βLg-TEG-Br (50 mg, 0.0026 mmol) was added into 5 ml of 50 mM phosphate buffer (pH 7.5). HEMA-CA (61.35 mg, 0.21 mmol), $CuBr_2$ (46.92 mg, 0.21 mmol), and TPMA (61.00 mg, 0.21 mmol) were added to the mixture, which was maintained at 4 °C under nitrogen atmosphere for 20 min. Polymerization was initiated using Ascorbic acid (4.63 mg, 0.0075 mmol) and continued for 12 h (Magnusson et al., 2010). Sodium azide (0.02%) was used to prevent microbial growth during a 72 h dialysis of crude samples. Samples were freeze dried to generate βLg-polyCA conjugate (Scheme 1). βLg-CA has been



Scheme 1. The synthetic pathways of Step 1: 2-((3-(3,4-dihydroxyphenyl)acryloyl)oxy)ethyl methacrylate, HEMA-CA; and Step 2: 2(2(2(2-Hydroxyethoxy)ethoxy) ethoxy)-ethyl 2-bromo-2-methylpropanoate, and (1-(2,5-Dioxopyrrolidin-1-yloxy)-1-oxo-2,5,8,11-tetraoxatridecan-13-yl-α-bromo-2-isobutyrrate), and grafting caffeic acid to βLg via ATRP initiated polymerization of HEMA-CA.

synthesized according to our previous study (El-Maksoud et al., 2018).

2.2.2. Molecular weight determination of conjugates by use of MALDI-TOF-MS

MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight-Mass Spectrometry) was used to determine the molecular weight of conjugates. About 1–5 µl of lyophilized proteins in 2 µl trichloroacetic acid (2% TCA) was added to 2 µl 2, 5-dihydroxyacetophenone (0.1 M in 20 mM ammonium dihydrogen citrate and 75% (v/v) EtOH) (Wenzel, Sparbier, Mieruch, & Kostrzewa, 2006). One microliter of the resulting solution was spotted to dry on a stainless steel. Spectra were acquired in positive and linear modes by use of an Auto Flex Smart beam III instrument (Bruker) (Peptide calibration standard I; Bruker Daltronics). Centroid masses were analyzed using GPMAW software (gpmaw.com).

2.2.3. Fourier transform infrared (FTIR) spectroscopic measurements

FTIR spectra in the range 3500–600 cm^{-1} were scanned for all samples at room temperature on an attenuated total reflection FTIR spectrophotometer (QFA Flex, USA). 10 scans were acquired for samples at a resolution of 4 cm^{-1}.

2.2.4. Differential scanning calorimetry (DSC) analysis

DSC scans were made using Pyris 6 DEC equipment (PerkinElmer, USA). Approximately 4–6 mg of samples were introduced into empty aluminum pans and sealed. The system was purged with nitrogen before analysis. Heating was from 20 °C to 350 °C at 10 °C min^{-1}.

2.2.5. Circular dichroism spectroscopy (CD) analysis

CD spectra from 190 nm to 260 nm were acquired at 25–95 °C. The concentrations of samples were 0.2–0.12 mg/ml.

2.2.6. Antioxidant capacity assay

DPPH (2,2-Diphenyl-1-picryl-hydrazil) assay was performed as described before (Wang et al., 2008). Two milliliters of sample solutions and 2 ml of DPPH solutions were mixed and incubated in the dark at room temperature for 30 min. Sample absorbances at 517 nm were read on a UV/Vis spectrophotometer (Varian, Cary 50 Bio). DPPH free radical scavenging activity was calculated as:

$$\%\ Radical\ scavenging\ percentage\ =\ [(A0\ -\ A1)/\ A0] \times 100 \quad (1)$$

where A0 = the absorbance of blank without test samples. A1 = absorption of test samples.

