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Preventing the thermal degradation of astaxanthin through nanoencapsulation

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ABSTRACT

The encapsulation of astaxanthin into polymeric nanospheres by solvent displacement was compared for three chemically diverse polymers, namely; poly(ethylene oxide)-4-methoxycinnamoylphthaloyl-chitosan (PCPLC), poly(vinylalcohol-co-vinyl-4-methoxycinnamate) (PB4) and ethylcellulose (EC). Although capable of forming nanospheres themselves, EC could not encapsulate astaxanthin at all, whilst PB4 yielded a poor encapsulation efficiency. In contrast, PCPLC yielded reasonably good encapsulation efficiency (98%) at a loading of 40% (w/w). Moreover, the freeze-dried astaxanthin-encapsulated PCPLC nanospheres showed good dispersibility in water yielding stable aqueous suspensions of 300–320 nm nanoparticles. A steady release of astaxanthin from the nanospheres up to a maximum of ~85% payload over 60 min was also demonstrated, at least in acetone. NMR analysis indicated that after a two-hour-heating at 70 °C in an aqueous environment, PCPLC nanoencapsulated astaxanthin showed minimal heat degradation of olefinic functionality in contrast to that of the unencapsulated pigment molecules which were almost completely destroyed.

1. Introduction

Astaxanthin, or 3,3′-dihydroxy-β-β′-carotene-4-4′-dione (Fig. 1), is one of many xanthophyll carotenoid pigments found in aquatic animals such as shrimps, crabs, salmon, and many other organisms (Shahidi et al., 1998). The utility of astaxanthin as a pigmentation source in the aquaculture industry is well documented (Lorenz and Cysewski, 2000). Recently, the application of astaxanthin as a nutraceutical (Hussein et al., 2006; Guerin et al., 2003) and a medicinal ingredient for the prevention and treatment of various diseases such as cancer (Tanaka et al., 1995; Jyonouchi et al., 2000; Nishino et al., 2005), age-related macular degeneration (Parisi et al., 2008; Santocono et al., 2007), inflammation (Kurashige et al., 1990), *Helicobacter pylori* infection (Wang et al., 2001; Bennedsen et al., 2000) and cardiovascular oxidative stress (Pashkow et al., 2008), as well as for general enhancement of immune responses (Jyonouchi et al., 1994), has gained in popularity. Research has demonstrated that astaxanthin is significantly more effective than β-carotene and lutein at preventing UV light photooxidation of lipids (Santocono et al., 2006; Nielsen et al., 1998), and it possesses 100 and 10 times greater antioxidant activity than vitamin E and β-carotene, respectively (Nishigaki et al., 1994; Jorgensen and Skibsted, 1993).

Astaxanthin is a highly unsaturated molecule that decomposes easily when being exposed to heat, light and oxygen (Christophersen et al., 1991; Jorgensen et al., 1992; Nielsen et al., 1996; Zhao et al., 2006). In addition, the intense dark purple color and the limited water solubility/dispersibility of astaxanthin have hampered its applications. As a result, astaxanthin derivatives with improved either solubility or stability have been prepared. Examples include various esters of astaxanthin, such as disodium disuccinate astaxanthin, tetrasodium diphosphate astaxanthin, divitamin C disuccinate astaxanthin and various fatty acid esters of astaxanthin (Nakao et al., 2008; Gloor and Simon, 2005, 2008; Sumida et al., 2005; Lockwood and Nadolski, 2007).

In addition to the above mentioned strategies based upon structural modification to impart a hydrophilic nature, improving the solubility and stability of astaxanthin has been attempted through various other strategies, such as inclusion complexation with hydroxypropyl-β-cyclodextrin (Yuan et al., 2008), complexation with calcium ions (Chen et al., 2007), microencapsulation into chitosan matrix (Higuera-Ciapara et al., 2004), embedding into half-centimeter-chitosan beads (Kittikaiwan et al., 2007), and incorporation into emulsions, suspensions (Moldt, 1996; Ribeiro et al., 2005) and liposomes (Matsushita, 2000).

To our knowledge, effective nanoencapsulation of astaxanthin has not yet been demonstrated, despite the many well-known advantages of nanosize-carriers (Liu et al., 2008). Thus, here, using solvent displacement, we have compared the encapsulation of astaxanthin into three different polymeric nanocarriers of

