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The Effects of Concentration Ratios on the Particle Size of CHI-ALG-$CaCl_2$ Nanocarriers

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Abstract. Nanotechnology is highly desirable in numbers of industry including cosmeceutical and pharmaceutical. However, the nanoparticle size is greatly varied by the method of synthesising them such as the addition of calcium chloride. Hence, the present study was carried out to investigate the effect of calcium chloride concentration on nanoparticle size when added to the chitosan and alginate nanocarriers. For this purpose, we have performed the preliminary study on the antioxidant activities of Cymbopogon sp. extracts obtained through decoction and infusion method. From this preliminary study, the optimal mixture ratio of extract, including five different ratios of CHI-ALG/$CaCl_2$ nanocarriers which were prepared with ratio of 1:3:0, 1:3:2, 1:3:5, 1:3:10 and 1:3:20 was used to formulate the nanocarrier. Comparing both methods, decoction gives the highest antiradical activity which indicating strong antioxidant potential compared to infusion, are selected to be formulated into nanoparticle. CHI-ALG/$CaCl_2$ nanocarriers were then synthesised to encapsulate antioxidant extracts for topical delivery. Each sample of nanoparticles was evaluated for its size distributions, polydispersity index (PdI) and zeta potential by dynamic light scattering using DelsaMax Pro (Beckman Coulter, USA). The experiment was conducted with five different ratios of CHI-ALG/$CaCl_2$ nanocarriers which were prepared with ratio of 1:3:0, 1:3:2, 1:3:5, 1:3:10 and 1:3:20 in a volume of 50 ml with 1:3:0 acts as a negative control. The best particle size was observed at 1:3:2 mixture ratio with 0.76-fold change (350.47 ± 36.12 nm) from the control (460.03 ± 107.17 nm) which falls in the acceptable range of desirable size of nanoparticles. The nanoparticles produced in this study showed PdI values less than 1, showing good uniformity. The zeta potential value for the all mixture ratios are high (>20 mV) which is able to prevent aggregation of the nanoparticle. As a conclusion, addition of calcium chloride at 1:3:2 to chitosan and alginate has improved the size of nanoparticles containing antioxidant extract of Cymbopogon sp.

1. Introduction

Plant antioxidants exhibit a range of applications as therapeutics in cosmeceutical and pharmaceutical industry [1–4]. The study of antioxidant properties from Cymbopogon genus including Cymbopogon citratus and Cymbopogon nardus are poorly elucidated, hence its exploitation as an antioxidant source is very limited. Cymbopogon sp. comes from the Graminae (Poaceae) family which consists of herbs that are widely known for their traditional medicine properties. Various species of Cymbopogon are considered beneficial for medicinal uses due to its biological activities of their secondary metabolites such as phenolics, alkaloid, saponins and terpenes. Other than their beneficial applications as therapeutic agents, Cymbopogon species also have a commercial value for their ability to grow in a moderate to extreme climatic conditions which explains the wide distribution in the tropical and subtropical regions of Asia, Africa, and America [3].

In particular, the extraction from Cymbopogon sp. has been mainly focused on the recovery of essential oil [3], [5–8] from different extraction methods of Cymbopogon sp. including supercritical solvent extract [9–14], ultrasound-assisted extraction [15–18], Soxhlet [19, 20] and steam distillation [12, 21]. On the other hand, aqueous extraction method such as decoction and infusion study were limited compared to other techniques. One common point in both proposed methods; decoction and infusion, are the used of temperature and time when applied to dried samples. However, both methods are basically introducing dried sample into a vessel with heated ultra-purified water at different techniques. Thus, these techniques reduce solvent consumption and time taken prior to the extraction. The purpose of this process is to select the best method to extract antioxidant compound from the Cymbopogon sp. before subjected into nanoparticle formulation. The selection of this response was justified by conducting DPPH radical scavenging activity of the extracts [22]. The potential use of this antioxidant is then subjected to be considered as most convenient for topical administration. Rationally, this administration may inherent limitation which could potentially be overcome by minimising its particle size. Hence, in this study, the antioxidant from the Cymbopogon sp. will be subjected to nanoparticle formulation. In this work, chitosan-alginate were used as it already has been developed and tested over the past decades [23–31].

