



Physicochemical characterization of astaxanthin-loaded PLGA formulation via nanoprecipitation technique

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ABSTRACT

Astaxanthin is a strong antioxidant with numerous medicinal properties. However, it is highly lipophilic and has low bioavailability which hinders its absorption capabilities. In order to improve the absorption of astaxanthin, the present study evaluates the physicochemical characteristics of astaxanthin-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles prepared via the nanoprecipitation technique. An optimum formulation of nanoparticles was then tested for particle size, zeta potential, encapsulation and entrapment efficiency, morphology, thermal behavior, crystallinity, and compound-polymer interaction. The size of the particles and zeta potential of the formed nanoparticle were reported at 142.23 ± 0.961 nm and -27.3 ± 4.67 mV, respectively. Scanning electron microscope (SEM) study through photomicrogram confirmed the nano-range size of the optimum formulation. The formulation shows low nanoparticle yield but recorded high encapsulation efficiency. Density scanning calorimetry and X-ray diffraction results proved that the nanoparticles were in an amorphous state. Fourier transform infrared spectroscopy analysis had shown that the stretching vibrations of ketone and hydroxyl functional groups were restricted, indicating that the astaxanthin was encapsulated inside the PLGA polymer. These findings provide valuable information regarding astaxanthin bioavailability enhancement through the nanoprecipitation technique.

INTRODUCTION

Free radicals are yields of normal cellular activities. They play an important role in the biological function of humans in which they may assist immunity by mediating cell signaling and play an important role in apoptosis. However, free radicals may lead to the impairment of important macromolecules in cells and have been shown to be involved in carcinogenesis and cardiovascular diseases (Sailaja Rao *et al.*, 2011; Tvrdá and Benko, 2020). Various reports have demonstrated the production of free radicals as an initial response to both pathogenic and

nonpathogenic microorganisms in plants (Macarisin *et al.*, 2010). Although known as mediators of intracellular signaling cascades, excessive production of free radicals may lead to a loss of cell function and eventually apoptosis (Alfarouk *et al.*, 2020). Another condition that is usually caused by the excessive production of free radicals is oxidative stress. Oxidative stress is the discrepancy between free radical development and the body's antioxidant defense capacity (Lobo *et al.*, 2010). Although the human body system is capable of generating its own antioxidants to fight against free radicals, the production will plunge as the human body ages.

Astaxanthin is well known for being a strong antioxidant. The antioxidant capacity of astaxanthin is approximately 10 times stronger than zeaxanthin, lutein, β -carotene, and tunaxanthin and is 100 times greater than α -tocopherol (Inoue *et al.*, 2019). The substance belongs to the xanthophylls group and does not possess a vitamin A activity (Meor Mohd Affandi, *et al.*, 2012; Tolba *et al.*, 2020). Astaxanthin is rich in antioxidant activity, is able to esterify, and is more polar than other carotenoids.

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These are some of the substance's distinctive features owing to the presence of hydroxyl (OH) and ketone group (C=O) on its ionone ring (Meor Mohd Affandi *et al.*, 2011). Numerous reports have illustrated various medicinal potentials of astaxanthin such as chemopreventive effects, anti-inflammatory properties, skin protection from damaging effects of ultraviolet, and the reduction in age-related macular degeneration (Ito *et al.*, 2018; Kavitha *et al.*, 2013; Li *et al.*, 2020; Yasui *et al.*, 2011). Astaxanthin is strongly lipophilic and has poor oral bioavailability, despite being a potent antioxidant, similar to other carotenoids (Yang *et al.*, 2020). There has been a surge of interest among researchers to unravel the best approach to boost astaxanthin absorption in the human body. For diagnostic and therapeutic uses, there are many techniques that can be used in pharmaceutical applications, such as carbon nanotubes, liposomes, micelles, polymer nanoparticles, and metallic nanoparticles. Among them, one promising approach to overcome the low bioavailability of astaxanthin is by incorporating the substance in nanoparticles by using certain polymers (polymeric nanoparticles). Nanoparticles have been reported to enhance drug delivery to the target site, control drug release, and reduce drug-associated adverse effects.

