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Research Article

DEVELOPMENT OF ASTAXANTHIN-LOADED BIODEGRADABLE NANOPARTICLES BY NANOPRECIPITATION METHOD

Ku Aizuddin K.A[1], Nurlina M.A[1], KhuriahA.H[2], C.S. Foo[3], M.M.R MeorMohd Affandi[1, 2]*
[1]Nanopharmacy Laboratory, Faculty of Pharmacy, UniversitiTeknologi MARA, 42300 Bandar PuncakAlam, Selangor, Malaysia.
[2]Department of Pharmaceutic, Faculty of Pharmacy, UniversitiTeknologi MARA, 42300 Bandar PuncakAlam, Selangor, Malaysia.
[3]ABH cosmeceuticalSdn. Bhd, 42200, Kapar, Klang, Selangor, Malaysia.
Email: meor@puncakalam.uitm.edu.my

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Abstract

Astaxanthin is a naturally occurring carotenoid abundantly found in *Haematococcuspluvaris* with strong antioxidant property (40 and 1000 times higher compared to β-Carotene and Vitamin E respectively) and it is found to be useful in many biological functions. Recent scientific findings indicate that Astaxanthin shows low oral bioavailability due to its lipophilicity. The use of advanced drug delivery system such as nanoscalecarrier is one of the efforts to design Astaxanthin-loaded biodegradable nanoparticles. In this study, nanoprecipitation method was applied to produceAstaxanthin-loaded biodegradable nanoparticles. The effect of several formulation parameters on the Astaxanthin-loaded nanoparticles properties (particle size and encapsulation efficiency) was investigated. The optimized nanoparticles with particle size of 0.231±0.006μm and high encapsulation efficiency 95.49±0.49% was obtained.

Keywords: Nanoparticles, Poly (lactide-co-glycolide acid), Astaxanthin, Nanoprecipitation.

Introduction

Astaxanthin can be generally classified as carotenoids which can be further divided into two distinct groups, Carotene and Xanthophyll[1]. The difference between these two groups lies in their chemical structure. Carotenes are merely consisting of carbon and hydrogen while Xanthophyllhas hydroxyl and oxygen groups in their component[2]. Astaxanthina member of Xanthophyll group, are usually present in bright colour vegetables, fruits and microalgae. Xanthophylls are also responsible for antioxidant properties of Astaxanthin.

Astaxanthin can neutralize several free radicals at a time compared to other types of antioxidant such asβ-Carotene or Vitamin C which can only neutralize one radical at a time[3]. Astaxanthin can only be synthesis in plant, thus the only way human can get benefit from it is through daily diet. The freshwater unicellular algae, *Haematococcuspluvialis* is one of the richest known sources of red secondary carotenoid Astaxanthin[4].Despite of having the most powerful antioxidant properties, Astaxanthinencounter bioavailability problem[5]. Its lipophilic characteristic and its incomplete release from food might be one of the factors that lead to this problem[6].

Various scientific researchers have reported wide range of methods to improve its bioavailability.These include by incorporating it into lipid based formulations, colloidal delivery system, modification of drug properties, nanotechnology approach and by using poly(D,L-lactic-*co*-glycolic acid) (PLGA)[6-10].Among these methods, nanoparticles approach is the most applicable for encapsulation of lipophilic drug. One of the best approaches in the development of nanoparticles is by using biodegradable polymer.

Table 1 represents various polymers and methods that have been used in the formulation of nanoparticles. One of the most commonly used biodegradable polymer is poly (lactic-co-glycolic) acid (PLGA).PLGA consideredas biodegradable polymer because it undergoes hydrolysis in the body to produce its original monomers, lactic acid and glycolic acid. These two monomers under normal physiological conditions are by-products of various metabolic pathways in the body. Since the body effectively deals with the two monomers, there is less toxicity by using PLGA for drug delivery.

Table-1: Summary of methods and polymer used for preparation of polymeric nanoparticles.