Chitosan that is known as biocompatible polysaccharide usually used in food industry [32–37], textiles [38–44] and also medicine [28, 45]. Chitosan is made from chitin by deacetylation [24]. Chitosan can perform ionic interaction with sodium alginate [23]. Calcium alginate was chosen because it does not produce toxicity and is known for its hydrophilic carrier [46]. Coating chitosan with alginate can reduce the interaction between chitosan and extracts [28]. This makes nanoparticle becomes less stable and dissociates better on the application on the skin [47]. However, limitations are found in which the size of the nanoparticle prepared by this technique is too large to be effectively applied on skin [28]. Thus, calcium chloride is used in order to harden the nanoparticle formed [48].

Although many studies have been extensively using chitosan-alginate, the studies on the effect of pH of alginate, sonication time and addition of calcium chloride are limited. pH is known to be played important roles in nanoparticles formulation but usually focusing on pH of chitosan. Adjusting pH values is an efficient method to support the needs of specific application [49]. As for sonication time, Pradhan et. al (2016) said it is important to investigate the sonication time effect on the nanoparticle as it can influence the physico-chemical properties of the nanoparticle [50]. Besides that, calcium chloride addition can help the chain to formed better and showed better distribution and smaller size of nanoparticle [51].

Thus, this present study focused on studying the effects of different formulation and parameters that influence the development of nanoparticle of antioxidant extracts core coated with chitosan.

2. Materials and Methods

2.1. Chemicals

Materials used for decoction and infusion extractions were ultra-purified water by Milli-Q Filtration system. Ultra-purified water was produced in the laboratory by Milli-Q gradient filtration system (Millipore, Bedford, MA, USA). All chemical used in this study were purchased in analytical grade. 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) was obtained from Merck Brand (Merck, Darmstadt, Germany). Other chemicals that were needed for scavenging assay such as gallic acid and ascorbic acid were purchased from Sigma-Aldrich (M) Sdn. Bhd. (Malaysia). Individual standard stock solutions from gallic acid and ascorbic acid were prepared in methanol (Sigma-Aldrich (M) Sdn Bhd., Malaysia) in 400µg/L.

Polymers used for the nanoparticle preparation are chitosan with low molecular weight (50 kDa – 180 kDa) (Sigma-Aldrich (M) Sdn. Bhd., Malaysia) and sodium alginate (Sigma-Aldrich (M) Sdn. Bhd., Malaysia). Other chemicals that were employed to formulate nanocarrier were acetic acid (Sigma-Aldrich (M) Sdn. Bhd., Malaysia), methanol (Sigma-Aldrich (M) Sdn Bhd., Malaysia), sodium

hydroxide (Sigma-Aldrich (M) Sdn. Bhd., Malaysia), calcium chloride (R&M, Malaysia) and hydrochloric acid (Fisher, Malaysia).

2.2. Cymbopogon citratus and Cymbopogon nardus sample preparations

Cymbopogon sp. sample used for this experiment were purchased from Pusat Teknologi Lepas Tuai (PHTC) (Forest Research Institute Malaysia (FRIM), Malaysia). The samples belong to two different species which were *Cymbopogon citratus* and *Cymbopogon nardus.* The grass was collected at their mature state and immediately placed into container, sealed and stored away from sunlight. Only the leaves part was used in this study. The samples were then cut into smaller pieces approximately 1.0 cm to facilitate the drying process performed at 105 °C. The samples were packed in containers made from paper and stored in a dark place for future used.

2.2.1. Aqueous Extraction. Extraction of Cymbopogon sp. were performed on two different methods; decoction and infusion. Extractions were performed at the following basic conditions: For decoction method, 10 g of dried sample was placed in a vessel and 100 mL of ultra-purified water were added. As for infusion, 100 mL of ultra-purified water was boiled. Then, dried sample (10 g) was placed into a vessel with boiled water. The process of extraction was performed for 5 minutes, 15 minutes and 30 minutes for each method. Fresh solvents were let to cool down to the room temperature.

2.2.2. Freeze-drying Process. After filtration process using vacuum filtration system, the obtained extracts were freeze-dried for 4 days. The obtained dry extracts were stored at 4 °C until analysis. The three replicates were collected in separated 100 mL collection bottle with lid. Both Cymbopogon sp. were then mixed in ratios.