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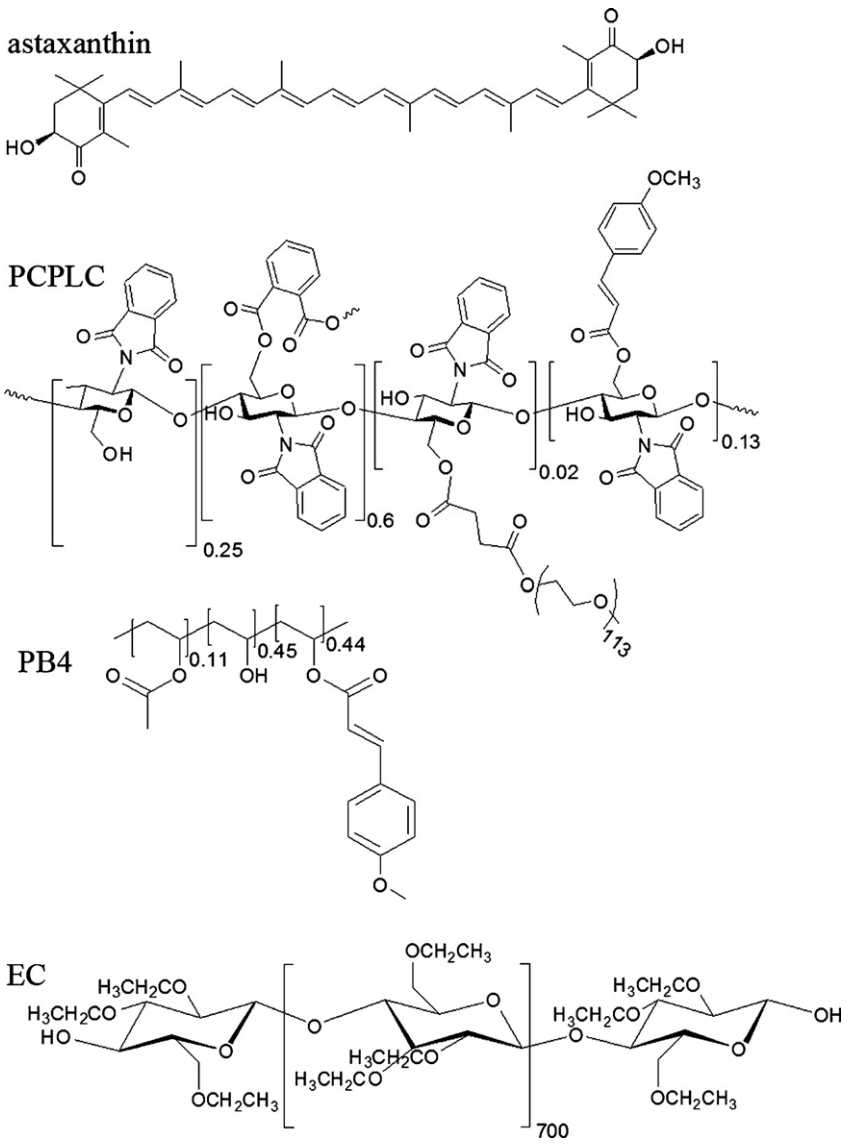

Fig. 1. The structure of astaxanthin and the three polymers; PCPLC, PB4 and EC.

varying size and hydrophilic nature, namely; (i) poly(ethylene oxide)-4-methoxycinnamoylphthaloylchitosan (PCPLC), (ii) poly(vinylalcohol-co-vinyl-4-methoxycinnamate) (PB4), and (iii) ethylcellulose nanospheres (EC) (Fig. 1). Astaxanthin-encapsulated-nanospheres were evaluated for encapsulation efficiency and loading and, if suitable, they were then evaluated for their aqueous dispersibility and stability in terms of non-agglomeration and their ability to release astaxanthin. Finally, the thermal stability of encapsulated astaxanthin was compared to the unencapsulated pigment molecules.

2. Material and methods

2.1. Materials and equipments

Astaxanthin (97% (w/w) purity) was purchased from Acros Organics (Geel, Belgium). Ethylcellulose or EC (ethoxy content 48% (w/w), MW 170,000) was purchased from Sigma–Aldrich (St. Louis, MO, USA). All chemicals were reagent grade and were used without additional purification. Poly(ethylene oxide)-4-methoxycinnamoylphthaloylchitosan and poly(vinylalcohol-co-vinyl-4-methoxycinnamate) were prepared as previously described from chitosan and poly(vinylalcohol), respectively (Anumansirikul et al., 2008; Luadthong et al., 2008). The ^{1}H and ^{13}C NMR analyses were performed using a Varian Mercury spectrometer, which operated at 400.00 MHz for ^{1}H and 100.00 MHz for ^{13}C nuclei (Varian Company, Palo Alto, CA, USA), in deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) with tetramethysilane (TMS) as an internal reference. UV Absorption spectra were acquired with a UV 2500 UV–vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using a quartz cell with a 1 cm path length. Transmission (TEM) and scanning electron micrographs (SEM) were obtained using a JEM-2100 (Jeol, Ltd., Japan) and JEM-6400 (Jeol, Ltd., Japan) electron microscopes, respectively. The particle size and zeta potential were acquired using ay Mastersizer S and Zetasizer nanoseries (Mulvern Instruments, Worcestershire, UK), respectively. The particle suspension was freeze-dried using a Freeze-Dry/Shell Freeze System Model 7753501 (Labconco Corp., Kansus, MI, USA).

