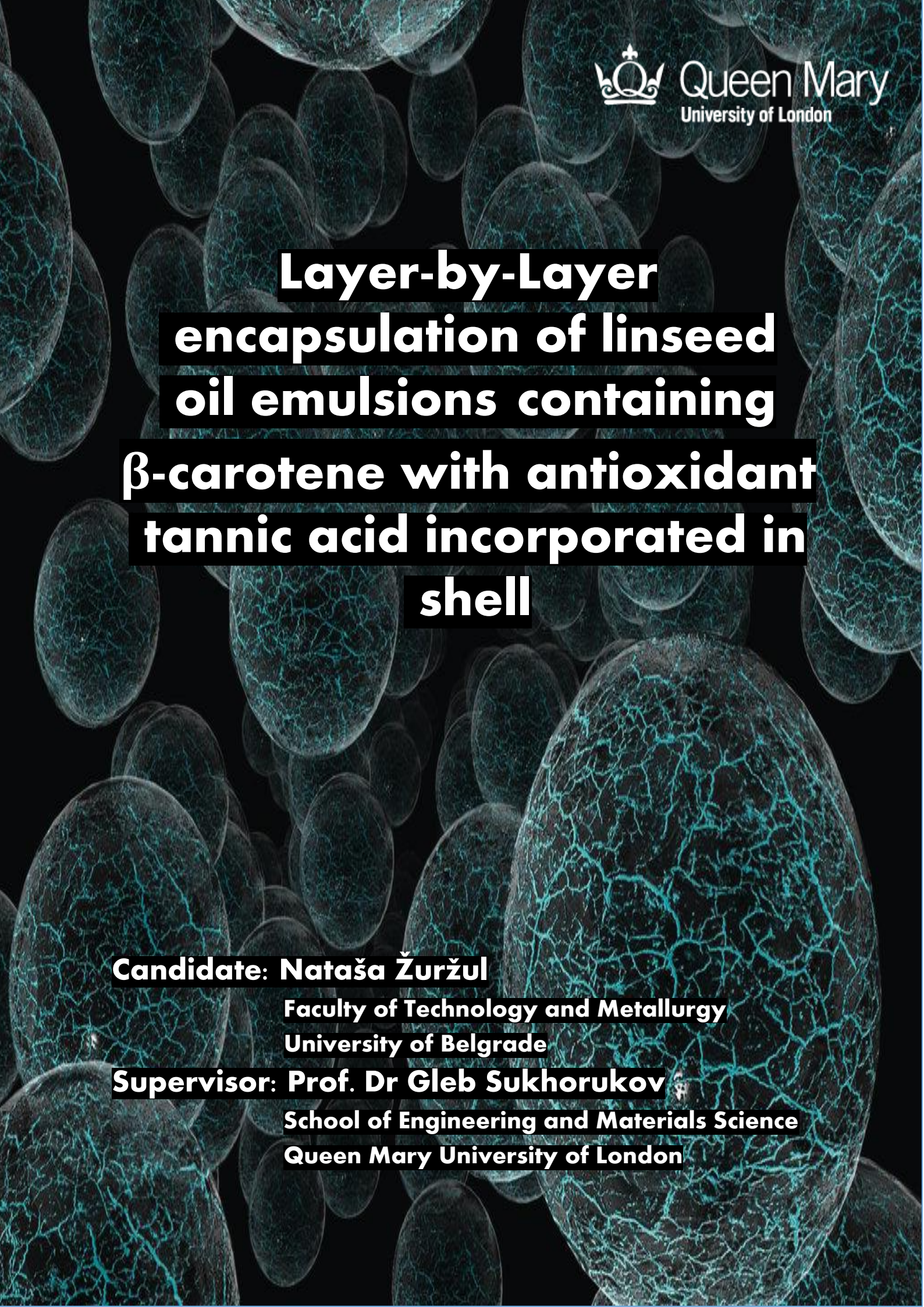

Queen Mary
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Layer-by-Layer encapsulation of linseed oil emulsions containing β-carotene with antioxidant tannic acid incorporated in shell

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INTRODUCTION

Incorporation of bioactive compounds into food systems provides a simple way to develop novel functional foods that may have nutritional benefits of reduce the risks of diseases. However, the production of these active ingredients and their incorporation in a variety of food products, pharmaceutics, and consumer care products require new and innovative technologies because such ingredients are sensitive to a variety of environmental processing factors that may cause the loss of biological functionality, chemical degradation and/or premature or incomplete release. Nanotechnology provides a good opportunity to improve the solubility and stability of such active ingredients and to increase bioavailability.