2.3. Determination of antioxidant capacity of extracts by DPPH.

The antioxidant capacity of extract was determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Final result was expressed in IC_{50} value. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was determined using modified method. DPPH 0.1 mM was dissolved in methanol. 1.8 mL of freeze-dried extracts were added to 1.2 mL of the methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 minutes. The decrease in absorbance of the solution was monitored at 517 nm by using UV-Visible Spectrophotometer (Shimadzu, Japan). The radical stock solution was prepared on daily basis. Condition which showing the highest antioxidant activity was chosen for further analysis. All tests were carried out in triplicates.

2.4. Preparation of CHI-ALG/$CaCl_2$ Nanocarrier

Sodium alginate and calcium chloride solutions were prepared by dissolving its powders in ultra-purified water in 0.03% w/v and 0.02% w/v, respectively. The pH of sodium alginate was adjusted to 5 by using hydrochloric acid (0.1N). Chitosan flakes was dissolved in 1% of acetic acid and the pH was modified to 5.4 using sodium hydroxide (0.1N).

Calcium chloride was added drop wise to sodium alginate while stirring for 30 minutes. After that, chitosan solution was added to ALG/$CaCl_2$ pre-gel and stirred for 1 hour. The resultant solution was let to equilibrate overnight.

2.5. Preparation of Cymbopogon sp. loaded CHI-ALG/$CaCl_2$ Nanoparticles

The sample (4 mg) was dissolved in 10 mL ultra-purified water then was introduced into the calcium chloride solution (0.02% w/o). Then, the following steps were the repeated as in preparation of CHI-ALG/$CaCl_2$ nanocarrier.

The factors of nanocarrier which is CHI-ALG/$CaCl_2$ ratio was studied. The CHI-ALG/$CaCl_2$ ratio was prepared at 3:1:0, 3:1:2, 3:1:5, 3:1:10 and 3:1:20 ratio. The ratio was prepared by adjusting the concentration calcium chloride.

2.6. Characterisation of Nanoparticles

Particle size distribution and zeta potential of *Cymbopogon* sp. loaded CHI-ALG/$CaCl_2$ nanoparticles was analysed by using dynamic light scattering with Particle Sizer (Beckmann-Counter, USA). The analysis was performed in triplicates at a temperature of 25 °C.

3. Result and Discussion

3.1. Determination of antioxidant activity

Free radical scavenging ability of the Cymbopogon sp. was tested by DPPH radical scavenging assay. The results were demonstrated in Figure 1 and Figure 2.

Figure 1 shows the comparison of antioxidant activities between the standard, gallic acid and ascorbic acid and Cymbopogon sp. that has been extracted by using distilled water. The IC50 of ascorbic acid and gallic acid were 1.71 and 0.86 ug/mL respectively which indicate a really strong antioxidant. It was observed that the extracts of 100N0C for decoction and infusion were 32.30 ± 3.36 and 48.78 ± 10.05, respectively. As for 0N100C, antioxidant activity for both decoction and infusion were 20.54 ± 1.23 and 25.57 ± 2.47. Comparing both methods, decoction of 0N100C is better compared to 100N0C with 0.64-fold, while for infusion 0.52-fold. It can be seen on 0N100C and 100N0C in Figure 1 and Figure 2. Generally, it is shown that the infusion containing lesser antioxidant activity as high as the decoction method. Comparing both methods, there is no considerable difference between decoction and infusion in terms of antioxidant activity in extracts. Furthermore, the Cymbopogon citratus showed a better antioxidant activity as compared to Cymbopogon nardus (0.64-fold and 0.52-fold). It can be seen that mixing both species does not improve the overall antioxidant activities at all. This may be due to the need of specific concentration and ratio to reach the synergistic effect while combining different species of herbs or plants [52]. This is because the synergistic effect is affected by the concentration ratio of the plant to be mixed.