In this study, poly(lactic-co-glycolic acid) (PLGA) was selected due to its biodegradable, biocompatible, and nontoxic characteristics (Fredenberg *et al.*, 2011). Previously, we have successfully formulated astaxanthin-loaded PLGA nanoparticles (average mean size of 0.231 ± 0.006) through the nanoprecipitation technique (Ku Aizuddin *et al.*, 2014). However, the development of an optimized formulation is not sufficient without the support of a physicochemical properties study. Conducting physicochemical characterization of nanoparticles is detrimental to uncover the reaction of the biological system toward these nanosized particles, particularly the size and surface area of nanoparticles which are two prominent deciding factors for nanoparticle's fate in the body. Absorption, distribution, retention, and removal of nanoparticles are dependent on particle size and surface which are important in their formulation. Other than that, the structural state of nanoparticles is also important to determine their solubility and, by an extent, their bioavailability. Nanoparticle characterization is also valuable in the industry for scaling up (Raval *et al.*, 2019). A previous physicochemical study has used the emulsion solvent evaporation technique but to the best of our knowledge the nanoprecipitation technique is yet to be explored (Hu *et al.*, 2019). Therefore, this study is aimed to evaluate the physicochemical characterization of the newly designed nanoparticles proposed for the oral route of administration. Assessment on entrapment efficiency, nanoparticle recovery, morphology, particle size, zeta potential, thermal properties, x-ray diffraction (XRD), and fourier transform infrared (FTIR) spectroscopy was conducted. The findings of this study will provide important information on the physicochemical characterization of astaxanthin-loaded PLGA formulation via nanoprecipitation technique as an alternative method to improve astaxanthin bioavailability.

EXPERIMENTAL METHOD

Materials

Astaxanthin was purchased from Sigma-Aldrich Chemical (St. Louis, MO). PLGA with a molecular weight ranging from 7,000 to 240,000 kDa (lactide:glycolide 50:50) was supplied

by Boehringer Ingelheim (Ingelheim, Germany). The surfactant Pluronic F-127 and analytical grade acetone were procured from Sigma-Aldrich Chemical and Merck (Darmstadt, Germany), respectively.

Preparation of nanoparticles

The nanoprecipitation technique was used to prepare astaxanthin-loaded PLGA as discussed in our previous work (Ku Aizuddin *et al.*, 2014). The surfactant was dissolved in water as an aqueous phase. Meanwhile, PLGA and astaxanthin were dissolved in acetone as an organic phase. In a dropwise manner (1 ml second^{-1}), the aqueous phase was added to the organic phase and mixed with a magnetic stirrer at the speed of 2. Then, the solvent was left evaporate for 90 minutes. For 15 minutes, the freshly prepared nanoparticles were then centrifuged twice at 5,000 rpm. The pellet was washed twice with 5 ml distilled water in which the astaxanthin, polymer, and surfactant were dissolved.

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Particle size and zeta potential

The particle size, polydispersity index (PDI), and zeta potential were evaluated by using the Zetasizer 1,600 (Malvern Instruments Ltd, Malvern, UK). Both size and zeta potential determination was carried out in triplicate following the dispersion of nanoparticles in distilled water at 25°C . The average particle size was expressed in mean diameter and the reported value was represented as $\pm\text{SD}$ ($n = 3$).

Scanning electron microscope (SEM)

The morphology of astaxanthin biodegradable nanoparticles was analyzed with environmental SEM (FEI Quanta 450 FEG). One drop of the dispersed nanoparticles was placed on the carbon-coated grid. The test was carried out at room temperature.

Nanoparticle yield and entrapment efficiency

Freeze-dried astaxanthin-loaded PLGA nanoparticles were accurately weighed and the nanoparticle recovery (%) was calculated using Equation (1). Meanwhile, the entrapment efficiency of astaxanthin-loaded PLGA nanoparticles was also calculated using Equation (2). All calculations and values were made in triplicate.