Drug	Method	Polymer	Size	Encapsulation efficiency	Reference
Vincristine sulfate	Solvent evaporation	PLGA	111nm	55.35%	Song *et al*. (2008)
Hydrophilic drug	Nanoprecipitation	PLGA	85-560nm	-	Bilati*et al*. (2005)
Paclitaxel	Interfacial deosition	PLGA	<200nm	-	Fonseca *et al*.[11] (2002)
Coenzyme Q10	Nanoprecipitation	PLGA	200nm	-	Nehilla*et al*.[12] (2006)
Spironolactone	Nanoprecipitation	Polycaprolactone	320nm	96.21%	Blouza*et al*.[13] (2006)

Vitamin E	Nanoprecipitation	Polycaprolactone	165nm	98%	Khayata*et al*.[14] (2012)
Insulin	Solvent evaporation	PLGA	223-243nm	-	Kumari*et al*.[15] (2010)

This study focuses on the development of Astaxanthin–loaded biodegradable nanoparticle by nanoprecipitation method. The main aim of this study is to develop optimum formulation of Astaxanthin-loaded biodegradable nanoparticle by studying the effect of various conditions on the physical characteristic of Astaxanthinnanoparticle. It is expected that the oral bioavailability of Astaxanthin can be increased and at the same time can have a sustained release characteristic.

Experimental

Material and Methods:

Asta REAL ® P2AF grade was purchased from Fuji Chemical Industry (Nakaniikawa, Toyama, Japan). Astaxanthin was supplied by Sigma-Aldrich Chemical (St. Louis, MO, USA).Poly (DL-lactide-co-glycolide) (PLGA) with molecular weight ranging from 7000-240000kDa (lactide:glycolide (50:50), (65:35) and (85:15) were supplied by BoehringerIngelheim(Ingelheim, Germany). The surfactant Pluronic F-68 and Pluronic F-127 were purchased from Sigma-Aldrich Chemical. Analytical grade acetone was purchased from Merck(Darmstadt, Germany).

Preparation of Astaxanthin loaded PLGA nanoparticles: Astaxanthin nanoparticles were prepared by nanoprecipitation method.Aqueous phase was prepared by dissolving surfactant in water. An organic phase was prepared by dissolving PLGA and Astaxanthin in acetone. The aqueous phase was then added drop wise, at the rate of 1 ml sec^{-1} into organic phase by using stirrer at speed of 2. The solvent was then evaporated for one and half hour. The freshly formed nanoparticles were then centrifugedtwiceat 5000 rpm for 15 minutes. The pellet was washed twice with 5 ml distilled water in which Astaxanthin, polymer and surfactant were dissolved.The optimization of the formulation in each step was determined by measuring the particle size and encapsulation efficiency using mastersizer and ultraviolet spectrophotometer at 474 nm.

Effect of polymer type and amount: Nanoparticle formed was further investigated for the effect of polymer type and amount.Formulations were firstly prepared with various amount of PLGA ranging from 50 milligram to 200milligram.

The effect of polymer type was then investigated with five types of PLGAwhich were RG 502, RG 503, RG 504, RG 653 H and RG 858 S. All other parameter of the formulation remains unchanged. Table 2 shows the molecular weight of resomerused in this study.

Table-2: Resomer biodegradable polymers.

Resomer type	Product name	Molecular weight range
RG 502	Poly(D,L-Lactide-co-glycolide) 50:50	7,000-17,000
RG 503	Poly(D,L-Lactide-co-glycolide) 50:50	24,000-38,000
RG 504	Poly(D,L-Lactide-co-glycolide) 50:50	38,000-54,000
RG 653 H	Poly(D,L-Lactide-co-glycolide) 65:35	24,000-38,000
RG 858 S	Poly(D,L-Lactide-co-glycolide) 85:15	190,000-240,000

Effect of surfactant type and amount: The effects of surfactant type and amount on the nanoparticles characteristics were further investigated. Different amount of surfactant were used in the formulation ranging from 25 milligram to 150milligram. The effects of surfactants type were then observed by using two type of surfactant namely pluronic F-68 and pluronic F-127.