2.2. Nanoencapsulation

Astaxanthin was encapsulated into nanospheres of three different polymers (EC, PPLC and PB4) by similar solvent displacement

process. Nanoencapsulation was carried out by dialyzing 100 ml of solution containing 0.06 g polymer and 0.04 g astaxanthin in dimethylformamide (DMF), against Milli-Q® water. The obtained suspension of astaxanthin-encapsulated nanoparticles in water was then subjected to SEM and TEM analysis. To obtain the loading capacity and encapsulation efficiency, the amounts of astaxanthin recovered in the dialysate were determined using UV absorption spectroscopy (at λ of 473 nm) with the aid of a calibration curve. The amount of astaxanthin that was encapsulated was determined by subtracting the amount of astaxanthin used with amount of astaxanthin found in the dialysate (after confirming the absence of any astaxanthin precipitate in the dialysis bag). Loading and encapsulation efficiency were then obtained as follows:

$$\%\text{loading} = \left[\frac{A}{(A+B)}\right] \times 100$$

$$\%\text{encapsulation efficiency} = \left(\frac{A}{C}\right) \times 100$$

A = weight of encapsulated astaxanthin = weight of astaxanthin used − weight of astaxanthin found in dialysate; B = weight of polymer used; C = weight of astaxanthin used.

Loading percentage was also confirmed by NMR analysis using the ratio of peak area representing PCPLC protons and astaxanthin protons. Dry particles were obtained by freeze-drying the suspension.

2.3. Redispersion of astaxanthin-loaded particles

Twenty milliliters of water were added to 6 mg of the dry astaxanthin-loaded nanoparticles and the suspension was sonicated (40 kHz, room temperature) for 10 min, and then subjected to TEM, SEM and dynamic light scattering (DLS) analyses.

2.4. Controlled release

The release of astaxanthin from PCPLC nanoparticles was measured by dialysis. The aqueous astaxanthin-loaded PCPLC nanoparticle suspension (10 ml, 6 mg polymer and 4 mg astaxanthin) was placed into a dialysis bag (CelluSep T4, MWCO 12,000–14,000, 45 mm flatwidth, 6.42 ml cm^{-1} volume capacity, Membrane Filtration Products, Seguin, TX, USA) and the bag was clipped with minimal void volume. The bag was then immersed into release medium (50 ml of acetone) with constant stirring at 25 °C. During the experiment, aliquots of the medium (3 ml) were withdrawn at 1, 2, 4, 6, 24, 30, 54 and 124 h with the replacement of the same volume of fresh medium. The release system was otherwise carefully closed to prevent evaporation. The amount of released astaxanthin was determined by UV–vis absorption spectroscopy at 473 nm using a calibration curve constructed from a series of astaxanthin solutions prepared in acetone. The results are expressed as a percent cumulative release of astaxanthin at each sampling time. The experiments were carried out in triplicate and are shown as the mean ± 1S.D.

2.5. Thermal stability of free and PCPLC encapsulated astaxanthin.

Forty five milliliters of astaxanthin-loaded PCPLC aqueous suspension (24 mg astaxanthin and 36 mg PCPLC) were refluxed at 70 °C for two hours, and then subjected to freeze-drying. The dry product was subjected to ^{1}H NMR (in DMSO-d_6) and UV–vis absorption (in DMSO) analyses. In an otherwise similar experiment, 45 ml of 8.94×10^{-4} M astaxanthin (i.e. not encapsulated) solution (in ethanol) was likewise heat-treated and then evaluated in the same manner.

2.6. Particle size, zeta potential, SEM and TEM analyses

TEM micrographs were acquired on a transmission electron microscope (JEM-2100, JEOL, Japan) with an accelerating voltage of 100–120 kV in conjunction with selected area electron diffraction (SAED). SEM micrographs were obtained using a scanning electron microscope (JSM-6400, JEOL, Japan) with an accelerating voltage of 15 kV. A drop of the nanoparticle suspension was placed on a glass slide and dried overnight. After mounting the slide on aluminum pin, the sample was coated with a gold layer under vacuum at 20 kV for 90 s. The coated sample was then mounted on an SEM stud for visualization. For both SEM and TEM, the micrographs shown are representative of at least 6 fields of view per sample, and 3 independent samples.

The particle size and zeta potential of astaxanthin-loaded particles in water were acquired by dynamic light scattering technique using a Zetasizer Nano series model (Malvern Instruments, Worcestershire, UK), equipped with a He–Ne laser beam at 632.8 nm (scattering angle of 173°). The concentration of nanoparticles in water was diluted to about 0.1 mg ml^{-1} prior to evaluation. Each measurement was repeated at least five times from which the mean value ± 1S.D. were evaluated and reported.