Layer-by-layer coating technique considerably advantageous for fabrication of complex and multifunctional coating films on plane surfaces and colloid particles has been applied for encapsulation of micrometer- and submicrometer size oil-in-water emulsions. Nanoemulsions are thermodynamically unstable systems that tend to breakdown over the time, thus this technique could improve their stability. Besides improved stability to coalescence and flocculation those double- or multilayer coated emulsions were characterized with better stability to oxidative stress.

β-Carotene is a well-established natural pro-vitamin and antioxidant agent which may provide protection against various serious diseases as lung cancer, heart disease, colorectal adenomas, but its poor water-solubility, low chemical stability and low bioavailability limit its application. Since β-carotene is a highly lipophilic non-polar organic compound bioavailability and solubility problems could be effectively solved by incorporation in linseed oil emulsion. Furthermore, linseed oil is having an antioxidant effect on β-carotene and vice versa.

Oil-in-water (o/w) emulsions are important ingredients of a wide range of functional foodstuffs, pharmaceuticals, 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. 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. The experiments were performed using **linseed oil**, the richest source of highly unstable omega-3-alpha linolenic essential fatty acid. There has been considerable commercial interest in providing deliverable forms of such components even though in many cases the component may be oxidative unstable. Fast lipid peroxidation in emulsified oils products leads to shortening their shelf life by formation of carcinogens and product rancidity. Therefore, oxidative stability is an essential requirement for lipid-based delivery systems.

Thus, therapeutic and healing properties of various products containing β-carotene and/or aqueous dispersed unsaturated lipids depend strongly on reliable protection against pro-oxidant damage. Therefore, choosing the right formulation could preserve relevant products during the formulation, production and storage.

Tannic acid (TA) known as a natural antioxidant and possessing antimicrobial properties was used as a protective remedy. Bovine serum albumin (BSA) was exploited as an emulsifier.

Therefore, with all the natural biocompatible materials and all examined and proved benefits this innovative multifunctional formulation has a high potential and possibility of wide application range.

EXPERIMENTAL SET-UP AND ENCAPSULATION PROCEDURE

Emulsion preparation

Emulsion of linseed oil was obtained by dispersing 10 % v/v linseed oil and 1% w/w β-arotene in 90 % v/v emulsifier (BSA, 9 mg/mL) water solution. For this purpose an oil/emulsifier mixture was treated in Vibra-Cell ultrasonicator 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.

A volume of 5mL of obtained mixture and 20 mL of TA (3 mg/mL) or DS (2 mg/mL) water solution was ultrasonicated in Vibra-Cell over 30 seconds to form the next layer. The mixture was transferred to a modified 50 mL stirred filtration cell followed by 2 washing cycles to remove uncoupled polyelectrolyte. In each washing cycle, the dispersion was placed into the cell, filled with DI water, and then 40 ml of aqueous phase were filtered through 0.22 µm hydrophilic surfactant free MF-Millipore membrane under pressure of compressed argon (20 psi). To adsorb a next layer of either TA or DS, 10 mL of filtered emulsion was topped-up with 20 mL of BSA (2 mg/mL) and vigorous stirred for 15 min followed by 2 washing cycles.

The described routine was repeated alternating BSA with TA to obtain the desired number of layers in the shell. The schematic diagram of the process is shown in the *Figure 1*.