Effect of the stirrer speed: Five different stirrer speeds were tested (2, 3, 4, 6, and 7) while all other constituents were remain unchanged. The effect of stirrer speed was evaluated by measuring the mean size.

Effect of the solvent used: The criteria for selecting the best solvents were depends on itsability to evaporate, high solubility of the polymer, absence of aggregation and the mean size of nanoparticle formed. Acetone, methanol, ethanol and dimethyl sulfoxide were used for this purposes.

Astaxanthin loading capacity and encapsulation efficiency:

Five amounts of Astaxanthin were tested ranging from 2% w/v to 10% w/v. The effect of Astaxanthin amount on nanoparticles was evaluated by measuring particle size and encapsulation efficiency. Total Astaxanthin concentration *(A)* was determined after solvent evaporated for one hour thirty minutes. Free Astaxanthin concentration *(B)* was determined after separation of loaded-nanoparticles from the aqueous medium by centrifugation. The free Astaxanthin concentration was determined in the supernatant. The various concentrations were measured at the absorbance of 474 nm with a UV-vis spectrophotometer. The Astaxanthin encapsulation was calculated as follow:

$$encapsulation\ efficiency\ (\%) = \frac{A - B}{A} \times 100$$

Size determination: Particle size was determined by using Mastersizer 5000 (Malvern Instrument Ltd., UK). All measurements were triplicate performed at room temperature.

Results and Discussion:

Effect of types and amount of polymer on properties of Astaxanthin nanoparticles:

The effects of PLGA type with different ratio of lactic acid and glycolic acid on the nanoparticle size were presented in Table 3. There is no significant difference on thenanoparticle average size produced by three different types of polymer. Notation 50:50 PLGA means 50% of copolymer is lactic acid and 50% is glycolic acid. The effects of PLGA type with different ratio of lactic acid and glycolic acid were also previously observed by other researchers. It was found that the release rate of drug increase with the decrease in lactic to glycolic acid proportion. PLGA 50:50 exhibited faster degradation followed by PLGA 65:35 and PLGA 85:15[16]. For these reason, PLGA 50:50 was chosen for the subsequent study.

Table-3: Effect of PLGA type with different ratio of lactic acid and glycolic acid.

Polymer type	Ratio Lactic acid:glycolic acid	Molecular weight	Average size ± S.D. (µm)	Average uniformity ±S.D.
RG 504	50:50	38,000-54,000	0.145 ± 0	0.349 ± 0.028
RG 653 H	65:35	24,000-38,000	0.139 ± 0.002	0.327 ± 0.021
RG 858 S	85:15	190,000-240,000	0.138 ± 0.003	0.308 ± 0.022

S.D.: standard deviation (*n=3*)

The effect of PLGA molecular weight with similar ratio of lactic acid and glycolic acid on the nanoparticle size is presented in Table 4.The smallest particle size of nanoparticles was obtained with RG 502.

This result similar to the study reported by Song*et al*.[17]which indicated that the particle size is slightly increased with the increase of PLGA molecular weight. This phenomenon was probably resulted from the increase of viscosity of internal phase, thereby decreasing the net shear stress and increasing the particle size[17].Smaller size of nanoparticles, approximately 100 nm, can be prepared with lower molecular weight polymer[18].For these reasons, further studies on effect of polymer amount were done for RG 502.

The effect of PLGA amount on the nanoparticle size was presented in Table 5.Significant particle size reduction was

observed when the polymer amounts reduced.This result was similar to the study reported by Khayata*et al.*[14] on Vitamin E-loadednanoparticles. High polymer amounts in the solvents prevent nanoparticle formation.