3. Results and discussion

3.1. Nanoencapsulation

Amongst the three polymers, PCPLC, PB4 and EC, only PCPLC could effectively encapsulate astaxanthin and give a stable aqueous suspension of astaxanthin-encapsulated nanospheres. Upon astaxanthin encapsulation, the hydrodynamic sizes of self-assembled nanospheres prepared from 600 ppm PCPLC increased from 68.3 ± 0.35 to 312 ± 5.83 nm (Fig. 2a–c). When the encapsulation process was carried out with a weight ratio of astaxanthin: PCPLC of 40:60, only a very small amount of astaxanthin could be detected in the dialysate. Thus, the encapsulation efficiency

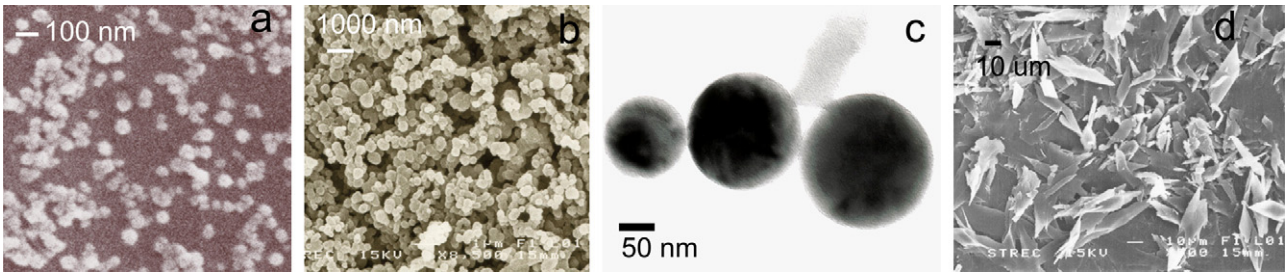

Fig. 2. Structure of PCPLC and astaxanthin-PCPLC nanospheres and astaxanthin precipitate.
Panel a, b and d are representative SEM micrographs of (a) PCPLC, and (b) astaxanthin-PCPLC, nanospheres, and (d) the astaxanthin precipitate from PB4-astaxanthin dialysis. Panel c is a representative TEM micrograph of astaxanthin-PCPLC nanospheres.

was 98% and the astaxanthin loading of the obtained spheres was ~40% (w/w). The color of the suspension was light pink and the dry particles were pinkish. SEM analysis (Fig. 2b) indicated no astaxanthin precipitate in the suspension. In other words, all astaxanthin inside the dialysis bag was encapsulated into PCPLC nanospheres. The smooth surface of all the nanospheres implies no accumulation of astaxanthin at the spherical surfaces. TEM micrographs of astaxanthin-encapsulated PCPLC show the distribution of dark patches inside the nanospheres, indicating the presence of solid astaxanthin in the polymer matrix at the particles' cores (Fig. 2c). The freeze-dry particles easily redispersed in water and the redispersed particles possessed similar hydrodynamic diameters (319 ± 7.87) to those from the freshly prepared suspension (obtained from the dialysis).

The loading level of astaxanthin at 40% (w/w) was also confirmed by ^{1}H NMR spectrum analysis (not shown; but see Fig. 5c) of the encapsulated particles (e.g. shown in Fig. 5c). The area of astaxanthin representative peaks at 6.20–6.75 (14H, m, olefinic-H of astaxanthin) or 1.9–2.1 ppm (1.94 (6H, s, 5,5′-Me), 1.99 (12H, s, 9,9′, 13,13′-Me of astaxanthin) not shown; but see Fig. 5a) were compared to area of PCPLC representative peaks at 6.9–7.6 ppm comprised of 6.95 and 7.59 (d, Ar–H of cinnamoyl group) or 7.48–7.72 (m, Ar–H and Ar–HC=CH–COOR of cinnamoyl group, and Ar–H of phthaloyl group) (not shown; but see Fig. 5c). The peak area ratio indicates a weight ratio between astaxanthin and PCPLC polymer of 40:60, in close agreement with the previous estimate of 40% (w/w) loading derived by dialysate analysis.

Although PB4 could partially nanoencapsulate astaxanthin by self-assembly with an astaxanthin: PB4 weight ratio of 40:60, a significant amount of astaxanthin (22% of astaxanthin used) could be detected in the dialysate. Moreover, dark purple precipitates of astaxanthin could also be clearly observed in the dialysis tube together with the pink spherical suspension. SEM (Fig. 2d) and ^{1}H NMR analysis of the dark purple precipitates confirmed that they were unencapsulated solid astaxanthin. These astaxanthin precipitates indicated excess of astaxanthin molecules in the encapsulation process. Although only 22% of the total amount of astaxanthin was found in the dialysate, the encapsulation efficiency had to be far less than 78% taking into account the precipitated astaxanthin. This low encapsulation efficiency implies poor interaction between astaxanthin molecules and PB4 polymer chains. In addition, the aqueous suspension of the obtained astaxanthin-encapsulated PB4 particles could not form a stable suspension in water, i.e., precipitation of the spheres could be observed clearly after two days.