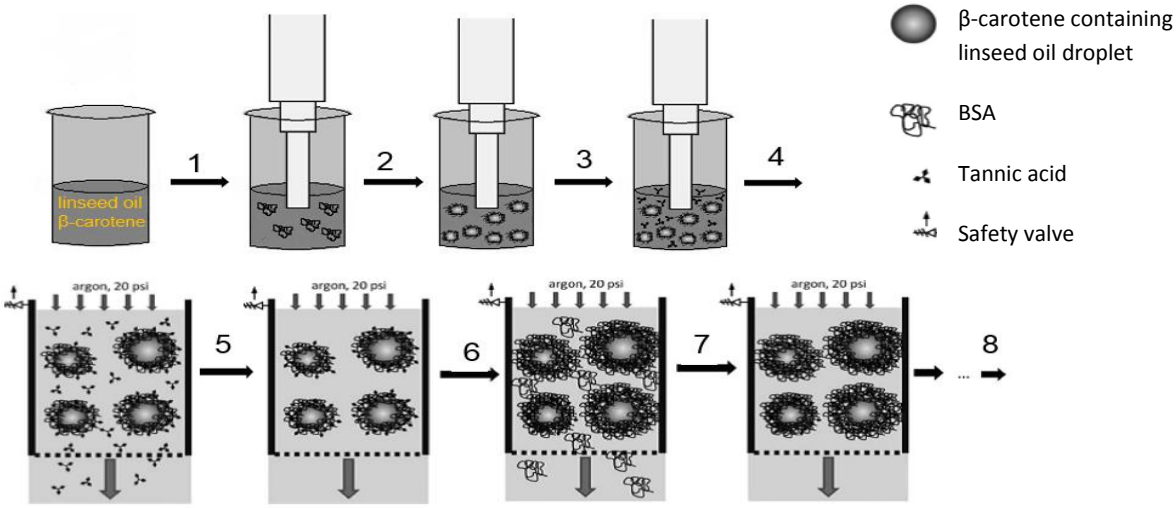

Figure 1. Schematic diagram of the process. (1) adding BSA; (2) ultrasonication; (3) adding TA; (4) ultrasonication; (5) washing out uncoupled TA; (6) coating with the second BSA layer; (7) washing out uncoupled BSA; (8) repeating the same steps for every new layer.

Sample extraction

To extract β-carotene from the o/w emulsion, first 0.4 mL of ethanol and then 0.6 mL of n-hexane were added to 0.2 mL of emulsion. The mixture was vortexed and left to rest until a complete phase separation was achieved. The n-hexane phase was taken out and another 0.6 mL of n-hexane were added to the ethanol/emulsion solution until a colorless n-hexane was extracted, indicating that β-carotene had been totally removed. Total β-carotene concentration in various samples and times was determined in the n-hexane/β-carotene solution using UV-vis spectrophotometry.

RESULTS AND CONCLUSIONS

ζ-Potential measurements

Electrostatic charge of the droplets' surface in emulsion and the hydrodynamic diameter of the water dispersed emulsion droplets were measured by Zetasizer Nano ZS utilizing dynamic light scattering (DLS) technique at 25°C. The measurement was carried out at a fixed angle of 90° with the samples diluted approximately 500 times with DI water.

Figure 2. shows changes of the surface charge of the particles after deposition of each layer. TA and BSA are both negatively charged, thus ζ-potential of the shell is remaining negative although changing the absolute value with every added layer.

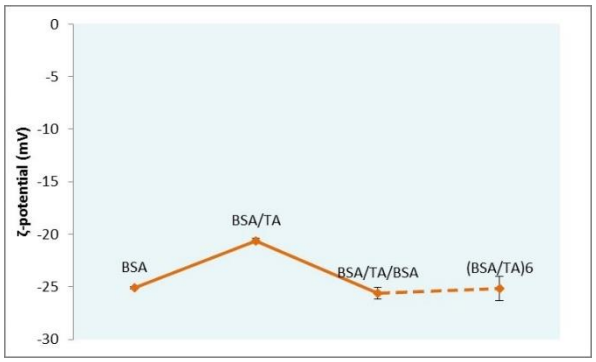

Figure 2. ζ-potential of encapsulated droplets of linseed oil-in-water emulsion containing β-carotene as a function of deposited layer

Particle size analysis

Additionally, the physical-chemical and mechanical stability of emulsion encapsulated into TA-containing multilayer shell in aqueous phase and over freeze-drying process was examined analyzing the particle size distribution. For this purpose, the DLS measurements were performed in BSA/TA/BSA and $(BSA/TA)_6$ coated o/w emulsion containing β-carotene 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 3.* and *Figure 4*.