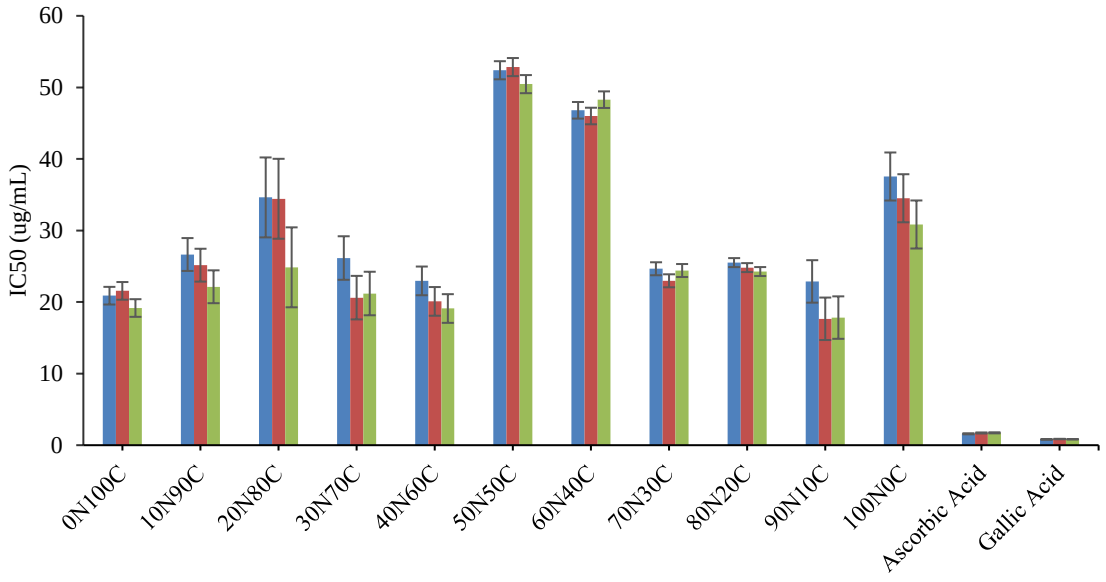

Figure 1. Mixture ratio of decoction extracts

It was observed that the decoction extract of 90N10C showed higher activity; 19.46 ug/mL compared to other decoction extracts. The activity obtained at 0N100C shows a slight decrease in antioxidant, although there were no substantial differences among the extracts obtained at 40N60C with 20.72 ug/mL, this shows that Cymbopogon nardus gives higher antioxidant activities even when combined with Cymbopogon citratus. The lowest activity was measured for the sample obtained at 50N50C which is 51.89 ug/mL. The obtained concentrations were in 50% of Cymbopogon nardus and 50% of

Cymbopogon citratus combined, indicating a weak antioxidant activity. This shows that both species are not compatible for producing synergistic effect at this mixture ratio due to the sensitivity on their ratio proportion [53]. The increase of activity was noticed between the extracts obtained at 60N40C, 100N0C and 20N80C as compared to 50N50C. The increase at 80N20C and became almost similar to the activity of the extracts obtained at 10N90C, 70N30C, and 30N70C which are 24.65, 24.01 and 22.66 ug/mL respectively.

For infusion extracts, the highest activity was measured for the sample obtained at 10N90C compared to other extracts (25.12 ug/mL). The obtained concentrations were in 10% of Cymbopogon nardus and 90% of Cymbopogon citratus combined, indicating a strong antioxidant activity. Other infusion extracts that has almost the same activity as compared to 10N90C are 30N70C, 0N100C and 20N80C. A significant decrease in activity is shown in 80N20C and 90N10C (36.97 ug/mL and 41.13 ug/mL respectively). The activity observed for 60N40C, 70N30C, 40N60C and 100N0C also show only slight different to compare with. The least activity for infusion method was measured in 50N50C which is 68.68 ug/mL hence it is similar to those obtained by 50N50C for decoction. Table 1 shows the comparison of fold-change of antioxidant activity all samples and standards which are gallic acid and ascorbic acid. The activities of all extracts were lower than antioxidant activity of standard compounds, ascorbic and gallic acid.

Table 1. Comparison of fold-change of antioxidant activity between extracts and standards.