- (1) Nanoparticle recovery (%) = $(\text{mass of nanoparticle}) / (\text{mass of polymer, surfactant and drug used}) \times 100$
- (2) Entrapment efficiency (%) = $(\text{mass of drug in nanoparticle}) / (\text{mass of drug used in formulation}) \times 100$

Differential scanning calorimetry (DSC)

Astaxanthin-loaded PLGA nanoparticles were analyzed with DSC 8,500 (Perkin Elmer Inc., America). Powdered PLGA, pure astaxanthin, and astaxanthin-loaded PLGA nanoparticles were placed in a separate pan and their tested weight was recorded. The samples were then crimped before the test resumed. The entire DSC tests were carried out in triplicate (scanning rate: $10^\circ\text{C second}^{-1}$, $0^\circ\text{C} - 250^\circ\text{C}$). The flow of nitrogen gas is fixed at $20 \text{ ml second}^{-1}$.

Powder XRD study

XRD analysis was completed on an X-ray diffractometer (Ultima IV, Rigaku, Japan) using K 2α rays with a current of 20 mA and a voltage of 40 kV in flat plate geometry of 2 θ , ranging from 3 to 70 $^{\circ}$, and a scan rate of 20. Pure astaxanthin, PLGA, and astaxanthin-loaded PLGA nanoparticles were tested and analyzed.

FTIR spectroscopy

The functional group of astaxanthin-loaded PLGA nanoparticles was determined using FTIR. Pure astaxanthin, unprocessed PLGA, and freeze-dried astaxanthin-loaded PLGA nanoparticles were mixed with KBr at the ratio of 1:3, respectively. The mixture was then pressed into a pellet using manual hydraulic press. The disks were then analyzed by FTIR (Varian 640-IR). FTIR spectra were recorded as a mean of 16 scans, in the spectral region ranging from 400 to 4,000 cm^{-1} .

RESULTS AND DISCUSSION

Particle size and zeta potential analysis

Nanoparticles can be defined as any particulate that recorded particle size ranging from 1 to 1,000 nm (Zhao *et al.*, 2014). In order to ascertain the particle size of an optimal formulation, it is crucial to evaluate its size and morphological characteristics. Zeta potential is the surface charge value of a particle of interest. Generally, the more positive or negative the value, the more stable the system. As shown in Table 1 and Figures 1 and 2, the particle size of astaxanthin-loaded PLGA was recorded to be at 142.23 ± 0.961 nm and the zeta potential value was -27.3 ± 4.67 mV. Both values indicated that the astaxanthin nanoparticle particle size was in the nano-range and showed good colloidal stability. These results coincided with a previous study on polymer-based nanoparticles (Mora-Huertas *et al.*, 2010). Due to the high-energy barrier between particles, a zeta value of less than -10 mV granted good colloidal stability.

SEM

The morphology analysis was conducted on the newly developed nanoparticles. The SEM photomicrograph in Figure 3 shows that astaxanthin-loaded PLGA nanoparticle size was in between 138.5 and 251.2 nm. Most of the particles were found

Table 1. Analysis by Zetasizer of particle size, zeta potential, and PDI of astaxanthin-loaded PLGA nanoparticle ($n = 3$).

Particle size (nm)	Zeta potential (mV)	PDI
142.23 ± 0.961	-27.3 ± 4.67	0.146 ± 0.023

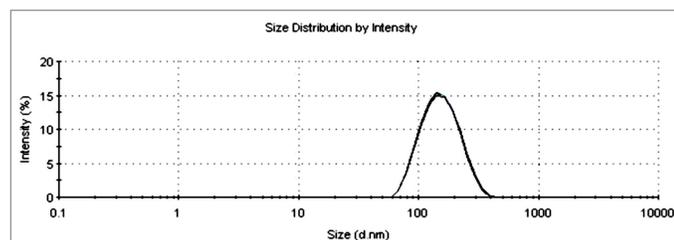


Figure 1. Particle size analysis of astaxanthin-loaded PLGA nanoparticles ($n = 3$).

to be spherical in shape with a smooth surface. These results are consistent with the particle size analysis results reported earlier.