Thus, it can be concluded that PCPLC possesses a higher capacity for astaxanthin encapsulation than PB4. This is in accordance with the higher substitution degree of the hydrophobic phthalimido and 4-methoxycinnamoyl moieties in the PCPLC structure and the lower substitution of 4-methoxycinnamoyl moieties in the PB4 structure (Fig. 1). Excellent astaxanthin encapsulation by PCPLC is therefore likely to be a result of hydrophobic interactions between PCPLC and astaxanthin (see a model in Fig. 3).

A better dispersibility in water of astaxanthin-encapsulated PCPLC particles over astaxanthin-encapsulated PB4 particles probably stems from the fact that PCPLC particles contain grafted poly(ethylene oxide) (PEO) chains and these grafted PEO hydrophilic chains can arrange themselves as coronas of the spheres. Good solvation of water molecules around PEO coronas would help to stabilize the nanospherical suspension by providing repulsion forces against other particles and allowing interaction with water molecules and thus reducing aggregation. Good colloidal stability of the astaxanthin-encapsulated PCPLC nanospheres in water was confirmed with the zeta potential value of −30.2 mV. In contrast, the lower surface interaction with water molecules of the crewcut PB4 particles results in the particles' agglomeration and precipitation. It should be noted here that, unencapsulated PB4 particles prepared at similar concentration (600 ppm) were smaller and, as a result, showed better water dispersibility compared to the encapsulated ones. The result agrees well with the zeta potential values of −18.2 and −29.1 mV for aqueous colloidal suspensions of astaxanthin-encapsulated PB4 nanospheres and unencapsulated PB4 particles, respectively.

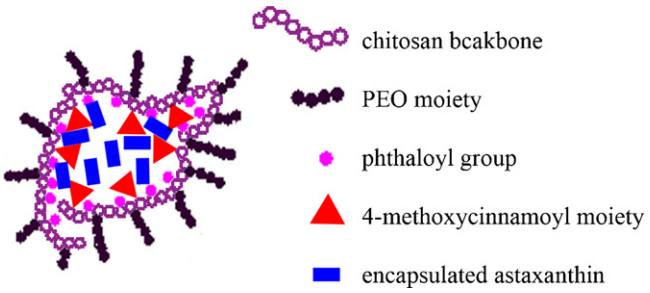

Fig. 3. A representative model showing interactions among hydrophobic phthaloyl moieties, 4-methoxycinnamoyl groups and the encapsulated astaxanthin molecules. The hydrophilic PEO chains arrange themselves to get maximum interaction with outside water molecules.

Although EC itself could form nanospheres by self-assembly, the polymer failed to encapsulate astaxanthin. Not only was a significant amount of astaxanthin detected in the dialysate during the nanoencapsulation process, but prominent astaxanthin precipitates were also found in the dialysis tube. The inability of EC to encapsulate astaxanthin is probably a result of the incompatibility between the interior (ethoxy groups) of the EC nanosphere and the astaxanthin molecule. The hydrophobicity of the ethoxy moiety, although enough to facilitate the self-assembling of nanospheres by EC alone, is probably too small to accommodate stable astaxanthin–EC interactions.

3.2. Controlled release

Since PCPLC is not soluble in acetone whilst astaxanthin is, acetone was used as a medium to investigate the release of astaxanthin from PCPLC. The experiment revealed the controlled release of astaxanthin from PCPLC particles (Fig. 4). This time release characteristic, with no burst of astaxanthin at the beginning, indicates that all the pigment molecules were well encapsulated inside the spheres. The release curve also indicates that encapsulated astaxanthin can relatively slowly diffuse out of the PCPLC spheres, at least in acetone, attaining an equilibrium release of ~85% within

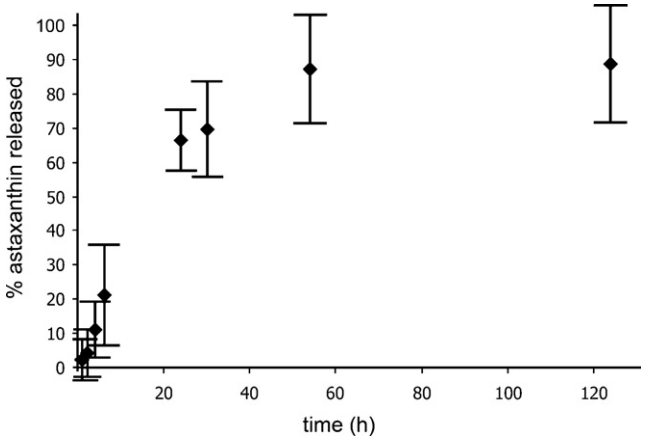

Fig. 4. Astaxanthin release kinetics from astaxanthin-PCPLC nanospheres in acetone suspension.
Data are the mean ± 1S.D.