Extraction Method	Sample	Standard	
		Gallic acid	Ascorbic acid
Decoction	0N100C	23.88	12.01
	10N90C	28.66	14.42
	20N80C	36.40	18.30
	30N70C	26.35	13.25
	40N60C	24.09	12.12
	50N50C	60.35	30.35
	60N40C	54.69	27.50
	70N30C	27.92	14.04
	80N20C	28.91	14.54
	90N10C	22.63	11.38
	100N0C	39.88	20.06
Infusion	0N100C	29.73	14.95
	10N90C	29.21	14.69
	20N80C	31.74	15.96
	30N70C	29.34	14.75
	40N60C	58.62	29.48
	50N50C	79.86	40.16
	60N40C	60.34	30.35
	70N30C	59.27	29.81
	80N20C	42.99	21.62
	90N10C	47.83	24.05
	100N0C	56.72	28.53

The lower IC_{50} value indicates higher antioxidant capacity and hence comparing both methods of extractions, decoction method gives the higher radical scavenging activity indicating highest activity as compared to infusion. By comparing both species, *Cymbopogon* citratus contain higher antioxidant properties as compared to *Cymbopogon* nardus.

Antioxidant activity can be increased by combining different extracts but in this study, it is limited to those ratios that were conducted in this study which are 0N100C, 10N90C, 20N80C. 30N70C, 40N60C,

50N50C, 60N40C, 70N30C, 80N20C, 90N10C and 100N0C. Thus, the mechanism that is underlying the synergistic interaction needs to be elucidated further in order to perform optimisation of the multi-extract preparation [53]. Hence, the complex synergistic interaction and nature of both species can be explained further, thus maximising the effect of the mixture. One of the critical parameters that are needed to be paid attention is the concentration proportion of the mixture. On top of that, the combinations of the herbs or plant species also need to be considered [53].

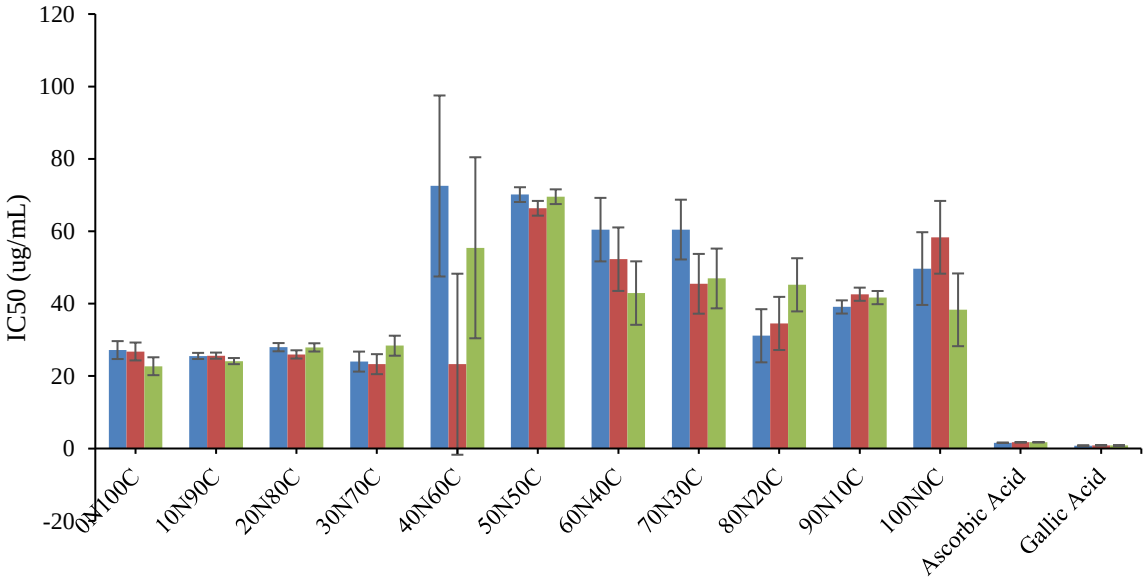

Figure 1. Mixture ratio of infusion extracts

3.2. Effects of nanoparticle preparation on the particle size

The present study was designed to formulate the potential cream containing nanoparticles with antioxidant activity for skin penetration. The nanoparticles are presence in the colloidal system which is difficult to be maintained as they usually agglomerate with time and the fact that sedimentation of nanoparticles may occur. The nanoparticles prepared were free of sedimentation at almost 20 days post preparation. This can be observed visually and has been proven by conducting particle size analysis which has shown larger particle sizes (2675 ± 95 nm).