2.2.7. 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) ABTS scavenging assay

A modified version of the method by Li et al. (2015) was used. ABTS di-ammonium salt (0.35 ml, 7.4 mmol/L) was mixed with 0.35 ml of potassium persulfate (2.6 mmol/L) to generate the ABTS radical. Incubation in the dark for 12 h allowed complete generation of the radical. Further dilution with 95% ethanol was necessary to keep the absorbance reading at 734 nm in the range of 0.05–0.70. The ABTS solution (3.9 ml) was mixed with 100 µl of test samples and the absorbance at 734 nm using ethanol as blank. The percentage inhibition was determined as:

$$Inhibition\ \%\ =\ [(A0\ --\ A)/A0)] \times 100 \quad (2)$$

where A0 is the absorbance at 734 nm of the control, A is the absorbance at 734 nm of the mixture with the samples.

2.2.8. Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity (%HA) of samples was determined using a procedure described by Li et al. (2012). One milliliter of protein solutions (1 mg/ml) was mixed with 1 ml of 9×10^{-3} M $FeSO_4$, 1 ml of 8.8×10^{-3} M H_2O_2 and 1 ml of 9×10^{-3} M salicylic acid-ethanol solution in that sequence with vortexing in-between. Mixtures were incubated for 1 h at 37 °C, diluted where necessary and the absorbance at 510 nm read using a UV/Vis spectrophotometer (Varian-Cary-50 Bio, USA). %HA was calculated as:

$$\%HA = x = \frac{A0 - (Ax - Ax0)}{A0} \times 100 \quad (3)$$

where %HA is the hydroxyl radical scavenging activity of samples, A_0 is the absorbance of equal volumes (1 ml) of distilled water, $FeSO_4$, H_2O_2, and salicylic acid-ethanol solution. A_x is the absorbance of mixtures containing sample solutions without distilled water. A_{x0} is the absorbance of sample solutions without salicylic acid-ethanol solution, respectively.

2.2.9. Application of βLg and βLg-polyCA in oil-in-water emulsions

2.2.9.1. Formulation of delivery emulsions. Emulsions enriched with fish oil (20 or 50% fish oil) were formulated using 3.0% (w/w) βLg-polyCA, 0.5% (w/w) pysllium husk powder, 0.02% (w/w) sodium azide, and aqueous phase. Homogenization was carried out by use of an Ultra-Turrax T25 homogenizer (IKA Instruments, Stanten in Breigan, Germany) for 2 min at 1000 rpm.

2.2.9.2. Emulsion characterization. Emulsion properties (*zeta potential and droplet size*) of βLg-polyCA were assessed by dynamic light scattering studies using non-invasive backscatter optics. Emulsions were diluted (1:1000) in Milli Q water before measuring. No destabilization occurred. Zeta potential and size of oil droplets were determined in triplicates.

2.2.9.3. Creaming index (CI) of emulsions. Emulsions stabilized by βLg, βLg-CA and βLg-polyCA were transferred to clean and clear 15 ml vials. Samples were screw capped and stored under refrigeration conditions. CI was monitored over a period of 7 days according to the equation below (El-Maksoud et al., 2018):

$$CI\ =\ (Ha/He)\ \times\ 100 \quad (4)$$

where Ha is the height of the lower aqueous phase and He is the total height of emulsion. Determinations were in triplicates.

2.2.9.4. Imaging of emulsion droplets by Wide field florescence microscopy (WFM). A WFM was used to assess microstructures and distributions of native βLg, βLg-CA and βLg-polyCA in emulsions. Nile red was used to stain oil droplets in emulsions. Fast green was used to tag proteins. Excitation of Nile red was at 488 nm. Emission light of Nile red was collected at 570–640 nm. Fast green was excited using a fluorescence lamp (667/30 band pass filter) and emission spectra was collected using 720/60 band pass filter.

2.2.9.5. Oxidative stability in emulsions. Thiobarbituric acid reactive species (TBARS) as described by Falkeborg and Guo (2015) was used to determine lipid oxidation in emulsions. Peroxidation of lipids was started by addition of 250 µl of 25 mM $FeSO_4$ to emulsions. Emulsions containing iron were stirred at 200 rpm for 15 min at room temperature. To 2 ml of TBARS reagent, 1 ml emulsions were added in test tubes and boiled for 15 min. The test tubes were cooled to room temperature in 5 min, and centrifuged at 20 °C and 4000 rpm for 2 min. The absorbance at 532 nm was read using UV visible spectrophotometer (Cary 50Bio, Varian, Australia). The percentage oxidation in emulsions was calculated as follows:

$$Oxidation\% = As/A0*100 \quad (5)$$

where A_S is the absorbance of samples with addition of βLg, βLg-CA or βLg-polyCA and A_0 is the absorbance of Tween 20 as a control.