60 h under these conditions. We did not attempt to perform the release experiment using physiological buffered saline as a release medium simply because astaxanthin is not at all soluble in such media. In fact, astaxanthin is too hydrophobic to dissolve in an aqueous medium and thus once encapsulated in PCPLC it may remain so in an aqueous environment and not be released as shown in the acetone environment in which it is soluble. This hydrophobic pigment has been found in lipid bilayer membranes and its presence was demonstrated to help preserve the membrane structure (McNulty et al., 2008). However, the mechanism of biological transportation of astaxanthin within the human body has yet to be elucidated but likely involves lipophilic carrier complexes or proteins. To this end, it will be interesting to evaluate the stability, that is the release rate of astaxanthin from PCPLC encapsulated nanospheres, in both digestive tract systems (with both food and bile salt derived emulsifiers and fats) and plasma based solutions.

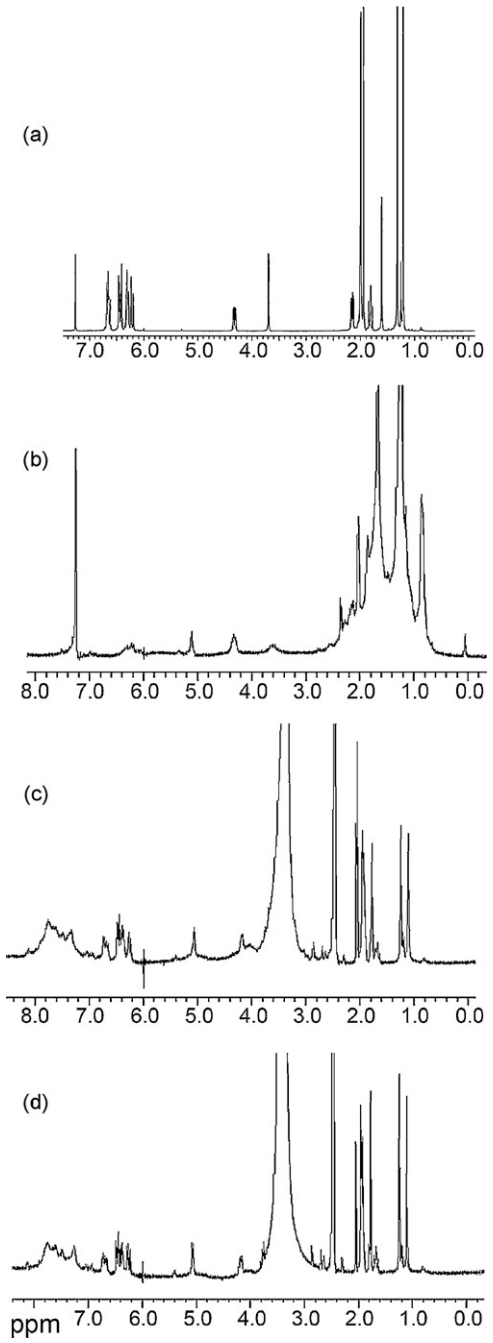

Fig. 5. Heat labiality of free astaxanthin and astaxanthin-PCPLC nanospheres. Representative ^{1}H NMR spectra of free astaxanthin (a, b), and astaxanthin-PCPLC nanospheres (c, d) with (b, d) and without (a, c) treatment at 70 °C for two hours.

3.3. Thermal stability

Although *trans* to *cis* isomerization of astaxanthin has been reported (Zhao et al., 2006), our preliminary study indicated an obvious loss of the olefinic functionality when the pigment was kept at 40–50 °C for more than one month. As a result, we investigated if PCPLC nanoencapsulation could prevent heat degradation of astaxanthin. However, to shorten the experimental time, the thermal degradation was investigated at 70 °C. When astaxanthin solution (in ethanol) was heated to 70 °C for two hours, most astaxanthin molecules were degraded. The UV–vis absorption spectrum of the heated solution showed a disappearance of the absorption at 475 nm, whilst the ^{1}H NMR spectrum of the heated product revealed a significantly decreased level of resonances from the olefinic protons at 6.2–6.8 ppm, together with the obvious increase in resonance from protons of saturated alkyl functionality (0.8–2.0 ppm) (Fig. 5a and b). However, in contrast, the ^{1}H NMR spectrum of the heated astaxanthin encapsulated in PCPLC nanospheres (aqueous suspension) indicated no obvious change (Fig. 5c and d). The resonances of olefinic protons at 6.2–6.8 ppm were preserved. It should be noted here that the resonances at 6.8–8.2 ppm correspond to protons from the phthalimido and cinnamoyl moieties of the PCPLC polymer. The result, thus, indicates that thermal degradation of astaxanthin which involves the loss of olefinic functionality, can be prevented by encapsulation of the pigment into the PCPLC nanospheres.