Two maximum points on the PSD curve of the initial BSA/TA/BSA coated sample (*Figure 3, curve a*) indicate the presence of predominant fractions of coated microdroplets with the cumulants mean diameter of (261.1 ± 52.79) nm and (1739 ± 913.7) 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). Thus, the mean particle diameter in the fraction was determined to be 788.3 nm and polydispersity index (PdI) 0.512.

The freeze-drying process had negligible effect on the fraction of smaller particles. In contrast, the average size of bigger particles decreased their diameter significantly or were partly damaged after freeze-drying (*Figure 3, curve b*).

Sample of $(BSA/TA)_6$ coated emulsion (*Figure 4, curve a*) showed less particles' polydispersity, only one fraction is noticeable and PdI is 0.093. The mean particle diameter was determined to be 1655 ± 340.5 nm. Results revealed high stability over the freeze-drying process (*Figure 4, curve b*).

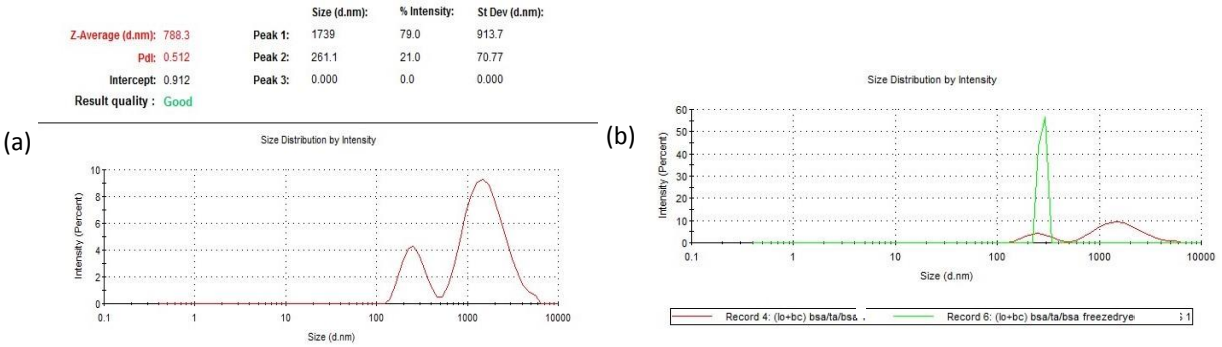

Figure 3. Size distribution (a) linseed oil containing β-carotene BSA/TA/BSA; (b) before and after freeze-drying

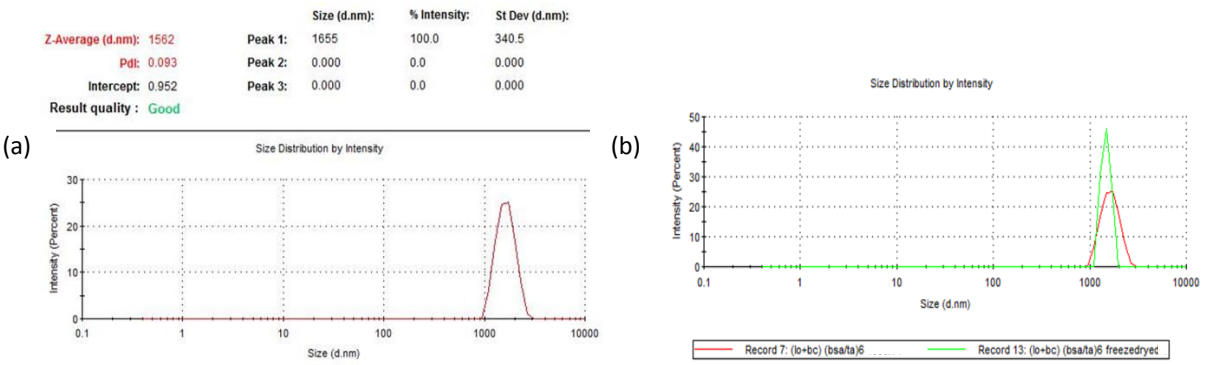

Figure 4. Size distribution (a) linseed oil containing β-carotene $(BSA/TA)_6$; (b) before and after freeze-drying