In this study, the particle size and zeta potential of the nanoparticles formed were studied. The size of the particle is important properties to be analysed because it will determine the ability of the particle to penetrate the skin as applied in the form of cream formulation.

Zeta potential is the parameter measured for sample stability in which a greater value is desirable [13]. The value between |−25 mV| and |−30 mV| of zeta potential is enough to create an energy barrier between the droplets to avoiding coalescence [54]. This analysis is important in cosmetic such as cream product as it might affect the properties like spread-ability of final product [54]. Both of these analyses are important as it serves as critical factor in indicating the efficiency of the process and product [28]. This study was performed to investigate the effect of CHI-ALG/$CaCl_2$ ratio on the nanoparticle size. Chitosan nanoparticles were prepared by ionotropic gelation with the dropwise addition to sodium alginate solution (Alg). The size distributions and zeta potential of particles were measured using DelsaMax Pro (Beckmann-Coulter, USA). Particles with diameter below 800nm are desirable as they have better absorption on the skin [55].

3.2.1. CHI-ALG/$CaCl_2$ ratio.

Table *2* represents the effects of calcium chloride addition on particle size. Previously, it was shown from the result that the nanoparticle sizes were in the desired range regardless of the presence of calcium chloride. It was observed that the presence of calcium chloride did enhance the particle size reduction [25]. This is due to the interaction between Ca2+ and alginate [51]. Therefore, it did act as nanoparticles enhancer. For formulation without calcium chloride which is 3:1:0, the size is 460.03 ± 107.17 nm while for 3:1:2 ratio, the size is 350.47 ± 36.12 nm. The concentration of calcium chloride has decreased the particle size by 1.31-fold. It is also shown that only suitable concentration of calcium chloride helps to decrease the particle size. Therefore, suitable concentration is needed to be determined and minimum ratio of CHI-ALG-$CaCl_2$ for nanoparticle formation should not be higher than 3:1:2. However, without calcium chloride the particle size is still acceptable for human skin penetration which is 2000nm. The increase in particle size due to the increase in the concentration of calcium chloride may be due to the agglomeration of the solution resulting from the changes in the solution pH [49]. The zeta potentials of the formulation prepared was 33.85 ± 2.94 mV the test for 3:1:0 formulations, so the formulations were considered stable to extend the stability test. As we include calcium chloride in 3:1:2, the zeta potential did decrease to 27.26 ± 1.93 mV but it is still in the range that was stable. For 3:1:5 and 3:1:10, the zeta potentials are not in the range but it is also can be considered as stable enough. In contrast, 3:1:20 shows high zeta potential (29.50 ± 4.67 mV) as compared to 3:1:5 and 3:1:10, but the size of the particle is too big (1413.35 ± 382.33 nm) to be considered as the best ratio to be formulated into topical cream.

Table 2. Average particle size of nanoparticles prepared at different CHI-ALG/$CaCl_2$ ratio.

CHI:ALG:$CaCl_2$	Average particle size (nm)	Zeta Potential (mV)
3:1:0	460.03 ± 107.17	33.85 ± 2.94
3:1:2	350.47 ± 36.12	27.26 ± 1.93
3:1:5	697.53 ± 60.82	22.08 ± 7.96
3:1:10	903.10 ± 167.51	18.41 ± 11.17
3:1:20	1413.35 ± 382.33	29.50 ± 4.67

4. Conclusion

The extraction method and ratio of sample were studied to extract major antioxidant properties from *Cymbopogon* sp. Decoction is also known for its low solvent usage. Antioxidant properties positively identified as high in the 10N90C combination while for infusion, the antioxidant activity is at 90N10C. Such extracts have several potential ways of exploitation since they contain radical scavenging activity.

In this study, the nanocarrier formed was based in terms of topical delivery system for cosmetic purposes. Formulation of nanoparticle was studied by varying the concentration ratio of CHI-ALG/$CaCl_2$. The reduced size of nanoparticle achieved in 3:1:2 (350.47 ± 36.12nm) ratio indicates the potential use of calcium chloride in the respective nanoparticles thus allowing it for a better topical application. A full optimisation of nanoparticles size is in progress and will be submitted as separated manuscript.

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