2.2.9.6. Data analysis. One-way analysis of variance (ANOVA) was performed using SPSS 11.5 (SPSS Inc, Chicago, IL, USA) at 0.05 significance level.

3. Results and discussions

3.1. Synthesis of βLg-PolyCA

Graft copolymerization is used to modify polymers' surface, and their physico-chemical properties. In this study emphasis is given to protein-polymer conjugates prepared with carbamate cross linker originated from βLg with a modified moiety of chain initiator. Grafting was achieved by creating reactive sites on the backbone (Tetra ethylene glycol) of the substrate polymer, which allowed attachments of grafted moieties on the polymer backbone. Creation of active sites for initiation of the polymerization was achieved through attaching a free radical initiator (isobutyryl bromide) via acylation. Free radicals were produced from ascorbic acid (initiator) and transferred to the substrate (βLg-TEG-Br) facilitating the polymerization reaction with HEMA-CA to form the βLg copolymer grafted with caffeic moiety (Scheme 1).

The ATRP initiator linker (succinimidyl-TEG-Br) reacted with βLg at pH 7 (Scheme 1) to allow conjugation at amine and lysine residues on βLg (15 lysine residues according to propka analysis of the cystal structure (pdb code: 2Q2M), and the N-terminus. Excess of succinimidyl-TEG-Br permitted conjugation of all amino acid residues compared to βLg. Succinimidyl carbonate was used as coupling reagent to conjugate TEG-Br molecules with the accessible primary amino groups. βLg-TEG-Br was regarded as substance to grow the polymerization reaction using HEMA-CA as monomer. Carboxylic group of CA was activated by thionyl chloride treatment and coupled with HEMA to yield HEMA-CA (Lu, Lin, & Yao, 2004; Bas, Rogers, & Jensen, 2008), which was used as monomer to graft caffeic acids through polymerization with βLg-TEG-Br (Scheme 1).

MALDI-TOF spectra of native βLg, conjugated βLg with succinimidyl-TEG-Br and βLg-polyCA after polymerization are shown in Fig. 1

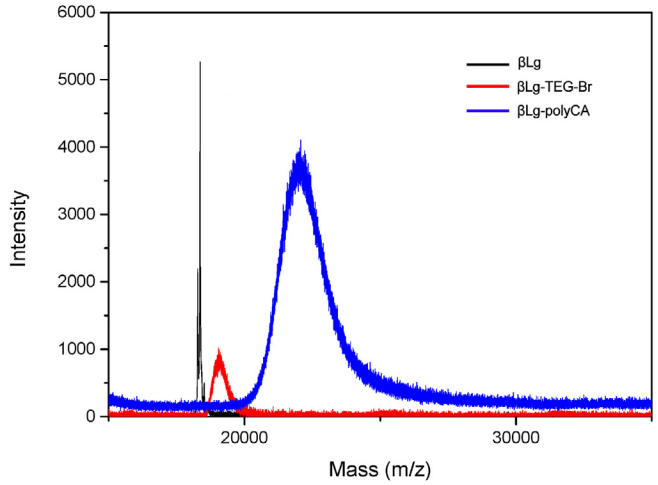


Fig. 1. Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) of βLg, βLg-TEG-Br and βLg-polyCA.

(18,366, 19,039 and 22,070 Da, respectively) revealing that the linker conjugation yielded up to 5 units of initiator (succinimidyl-TEG-Br) and up to 28 units of HEMA-CA monomers in each βLg (Fig. 1). An ^{1}H NMR spectra (400 MHz Bruker) of βLg and βLg-polyCA indicated saturated protons in the aliphatic region showing the presence of poly CA. Protons next to oxygen in TEG were within 3.00–4.00 ppm. Methacrylate unit protons were present around 4.50 ppm while multiplicities from 5.00 to 7.50 ppm indicate the presence of aromatic units (See supporting information Fig. S7).