Cryo Scanning Electron Microscopy (Cryo-SEM)

As capsules are highly sensitive to usual SEM, images were obtained with cryo-cooled sample on a Quanta 3D FEG. Results of capsules size and polydispersity are in accordance with those obtained with DLS technique.

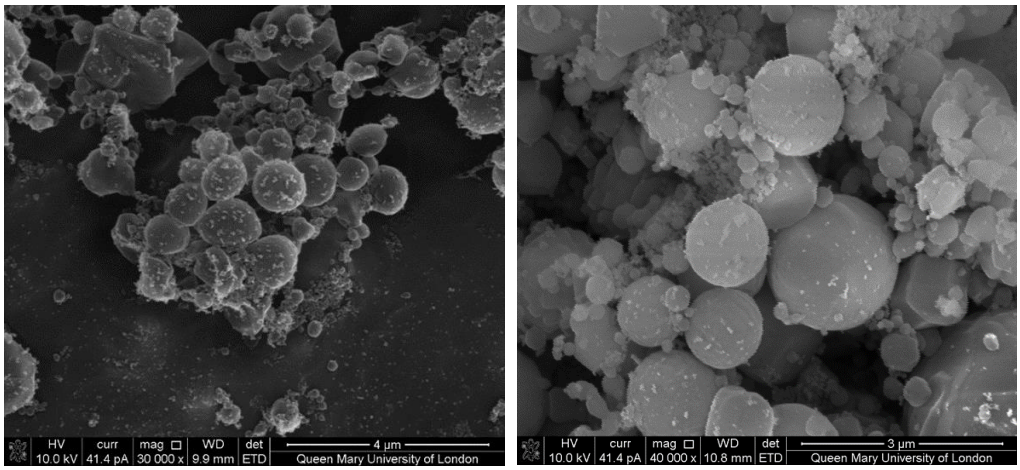

Figure 5. CryoSEM images of linseed oil containing β-carotene emulsion with BSA/TA/BSA layers

UV-vis determination of β-carotene

β-carotene has unique and characteristic spectra when studied by UV-vis spectrometry shown in the *Figure 6*.

The n-hexane suspension of β-carotene can be subjected to spectrophotometry, and the obtained values and spectra can be compared to those of pure β-C in known concentrations (*Figure 7* and *Figure 8*). This method provides both a qualitative answer to whether or not the desired compound is present and a qualitative indication of the concentration in the samples.

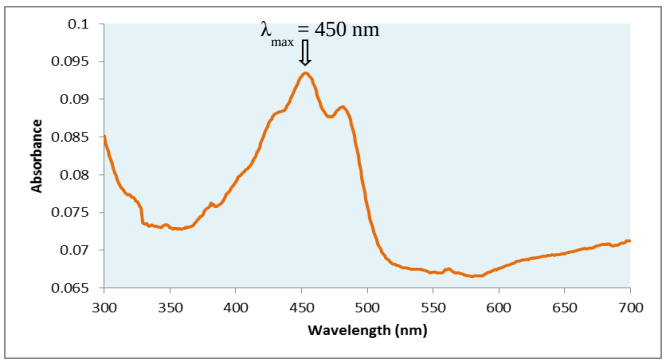

Figure 6. UV-Vis spectrum of β-carotene dissolved in hexane at concentration 4 µg/mL