Nanoparticle yield and entrapment efficiency

The yield of nanoparticle and its entrapment efficiency was analyzed to determine the mass of the nanoparticle formed and the efficiency of astaxanthin that is entrapped by PLGA. It was found that the yield achieved for nanoparticle was $18.9\% \pm 1.08\%$. Despite the low yield reported, the entrapment efficiency showed a high value of $84.375\% \pm 0.002$ ($n = 3$). Based on the high entrapment efficiency, astaxanthin is suitable to be incorporated into PLGA to form polymeric nanoparticle. This correlates with the study conducted by Mittal *et al.* (2007), which showed that even at 67% entrapment efficiency of a drug with almost similar nanoparticle size could result in a significant improvement in drug absorption.

DSC

DSC is a convenient method to explore the thermal activity of the formulation by providing information on the crystallinity of a particular drug in a system. The analysis was carried out with the curve as a function of temperature instead of time. As shown in Figure 4, pure astaxanthin has the endothermic peak of $227.93^{\circ}\text{C} \pm 0.366^{\circ}\text{C}$, while unprocessed PLGA was at $47.54^{\circ}\text{C} \pm 0.563^{\circ}\text{C}$. However, in the nanoparticle sample, only the

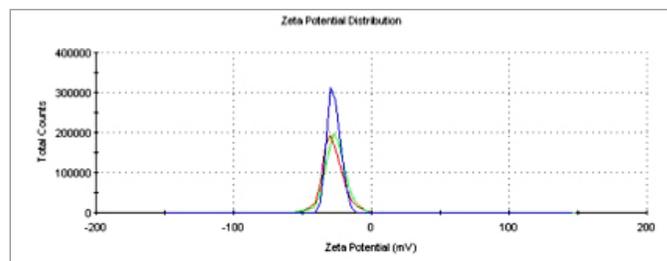


Figure 2. Zeta potential of astaxanthin-loaded PLGA nanoparticles ($n = 3$).

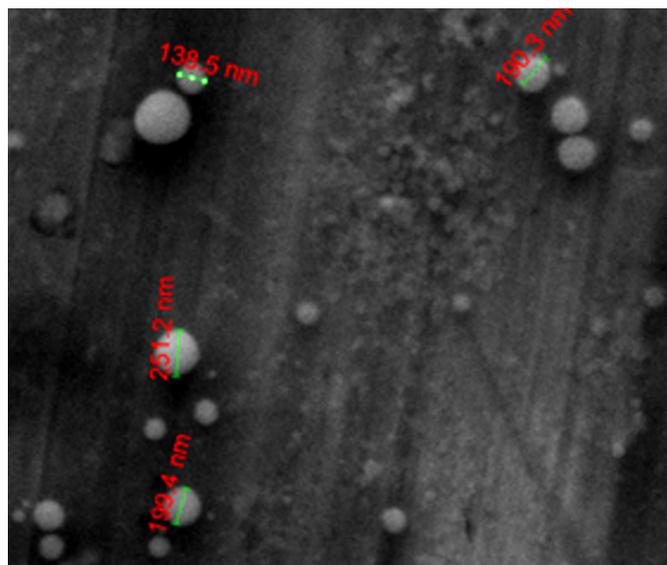


Figure 3. SEM analysis of astaxanthin-loaded PLGA nanoparticles.

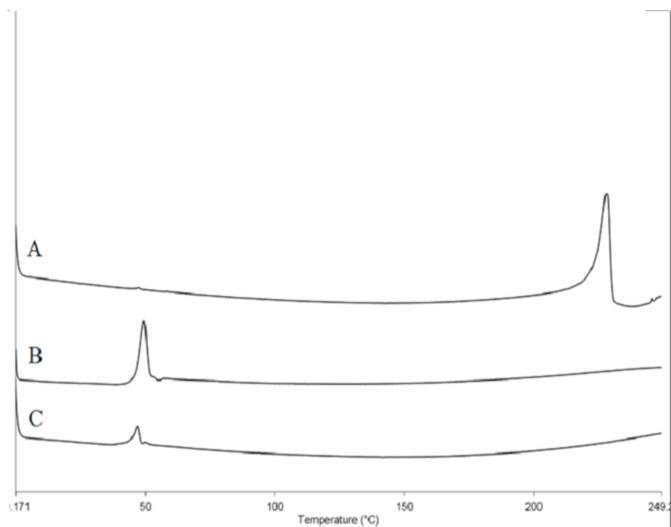


Figure 4. DSC thermogram representing (A) pure astaxanthin, (B) unprocessed PLGA, and (C) astaxanthin-loaded PLGA nanoparticles.