3.2. Fourier transform infrared spectroscopy (FT-IR)

The functional groups in βLg and βLg-polyCA were characterized by FTIR (Fig. S8). FTIR spectra of βlg-polyCA displayed a signal at 1257 cm^{-1}, and 2034 cm^{-1}attributed to the carboxylic group C═O bending and benzene ring of CA. In addition, there is a new sharp band around 1139 cm^{-1} and a broad band around 3250 cm^{-1}, which are related to the phenol hydroxyl groups of CA (Chen et al., 2010). The ester bond that formed from linking HEMA to isobutyryl bromide of the TEG branch appeared at 1720 cm^{-1}.

Furthermore, FTIR spectra showed a sharp signal at 1143 cm^{-1} and a broad band around at 3275 cm^{-1} for OH group and H_2O (Mauricio-Sánchez, Salazar, Luna-Bárcenas, & Mendoza-Galván, 2018), two split peaks were observed around 2050 and 2200 cm^{-1} for benzene rings of CA grafted to βLg via co-polymerization with HEMA-CA. The grafting of the polymer was noted by the appearance of characteristic peak at 1720 cm^{-1}, which is from the stretching vibration of the ester groups (Chen et al., 2010).

3.3. Differential scanning calorimetry

DSC was used to study the thermal stability of βLg and βLg-polyCA (Konieczny et al., 2016) (Fig S9). The endothermic peak started at 50 °C for both βLg and βLg-polyCA, while the endothermal denaturation peak started around 108.9 and 157.5 °C in the cases of βLg and βLg-polyCA, respectively. This may suggest that grafting of phenolic acids into proteins could alter the thermal properties of proteins (El-Maksoud et al., 2018; Labuckas, Maestri, Perello, Martínez, & Lamarque, 2008). Also, multiple peaks between 245 and 350 °C were suggestive of the degradation of native βLg, but no peaks between 245 and 350 °C were observed for βLg-polyCA. In summary, chemical conjugation of βLg