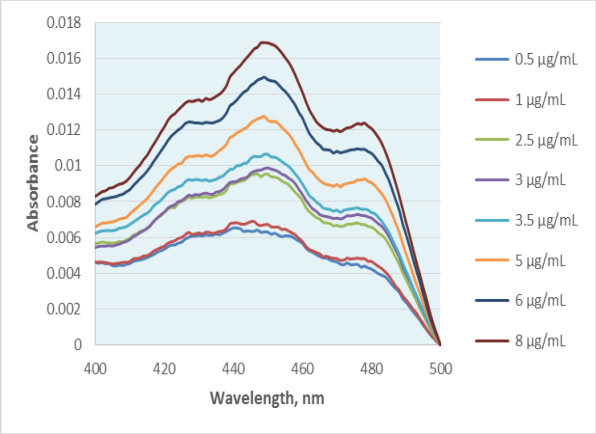

Figure 7. UV-Vis of β-carotene dissolved in n-hexane in different concentration

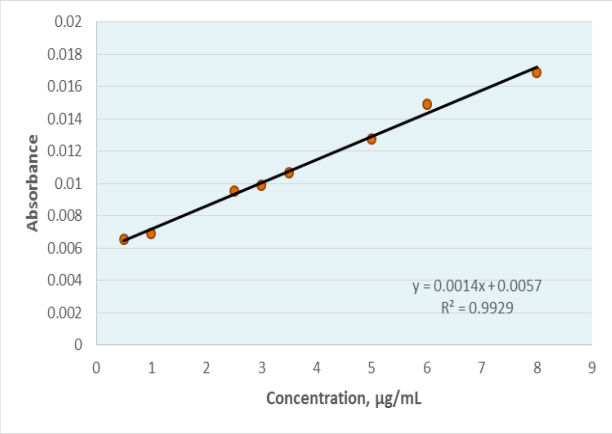

Figure 8. Calibration curve of β-carotene at 0.5-8 µg/mL

Formulation with six bilayers $(BSA/TA)_6$ showed visually unchanged constituency and stability over three weeks, while emulsion coated with BSA/TA/BSA had β-carotene crystals separated on the bottom of the vial after preparation. Separated amount of β-carotene was dissolved in n-hexane and quantified by UV-vis spectrometry. Efficiency of encapsulation was determined to be 81.05%.

Linseed oil emulsion containing β-carotene coated with BSA/TA/BSA and corresponding emulsion stabilized with single BSA layer were exposed to temperature treatment for 2h and 45min at 90°C (*Figure 9*). Results show that the β-carotene is better preserved when encapsulated with antioxidant TA present in shell. In the first part of the experiment it appears that control sample is exhibiting better stability against termo-oxidation. This deviation on the diagram could be explaned by the fact that β-carotene in this formulation is degrading in exponencial rather than linear trend as shown in *Figure 10* and due to experimental data deficiency during that period.

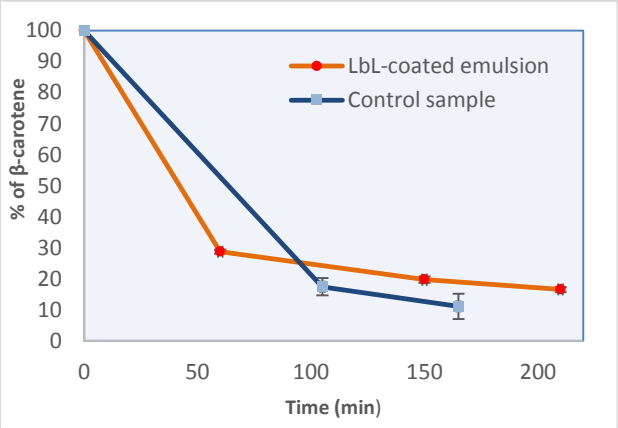

Figure 9. Profiles of β-carotene concentration changing for LbL-coated emulsion and corresponding emulsion stabilized with single BSA layer upon heating on 90°C