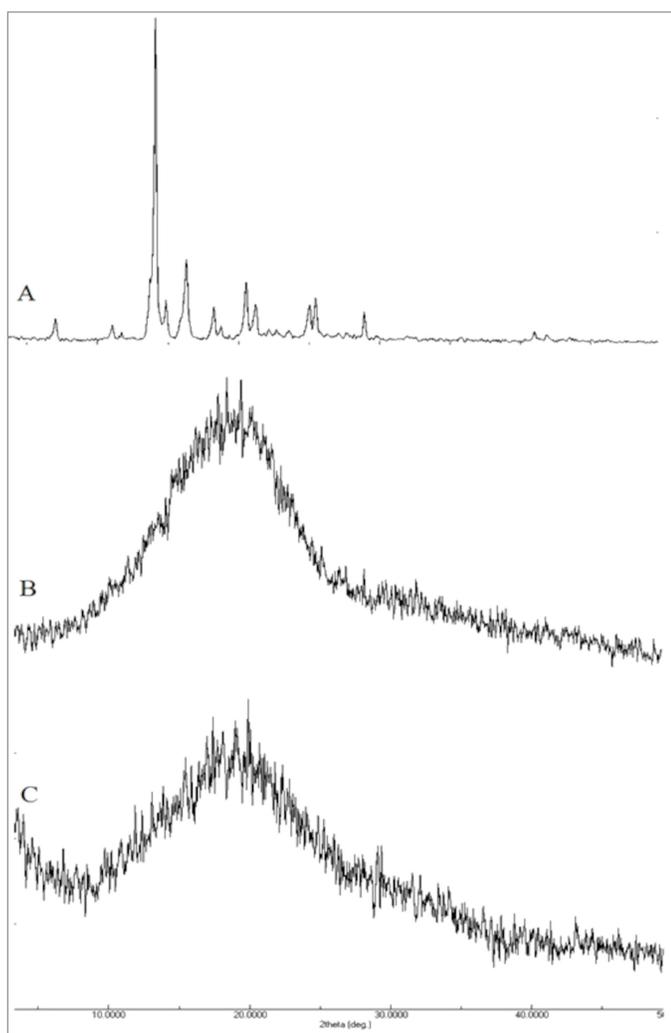


Figure 5. XRD analysis of (A) pure astaxanthin, (B) unprocessed PLGA, and (C) astaxanthin-loaded PLGA nanoparticles.

PLGA peak was present while the astaxanthin peak was absent. The PLGA melting point in the nanoparticle sample was recorded at 46.74 ± 0.165 , almost a similar temperature as in unprocessed PLGA. The absence of astaxanthin's peak in the nanoparticle curve suggests that the astaxanthin in the system was in the amorphous state or disordered-crystalline phase of a dispersion (Surolia *et al.*, 2012). This phenomenon where the hydrophobic compound or drug entrapped in PLGA becomes amorphous is consistent with the findings reported by other authors (Averineni *et al.*, 2012; Crucho and Barros, 2017).

Powder XRD study

XRD analysis provides critical information on the crystallinity degree of the formulation, thereby complimenting the DSC analysis results. Figure 5 summarizes the results obtained from the XRD analysis. Astaxanthin showed a highly crystalline structure which is characterized by intense peaks. However, these peaks were diminished in astaxanthin-loaded PLGA nanoparticles. The diminished peaks were due to the fact that the drug has changed from being crystalline to being in an amorphous state in the matrix of the polymer (Sun *et al.*, 2015). This confirmed the results derived from the DSC analysis. In a study carried out by Kim *et al.* (2008), the amorphous state formed in nanoparticles showed significantly higher solubility compared to unprocessed drug and thereby may improve its bioavailability (Jog and Burgess, 2017).