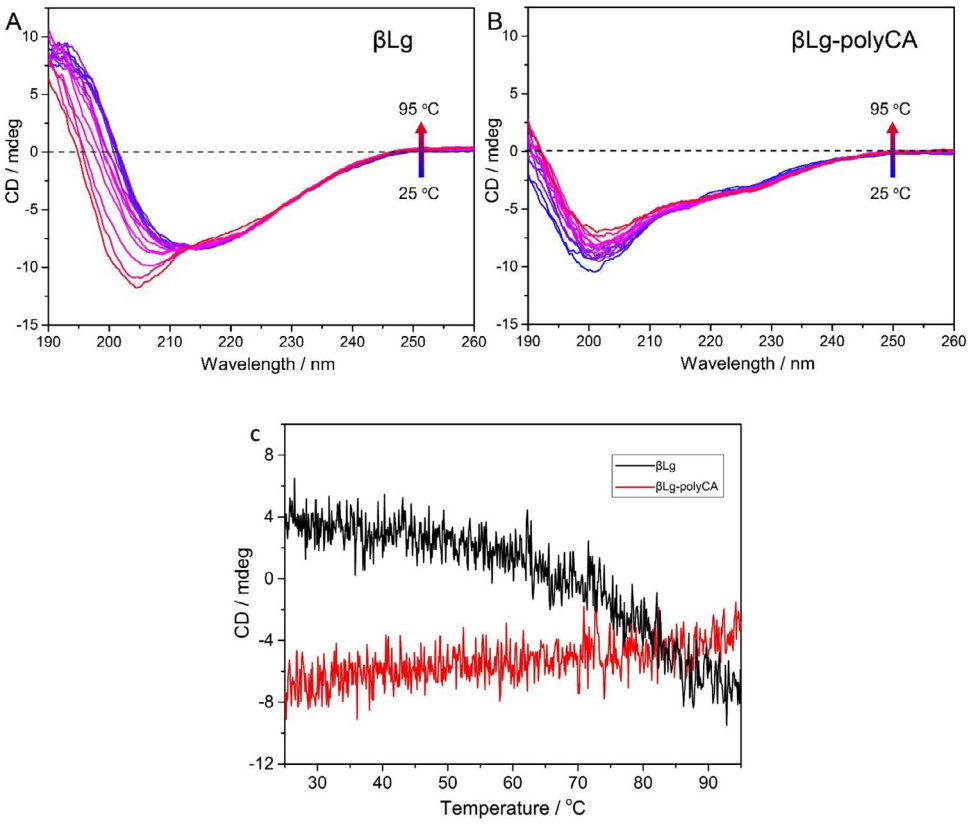


Fig. 2. Circular dichroism spectra of heat stability of βLg (A) and βLg-polyCA (B) at different temperature and (C) the heat scan of βLg and βLg-polyCA from 25 to 95 °C at 198 nm.

with TEG and CA provided greater thermal stability, which is of relevance for application in food processing.

3.4. Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy was performed to determine the secondary structure and thermostability of βLg and βLg-polyCA. As shown in Fig. 2 (A and B), circular dichroism at 222 nm collapsed and new negative peak occurred at ~200 nm when βLg was conjugated, indicative of decreased α-helix domains associated with increased random coil domains.

Thermal unfolding transitions of βLg and βLg-polyCA were monitored by collecting far-UV CD spectrum as a function of temperature. As shown in Fig. 2 (A and B), no change was observed at 222 nm, indicating no change in α-helix structure. Hence ellipticity at 198 nm was constantly recorded as the temperature was elevated (Fig. 2C).

Interestingly, decrease and increase in ellipticity at 198 nm were observed for βLg and βLg-polyCA, respectively, as a function of temperature, indicating opposite thermal unfolding behaviors between βLg and its conjugated counterparts. The thermal denaturation monitored by CD at 198 nm is characterized by a change in ellipticity against temperature in the case of βLg-polyCA, suggestive of the absence of a distinct transition during thermal unfolding.

3.5. Antioxidant properties of βLg, βLg-CA and βLg-polyCA

In this study, different radical scavenging abilities of βLg, βLg-CA and βLg-polyCA in preventing oxidation, was screened using DPPH, ABTS and HS% at different concentrations. As showed in Fig. 3, the antioxidant capacity for the three assays is improved whenever the concentration of samples increased.

The radicals scavenging activity of βLg increased significantly ($p < 0.05$) when coupled with CA. The scavenging of DPPH radical by βLg was 56.22% (1.5 mg/mL). DPPH radical scavenging was significantly increased in the case of βLg-CA (88.31%) and βLg-polyCA (92.40%) at the same concentration. Also, the ABTS radical scavenging of βLg (58.01%) increased when CA was linked to the structure (Fig. 3b). Except for the result at 1.5 mg/ml, ABTS% of βLg-CA (88.53%) and βLg-polyCA (87.92%) were statistically comparable. At concentrations of 0.5 and 1.0 mg/mL, ABTS% of βLg-polyCA was significantly higher than that of βLg-CA (Fig. 3b).

The ability of βLg-polyCA to scavenge hydroxyl radicals was more than doubled compared to βLg, and almost 60% increase of the HS% of βLg-CA at 1.5 mg/mL. βLg-polyCA had the highest scavenging index against different radicals (DPPH, ABTS and HS %). The superior performance of βLg-polyCA as antioxidant compared to βLg and βLg-CA may be due to its high density copy of caffeic acid units grafted onto βLg via the arm linker. The stretching branches of HEMA-CA polymer out of the protein matrix ensured a sufficient exposure for easy access and interaction with surrounding radicals (Liu et al., 2007; Li, Ma, & Ngadi, 2013).