The smallest particle size was obtained with 50 mg amount of polymer. Hence, 50 mg of polymer amount was retained for the determination of surfactant type and amount.

Table-4: Effect of PLGA type with similar ratio of lactic acid and glycolic acid on the nanoparticle size.

Polymer type	Ratio Lactic acid:glycolic acid	Molecular weight	Average size ± S.D. (µm)	Average uniformity ± S.D.
RG 502	50:50	7,000-17,000	0.184 ± 0.004	0.431 ± 0.019
RG 503	50:50	24,000-38,000	0.304 ± 0.005	0.185 ± 0.002
RG 504	50:50	38,000-54,000	0.384 ± 0.004	0.425 ± 0.022

S.D.: standard deviation (*n=3*)

Table-5: Effect of PLGA amount on the nanoparticle size.

Polymer type	**Polymer amount**	**Average size (µm) ± S.D**	**Average uniformity ± S.D.**
RG 502	50	0.177 ± 0.009	0.431 ± 0.019
	100	1.135 ± 0.153	3.357 ± 0.490
	150	27.595 ± 0.054	62.4 ± 0.3
	200	184.785 ± 11.651	0.305 ± 0.011

S.D.: standard deviation (*n=3*)

Effect of surfactant type and amount:

Fontana *et al*.[19]reported that different types of surfactant used in the formulation will give different impact on the size of nanoparticles. For example, particles prepared with Pluronic F-68 were smaller than particles prepared with Pluronic F-108[19]. The amount of surfactant used will also have an effect on the properties of the nanoparticles. If the surfactant is too low, aggregation of polymer droplet will occurand if the amount of surfactant used is too much, the drug incorporation could be reduced due to the interaction reduction[18].

It can also be noticed that the surfactant type played a role in changing the mean size of nanoparticles (Table 6). Using surfactant type Pluronic F-127 led to the production of more stable nanoparticles with all different amount of surfactant below 0.5 µm. It can be concluded that 25 µg of surfactant amount not sufficient to form smaller size of nanoparticles

as compared to 50 µg of Pluronic F-127. This result is very similar to the study reported by Yoo*et al.*[20]. Hence, 50 mg Pluronic F-127 was retained for further study.

Table-6: Effect of PLGA amount on the nanoparticle size.

Surfactant type	Surfactant amount (mg)	Average size (µm) ± S.D	Average uniformity ± S.D.
Pluronic F-68	25	13.579 ± 0.938	71.167 ± 3.879
	50	0.174 ± 0.009	0.483 ± 0.011
	100	6.394 ± 0.319	29.967 ± 1.419
	150	0.795 ± 0.031	2.763 ± 0.125
	200	0.355 ± 0.012	1.667 ± 0.078
Pluronic F-127	25	0.237 ± 0.001	0.268 ± 0.002
	50	0.184 ±0.004	0.431 ± 0.019
	100	0.250 ± 0.001	0.274 ± 0.012
	150	0.213 ± 0.006	0.448 ± 0.007
	200	0.475 ± 0.005	1.607 ± 0.032

S.D.: standard deviation (*n=3*)

Effect of stirrer speed:

Five different stirrer speeds were tested (2, 3, 4, 6, 7) and the result are shown in Table 7. Result show that, there were no significant differences on the average particle size of nanoparticles as the stirrer speed increased. However, formation of bubbles increased as the speed of stirrer increased. Hence, lowest speed of stirrer was retained for further study.

Table-7: Effect of stirrer speed.