4. Conclusions

Encapsulation of astaxanthin into PCPLC nanospheres by solvent displacement resulted in a 98% encapsulation efficiency and yielded 312 ± 5.83 nm nanospheres with a 40% (w/w) astaxanthin loading. Two other amphiphilic polymers, EC and PB4, were less suitable and, with reference to their chemical structure (Fig. 1), suggest that a suitable compatibility between astaxanthin and polymeric nanospheres is required for successful encapsulation. The freeze-dried astaxanthin-loaded PCPLC nanospheres dispersed well in water forming a stable aqueous colloidal suspension. The thermal stability of astaxanthin was greatly improved upon PCPLC nanoencapsulation, i.e., the loss of olefinic functionality observed when unencapsulated astaxanthin was heated at 70 °C for two hours could be prevented by PCPLC encapsulation.

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References

Anumansirikul, N., Wittayasuporn, M., Klinubol, P., Tachaprutinun, A., Wanichwecharungruang, S.P., 2008. UV-screening chitosan nanocontainers: increasing the photostability of encapsulated materials and controlled release. Nanotechnology, 19.

Bennedsen, M., Wang, X., Willen, R., Wadstrom, T., Andersen, L.P., 2000. Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol. Lett. 70, 185–189.

Chen, C.S., Wu, S.H., Wu, Y.Y., Fang, J.M., Wu, T.H., 2007. Properties of astaxanthin/Ca^{2+} complex formation in the deceleration of cis/trans isomerization. Org. Lett. 9, 2985–2988.

Christophersen, A.G., Jun, H., Jorgensen, K., Skibsted, L.H., 1991. Photobleaching of astaxanthin and canthaxanthin—quantum-yields dependence of solvent, temperature, and wavelength of irradiation in relation to packaging and storage of carotenoid pigmented salmonoids. Zeitschrift fur Lebensmittel-Untersuchung und -Forschung 192, 433–439.

Gloor, A., Simon, W., 2005. Astaxanthin esters. Patent Application Number US 2005/0096477 A1.

Gloor, A., Simon, W., 2008. Salts of astaxathin esters. Patent Application Number US 2008/0008798 A1.

Guerin, M., Huntley, M.E., Olaizola, M., 2003. Haematococcus astaxanthin: applications for human health and nutrition. Trends Biotechnol. 21, 210–216.

Higuera-Ciapara, I., Felix-Valenzuela, L., Goycoolea, F.M., Arguelles-Monal, W., 2004. Microencapsulation of astaxanthin in a chitosan matrix. Carbohyd. Polym. 56, 41–45.

Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., Watanabe, H., 2006. Astaxanthin, a carotenoid with potential in human health and nutrition. J. Nat. Prod. 69, 443–449.

Jorgensen, K., Skibsted, L.H., 1993. Carotenoid scavenging of radicals—effect of carotenoid structure and oxygen partial pressure on antioxidative activity. Zeitschrift fur Lebensmittel-Untersuchung und -Forschung 196, 423–429.

Jorgensen, K., Stapelfeldt, H., Skibsted, L.H., 1992. Fluorescence of carotenoids. Effect of oxygenation and cis/trans isomerization. Chem. Phys. Lett. 190, 514–519.

Jyonouchi, H., Sun, S., Iijima, K., Gross, M.D., 2000. Antitumor activity of astaxanthin and its mode of action. Nutr. Cancer 36, 59–65.

Jyonouchi, H., Zhang, L., Gross, M., Tomita, Y., 1994. Immunomodulating actions of carotenoids: enhancement of in vivo and in vitro antibody production to T-dependent antigens. Nutr. Cancer 21, 47–58.

Kittikaiwan, P., Powthongsook, S., Pavasant, P., Shotipruk, A., 2007. Encapsulation of *Haematococcus pluvialis* using chitosan for astaxanthin stability enhancement. Carbohyd. Polym. 70, 378–385.

Kurashige, M., Okimasu, E., Inoue, M., Utsumi, K., 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. Physiol. Chem. Phys. Med. NMR 22, 27–38.

Lockwood, S.F., Nadolski, G., 2007. Water-dispersible carotenoids, including analogs. US 2007/0015735 A1.

Lorenz, R.T., Cysewski, G.R., 2000. Commercial potential for *Haematococcus microalgae* as a natural source of astaxanthin. Trends Biotechnol. 18, 160–167.