FTIR spectroscopy

FTIR analysis made it possible to identify several functional groups present in astaxanthin compound and PLGA

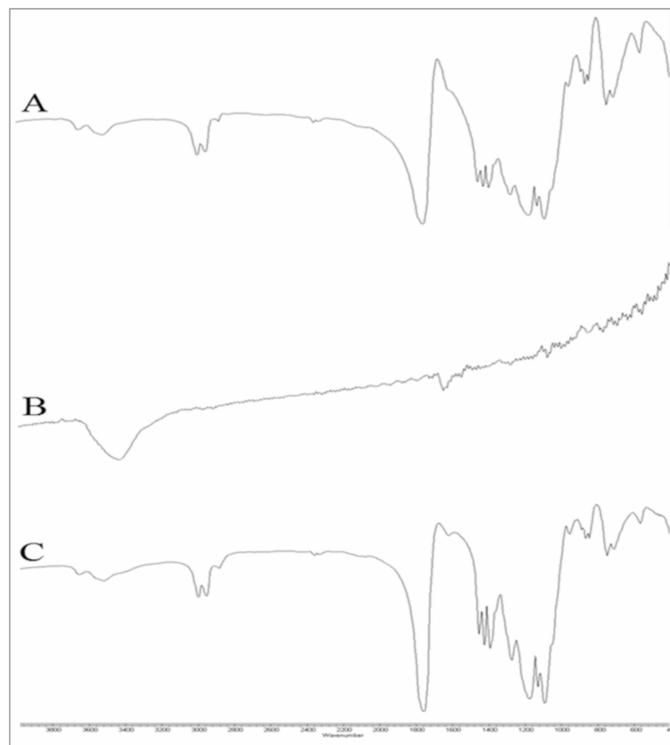


Figure 6. FTIR analysis of (A) unprocessed PLGA, (B) pure astaxanthin, and (C) astaxanthin-loaded PLGA nanoparticle.

polymer. As shown in Figure 6, there was a broad peak recorded at $3,431.909\text{ cm}^{-1}$, indicating the presence of the OH group in the astaxanthin compound. There was also a peak recorded at $1,653.8\text{ cm}^{-1}$ which indicates the presence of a ketone (C=O) group. Additionally, PLGA was shown to have a peak at $1,426.611\text{ cm}^{-1}$ which corresponds to the C–H deformation in the $-\text{O}-\text{CH}_2-$ structure, which is present only in the glycolic acid structure of PLGA (Kang *et al.*, 2008; Tavakoli *et al.*, 2015). As for the astaxanthin-loaded PLGA nanoparticles, the intensity of peaks that identify the purity of astaxanthin was almost diminished. Because of the environment of the compound, the wavenumber of the ketone group was slightly shifted from its normal range ($1,710-1,665\text{ cm}^{-1}$). This happens when there is another electronegative element attached to the neighbor carbon, and it is most likely that the electron cloud of the element will be affected by the vibration of the interest functional group through Van der Waals repulsion. The same condition was reported by Joshi *et al.* (2014) and Yuan *et al.* (2008) where peaks for the drug diminished after the formation of nanoparticle. The phenomenon occurs due to the restriction on the stretching vibrations of bonds in the ketone and OH group in the polymeric nanoparticle. This finding suggests that optimal incorporation of astaxanthin into PLGA nanoparticle was achieved.

CONCLUSION

This research focused on the analysis of the physicochemical characteristics of astaxanthin-loaded PLGA nanoparticles. Optimum formulation of astaxanthin-loaded PLGA recorded a particle size of $142.23 \pm 0.961\text{ nm}$ with a zeta potential value of $-27.3 \pm 4.67\text{ mV}$. The nano-range size of the optimum formulation was further confirmed by SEM photomicrogram. Nanoparticle yield and entrapment efficiency were recorded at $18.9\% \pm 1.08\%$ and $84.375\% \pm 0.002$ ($n = 3$), respectively. DSC thermograph of astaxanthin-loaded nanoparticle was diminished, while XRD study confirmed that the nanoparticles were in an amorphous state. FTIR analysis revealed that the astaxanthin was actually encapsulated inside PLGA, restricting the stretching vibration of the ketone and OH functional group. Results from these studies suggest that the astaxanthin-loaded PLGA nanoparticles can have the potential to enhance astaxanthin bioavailability.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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