3.6. Characteristics of emulsions stabilized by β-Lactoglobulin and its antioxidant conjugates

3.6.1. Particle size and zeta potential

Table S1 shows the dynamic light scattering results of emulsions stabilized by βLg, βLg-CA and βLg-polyCA. The average sizes of oil droplets ranged from 1.75 to 3.27 μm. In addition, the emulsions made with βLg-polyCA gave larger droplet size than those stabilized by native βLg. With an increased percent of oil in emulsion, the droplet size of oil increased.

The stability of emulsions increased in the case of the βLg-polyCA 50% fish oil. The PDI value for those emulsions was the lowest at 0.22, suggesting good homogeneity (Table S1). The zeta potential of oil droplets in 50% fish oil emulsions stabilized by βLg-polyCA (-69.9 Mv) was greater than the one stabilized by βLg (-56.1 Mv). Results suggest that the former presents a bigger electrostatic repulsion between droplets because of the large zeta potential, and as a consequence, more stable emulsions (Shpigelman, Cohen, & Livney, 2012).

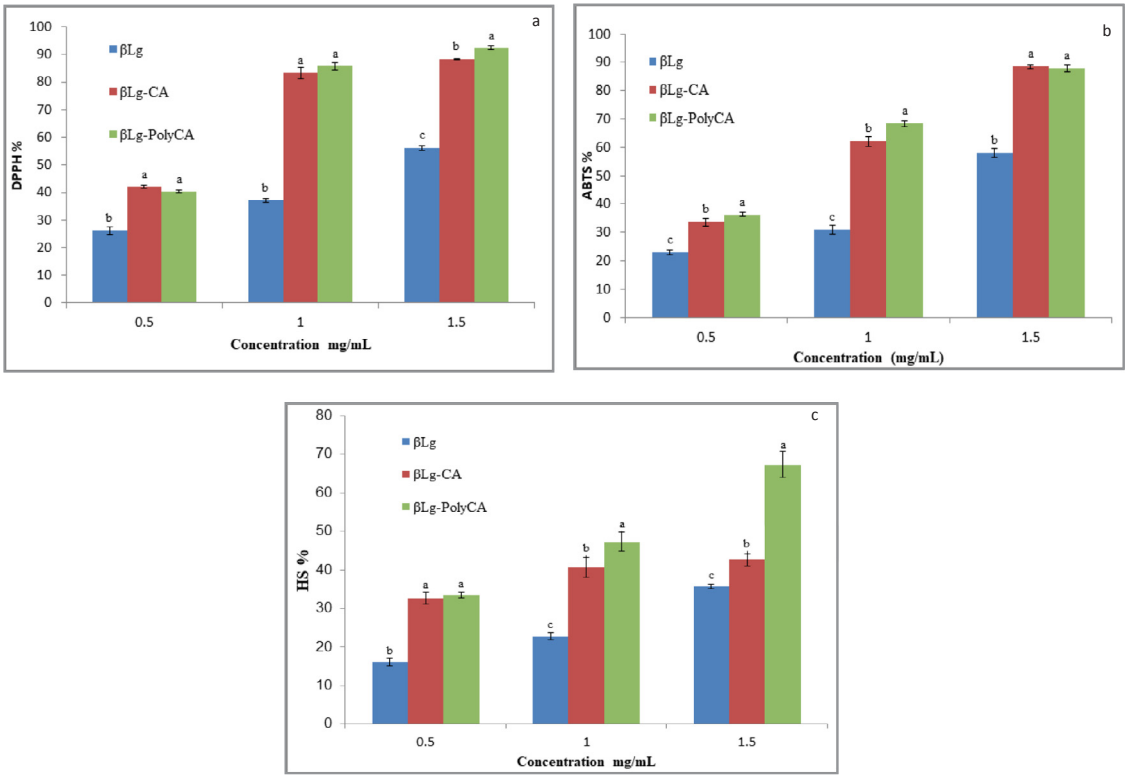


Fig. 3. Antioxidant properties: (a) DPPH free radical scavenging, (b) ABTS scavenging and (c) hydroxyl radical scavenging of tested βLg, βLg-CA and βLg-polyCA.