Luadthong, C., Tachaprutinun, A., Wanichwecharungruang, S.P., 2008. Synthesis and characterization of micro/nanoparticles of poly(vinylalcohol-co-vinylcinnamate) derivatives. Eur. Polym. J. 44, 1285–1295.

Liu, Z., Jiao, Y., Wang, Y., Zhou, C., Zhang, Z., 2008. Polysaccharides-based nanoparticles as drug delivery systems. Adv. Drug Deliv. Rev. 60, 1650–1662.

Matsushita, Y., 2000. Antioxidant activity of polar carotenoids including astaxanthin-?-glucoside from marine bacterium on PC liposomes. Fisheries Sci. 66, 980–985.

McNulty, H., Jacob, R.F., Mason, R.P., 2008. Biologic activity of carotenoids related to distinct membrane physicochemical interactions. Am. J. Cardiol., 101.

Moldt, P., 1996. Astaxanthin suspension. WO9623420 A1.

Nakao, M., Sumida, M., Katano, K., Fukami, H., 2008. Enzymatic synthesis of astaxanthin n-octanoic acid esters. J. Oleo Sci. 57, 371–374.

Nielsen, B.R., Jorgensen, K., Skibsted, L.H., 1998. Triplet-triplet extinction coefficients, rate constants of triplet decay and rate constants of anthracene triplet sensitization by laser flash photolysis of astaxanthin, ?-carotene, canthaxanthin and zeaxanthin in deaerated toluene at 298 K. J. Photochem. Photobiol. A: Chem. 112, 127–133.

Nielsen, B.R., Mortensen, A., Jorgensen, K., Skibsted, L.H., 1996. Singlet versus triplet reactivity in photodegradation of C40 carotenoids. J. Agric. Food Chem. 44, 2106–2113.

Nishigaki, I., Dmitrovskii, A.A., Miki, W., Yagi, K., 1994. Suppressive effect of astaxanthin on lipid peroxidation induced in rats. J. Clin. Biochem. Nutr. 16, 161–166.

Nishino, H., Murakoshi, M., Xiao, Y.M., Wada, S., Masuda, M., Ohsaka, Y., Satomi, Y., Jinno, K., 2005. Cancer prevention by phytochemicals. Oncology 69, 38–40.

Parisi, V., Tedeschi, M., Gallinaro, G., Varano, M., Saviano, S., Piermarocchi, S., 2008. Carotenoids and antioxidants in age-related maculopathy Italian Study. Multifocal electroretinogram modifications after 1 year. Ophthalmology, 115.

Pashkow, F.J., Watumull, D.G., Campbell, C.L., 2008. Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. Am. J. Cardiol. 101, S58–S68.

Ribeiro, H.S., Rico, L.G., Badolato, G.G., Schubert, H., 2005. Production of O/W emulsions containing astaxanthin by repeated premix membrane emulsification. J. Food Sci. 70, E117–E123.

Santocono, M., Zurria, M., Berrettini, M., Fedeli, D., Falcioni, G., 2006. Influence of astaxanthin, zeaxanthin and lutein on DNA damage and repair in UVA-irradiated cells. J. Photochem. Photobiol. B: Biol. 85, 205–215.

Santocono, M., Zurria, M., Berrettini, M., Fedeli, D., Falcioni, G., 2007. Lutein, zeaxanthin and astaxanthin protect against DNA damage in SK-N-SH human neuroblastoma cells induced by reactive nitrogen species. J. Photochem. Photobiol. B: Biol. 88, 1–10.

Shahidi, F., Metusalach, Brown, J.A., 1998. Carotenoid pigments in seafoods and aquaculture. Crit. Rev. Food Sci. Nutr. 38, 1–67.

Sumida, M., Nakao, M., Tomimori, N., Namikawa, K., Fukami, H., 2005. Astaxanthin medium-chain fatty acid ester, process for producing the same. US 2005/0228188 A1.

Tanaka, T., Kawamori, T., Ohnishi, M., Makita, H., Mori, H., Satoh, K., Hara, A., 1995. Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis 16, 2957–2963.

Wang, X., Hirmo, S., Willen, R., Wadstrom, T., 2001. Inhibition of *Helicobacter pylori* infection by bovine milk glycoconjugates in a BALB/cA mouse model. J. Med. Microbiol. 50, 430–435.

Yuan, C., Jin, Z., Xu, X., Zhuang, H., Shen, W., 2008. Preparation and stability of the inclusion complex of astaxanthin with hydroxypropyl-?-cyclodextrin. Food Chem. 109, 264–268.

Zhao, L., Zhao, G., Chen, F., Wang, Z., Wu, J., Hu, X., 2006. Different effects of microwave and ultrasound on the stability of (all-E)-astaxanthin. J. Agri. Food Chem. 54, 8346–8351.