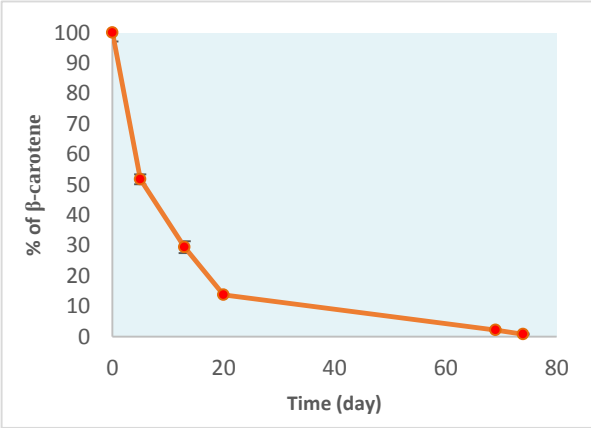

Figure 10. Degradation profile of β-carotene as a function of storage time at 5°C

Data presented in *Table1* give clear evidence that formulation containing antioxidant TA as a layer is giving considerably better protection comparing to control sample. Moreover, capsules with six bilayers have shown significant improvement in preserving β-carotene during two months storage.

Capsules composition	**Concentration of β-carotene in sample (μg/mL)**	**Percentage of β-carotene compared to fresh sample**
Core: linseed oil + β-C; Layers: BSA/TA BSA	6.45 ± 0.16	2.2 %
Control sample *Core: linseed oil + β-C; Layers: BSA/DS/BSA*	0.88 ± 0.54	0.2 %
Core : linseed oil + β-C; Layers: $(BSA/TA)_6$	67.33 ± 0.62	16.6 %

Table 1. Concentrations of β-carotene in samples after 2 months of storage at 5°C

All data represent the mean of three measurements of different trials, and results are reported as the means and standard deviations of these measurements.

Conclusions

- In this research work, we demonstrated an effective and innovative protection of β-carotene based on dissolving it in linseed oil, making emulsion and 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.
- Both constituents of the shell TA and BSA are negatively charged which is not usual for LbL encapsulation. Nevertheless, coated oil microdroplets 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 Cryo-SEM imaging.
- The emulsion of linseed oil with β-carotene having three layers and containing the antioxidant TA as a shell constituent enhanced stability and β-carotene protection than emulsion encapsulated into antioxidant-free biocompatible shell. Furthermore, formulation with six bilayers of BSA and TA demonstrated considerably better stability and β-carotene protection according to results obtained during two months observation.
- Termo-oxidative stability is proven by comparing the results of heating the emulsion of linseed oil with β-carotene protected by encapsulation of oil droplets inside antioxidant TA containing polyelectrolyte multilayer shell and non-encapsulated fresh prepared emulsion.
- Undoubtedly, this formulation has great potential for various applications. Besides offering solution for protection, stability and bioavailability problems to sensitive but highly beneficial compounds it is accordant with green production and healthy style of living where biocompatibility, usage of natural ingredients and noninvasive techniques are playing a crucial role.
- Next steps should be finding optimum for number of layers which could provide encapsulation of β-carotene without a loss, as well as variation of the concentration of β-carotene amount, so an optimal formulation in terms of protection, stability and utilization of ingredients could be determined.

CONTRIBUTION TO MY ONGOING RESEARCH AND WORK BACK AT HOME

Time spent at Queen Mary University of London has been rewarding and beneficial. Research done In UK resulted in a significant contribution to my doctorate research. I acquired substantial knowledge about innovative and versatile LbL method of encapsulation during this period that provided more quality to my research skills and work. Working in multicultural environment and professional interactions with experts in my field were truly precious.

Furthermore, work carried out in the UK gave an appreciable contribution to research and development project ''Novel encapsulation and enzyme technologies for designing of new biocatalysts and biologically active compounds targeting enhancement of food quality, safety and competitiveness'' of Serbian Ministry of Education, Science and Technological Development I'm engaged on. I believe it is contributing to the advancement of biotechnology field in Serbia and more effective usage and preservation of natural resources my country has.

Moreover, Dr Gleb Sukhorukov has offered scientific collaboration to Serbia, which we are trying to establish, so it would lead to further advancements and results.

ACKNOWLEDGMENT

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I wish to thank Professor Dr Gleb Sukhorukov for supervising my work at QMUL and for encouragement and support during my work.

I am furthermore grateful to all members of the Gleb's research group, visiting researchers and staff of Queen Mary University of London for creating a pleasant working atmosphere.