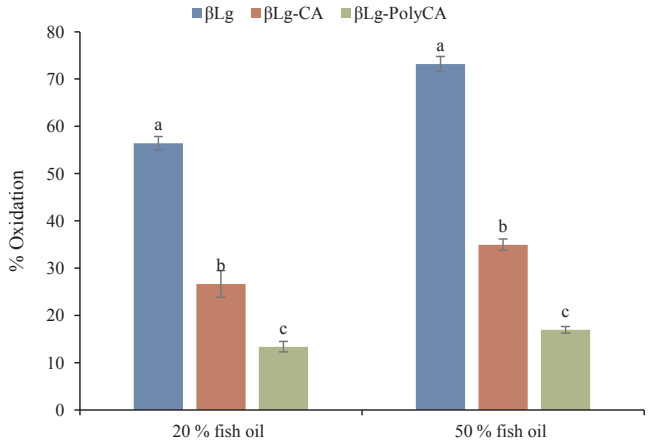


Fig. 4. Comparison of lipid oxidation levels of emulsified fish oils (20% and 50%) prepared using βLg, βLg-CA and βLg-polyCA as an emulsifier were detected using TBARS method (Tween 20 as control, defined as 100% oxidation).

3.6.2. Creaming stability

The creaming index, a parameter correlated with emulsion stability, was determined over time as shown in Table S1. No phase separation was detected in 50% oil-in-water emulsions after four days of storage. However, using βLg-polyCA as emulsifier resulted in a significantly higher emulsion stability (3.5%) than βLg (5.2%) in a 20% oil system, after seven days of storage. Thus, it appears that TEG-poly HEMA-CA grafted on the surface of βLg, stretching out as defensive arms, played a key role in preventing adjacent droplets from aggregation, therefore extending emulsion stability as proved in previous reports (Ye & Singh, 2006). The use of psyllium mucilage, a viscosity modifier (Farahnaky, Askari, Majzoobi, & Mesbahi, 2010), might have played a role in improving emulsion stability. No creaming was observed in 50% fish oil emulsions after the storage period. Perhaps the higher oil volume and presence of psyllium mucilage created a high viscosity, which impeded oil droplets movements.

3.6.3. Prevention of lipid peroxidation in delivery emulsions

The inhibition of lipid oxidation in fish oil delivery emulsions stabilized by βLg, βLg-CA and βLg-polyCA as emulsifiers were detected using TBARS method. Fig. 4 shows that the synthesized stabilizers prevented oxidation of the emulsified fish oil in both low and high loads.

Both βLg-CA and βLg-polyCA showed lower levels of oxidation than unmodified βLg. It is also clear that the βLg-polyCA is shows a stronger inhibition of oxidation of fish oil. At 50% load of fish oil oxidation was reduced by 4.3-fold compared to βLg (El-Maksoud et al., 2018) and around 1.8-fold compared to βLg-CA. βLg-polyCA emulsions also displayed lower levels of oxidation (13.36%) compared to βLg-CA emulsions (26.63%) or βLg (56.41%) in 20% fish oil emulsions. This result provided further evidence that grafting caffeic acid onto through stretching out polymers is a better strategy to inhibit lipid oxidation than direct covalent bonding to protein amino residues as in βLg-CA.

3.6.4. Fluorescence microscopic studies

Wide field fluorescence microscopy permitted determinations of interfacial locations of emulsifiers in emulsions (Fig. 5).

The wide field images suggested that the interfacial activity of βLg was maintained after polymerization with caffeic acid. Thus, the location of caffeic acid units in βLg-polyCA at the oil-water interface was responsible for its ability to inhibit lipid oxidation in emulsions. Similar results were reported in other studies (Anankanbil et al., 2017; El-Maksoud et al., 2018).