Stirrer speed	Average size (µm) ± S.D	Average uniformity ± S.D
2	0.146 ±0.004	0.389 ± 0.034
3	0.139 ± 0.005	0.338 ± 0.011
4	0.147 ± 0.016	0.360 ± 0.065
6	0.145 ± 0.008	0.346 ± 0.032
7	0.143 ± 0.007	0.347 ± 0.026

S.D.: standard deviation (*n=3*)

Effect of solvent used:

In the standard procedure, the organic phase containing the polymer that will form the nanoparticles are poured into the aqueous phase under slight stirring. When both phases are in contact, it is assumed that the solvent diffused from the organic phase into the water and carries with it some polymer chains which are still in a solution. Then, as the solvent

diffuse further into the water, the associated polymer chain aggregate forming nanoparticle. As noted, the mechanism of nanoparticle formation can be described based on the water-solvent, water polymer and solvent polymer interactions[21]. The result obtained using different solvents, are shown in Table 8. It's clearly showed that only acetone was evaporated and form the nanoparticles remain in the suspension resulting in particles size 0.246 μm. It should be noted that nanoprecipitation with methanol, ethanol and dimethyl sulfoxide was not possible. The main problem was due to the formation of aggregation and the slow rate of solvent evaporation. Boiling point is the important criteria that need to be taken into consideration for the determination of evaporation rate. Small amounts of low-boiling-point solvents like acetone will evaporate in seconds at room temperature, while high-boiling-point solvents like dimethyl sulfoxide need higher temperatures, an air flow, or the application of vacuum for fast evaporation. The boiling point of acetone 56.2°C, methanol 64.4°C, ethanol 78.5°C and dimethyl sulfoxide 189°C which means that the easiest solvent to evaporate is acetone followed by methanol, ethanoland dimethyl sulfoxide.

Table-8: Effect of solvent use.

Solvent	Conclusion
Acetone	Solvent evaporated after one and half hour. Average size 0.246 μm was obtained
Methanol	Formation of aggregation
Ethanol	Formation of aggregation
Dimethyl sulfoxide	Formation of aggregation and solvent not evaporated after one and half hour

Astaxanthin loading capacity and encapsulation efficiency:

The effect of Astaxanthin loading capacity on the nanoparticle size and encapsulation efficiency is shown in Table 9. Particle size of Astaxanthin-loaded PLGA did not vary much with the varying amount of Astaxanthin. The smallest particle size of Astaxanthin loaded nanoparticles was obtained when two percent of Astaxanthin was used. However, the encapsulation efficiency was high in the case of formulations prepared using low amount of Astaxanthin (2%) than those prepared by using higher amount (4, 6, 8 and 10%) of Astaxanthin. The study carried out byTrivedi*et.al.*[22]also revealed that encapsulation efficiency was significantly increased as the polymer amount increased.

Table-9: Astaxanthin loading capacity and encapsulation efficiency.

Drug loading capacity (%)	Average size (µm) ± S.D	Average uniformity ± S.D	Encapsulation efficiency (%) ± S.D
2	0.231 ± 0.006	0.297 ± 0.063	95.49 ± 0.49
4	0.254 ± 0	0.271 ± 0.001	36.62 ± 0.33
6	0.257 ± 0.005	0.273 ± 0.002	37.67 ± 0.15
8	0.239 ± 0.005	0.269 ± 0.001	31.81 ± 0.17
10	0.227 ± 0.001	0.256 ± 0.001	20.63 ± 0.11

S.D.: standard deviation (*n=3*)

Conclusions

The present study investigated the preparation of Astaxanthin-loaded nanoparticles by nanoprecipitation method. Different parameters were tested in order to obtain an optimized formulation. The optimized parameter (polymer type RG 502 50 mg, surfactant type Pluronic F-127 50 mg, stirrer speed 2, acetone as a solvent and 2% Astaxanthin loading capacity) produced Astaxanthin-loaded nanoparticle with an average mean size of 0.231±0.006 and with drug encapsulationof 95.49±0.49%.Nanoparticles were obtained with small average mean size and high encapsulation efficiency. Finally, it can be concluded that production of Astaxanthin-loaded nanoparticles prepared by nanoprecipitation method was possible and allowed production of nanoparticles in an easy and reproducible way.

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Corresponding Author:
M.M.R MeorMohd Affandi[1, 2]*
Email: *meor@puncakalam.uitm.edu.my*