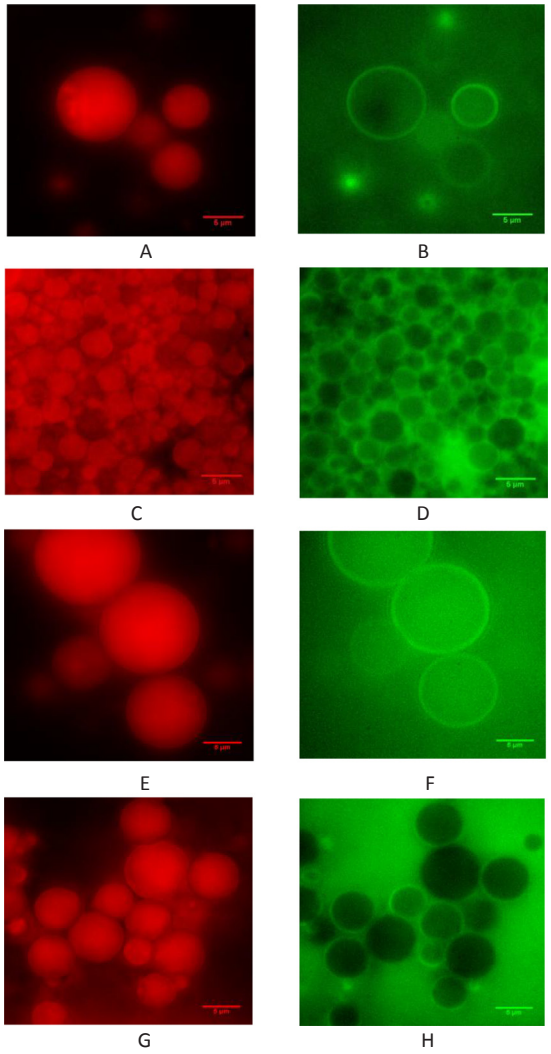


Fig. 5. Wide field fluorescence microscope images of emulsions (O/W) with native βLg at 20% fish oil (A and B) and 50% fish oil (C and D), and βLg-polyCA at 20% fish oil (E and F) and 50% fish oil (G and H) as emulsifier with nile red for oil phase imaging (A, C, E and G) and fast green for protein phase (B, D, F and H).

4. Conclusion

We hypothesized that atom transfer radical polymerization (ATRP) is a suitable technique for grafting multi-copies of phenolic acids into milk proteins. The resulting bio-conjugates are expected to outperform the native proteins, caffeic acid or their physical mixtures in emulsification/stabilization and antioxidant properties. To test the above hypothesis, this study successfully developed a novel type of bio-macromolecule derivative of milk protein (βLg-polyCA) via atom transfer radical polymerization. A natural antioxidant (caffeic acid) was thus grafted onto reactive sites of dairy protein scaffold via a linker. Structural verification by MALDI-TOF-MS, FTIR and NMR proved the generation of the targeted products. Compared to previous methods of bio-conjugation (Yi et al., 2016; El-Maksoud et al., 2018), the current method allowed grafting of more copies of phenolic units onto the reactive amino acid residues in βLg. DSC analyses indicated improved thermal stability compared to native βLg. Emulsions stabilized by βLg-polyCA displayed comparable physical stability as emulsions stabilized by native βLg.

Furthermore, the stretching-out polymer arms allowed sufficient exposure of caffeic moieties to scavenge free radicals in aqueous phase

and interface of o/w emulsion systems (Liu et al., 2007; Li et al., 2013). As a result, βLg-polyCA displayed superior antioxidant properties and inhibition of oxidative rancidity compared to native βLg and a βLg-CA bio-conjugate previously reported (El-Maksoud et al., 2018). In addition, βLg-polyCA efficiently inhibited lipid oxidation compared to other stabilizers previously reported (Falkeborg & Guo, 2015). The profound secondary structural changes of protein scaffold and molecular size/shape in βLg-polyCA generated a more flexible conformation that allowed superior surface-activity compared to other bio-conjugates (Yi et al., 2016; El-Maksoud et al., 2018) or stabilizers (Falkeborg & Guo, 2015; Guo, Kahveci, Özçelik, & Xu, 2009) in literature. Future studies are underway to assess cell toxicity of the bio-conjugates and bioavailability of encapsulated hydrophobic ingredients. Those studies will provide further enlightenment on the applicability of βLg-polyCA for stabilization of delivery emulsions meant for fortifying food and pharmaceutical products.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125298.

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