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SPECIAL ISSUE: ORIGINAL PAPER

Novel Analytical Approaches towards SDGs

Antioxidants encapsulated milk-derived exosomes for functional food development

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Abstract

Reactive oxygen species are known to be involved in various diseases, and antioxidant ingredients are expected to essentially prevent diseases and contribute to improving health. However, antioxidants are easily degraded by enzymes before being absorbed in the intestine, so a means of transport that prevents their degradation in the body is necessary. Exosomes, which play an important role in communication between individual cells, have attracted attention as a new transport carrier of miRNA and DNA, but not yet fully exploited in food research. More recently, exosomes extracted from bovine milk began to be widely used as a cost-effective transport carrier not in clinical medicine but also in functional food materials. To develop practical applications as carriers for functional foods, systematic studies are necessary to clarify the introduction efficiency and the properties of encapsulated substances. In this study, we applied electroporation and incubation to encapsulate antioxidants into the exosomes and studied the encapsulation efficiency into the exosomes and the anticancer activity.

Keywords Antioxidants · Milk-derived exosomes · Functional foods

Introduction

Antioxidants are expected to inhibit the generation and function of reactive oxygen species and are applied to functional foods [1]. Reactive oxygen species are highly reactive, and it has been reported that their excessive generation in the body causes cancer, arteriosclerosis, and other diseases due to mis-transcription caused by DNA damage and hardening caused by peroxidation of cell membranes [2]. Therefore, it is important to maintain the balance of reactive oxygen species in the body by taking antioxidants. However, antioxidants are difficult to be effective even when ingested due to their low absorption rate [3]. For example, approximately, 50% of β-carotene is degraded to retinol, which has low antioxidant properties, by β-carotene-15,15′-monooxygenase in small intestinal absorption epithelial cells [4]. Methods to prevent degradation are needed to increase absorption of antioxidants.

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As a method to improve the absorption rate of antioxidants, the use of transport carriers to protect antioxidants has been studied so far. Liposomes, which can encapsulate substances in a phospholipid bilayer, are useful transport carriers that can prevent degradation of antioxidants in the body [5]. Takahashi et al. observed a fivefold increase in the area under the concentration–time curve of curcumin in plasma after oral administration by introducing curcumin into liposomes [6]. On the other hand, unmodified liposomes are unstable in blood [7]. Although surface modification has been reported to improve stability, there is concern that it is not cost-effective [8].

Exosomes have attracted attention as a transport carrier that can overcome these issues (Fig. 1). Exosomes are 50–150 nm biogenic vesicles with a phospholipid bilayer that float in body fluids [9]. Recently, milk-derived exosomes have been reported [10]. Compared to cell-derived exosomes obtained from cancer patients or cancer cells, milk-derived exosomes are expected to be safer and more cost-effective carriers because they are obtained from commonly consumed foods Munagala et al. have introduced anthocyanidins into milk-derived exosomes and demonstrated increased stability in vitro and in vivo [11]. Not only antioxidants but also anticancer applications have been reported, and milk-derived

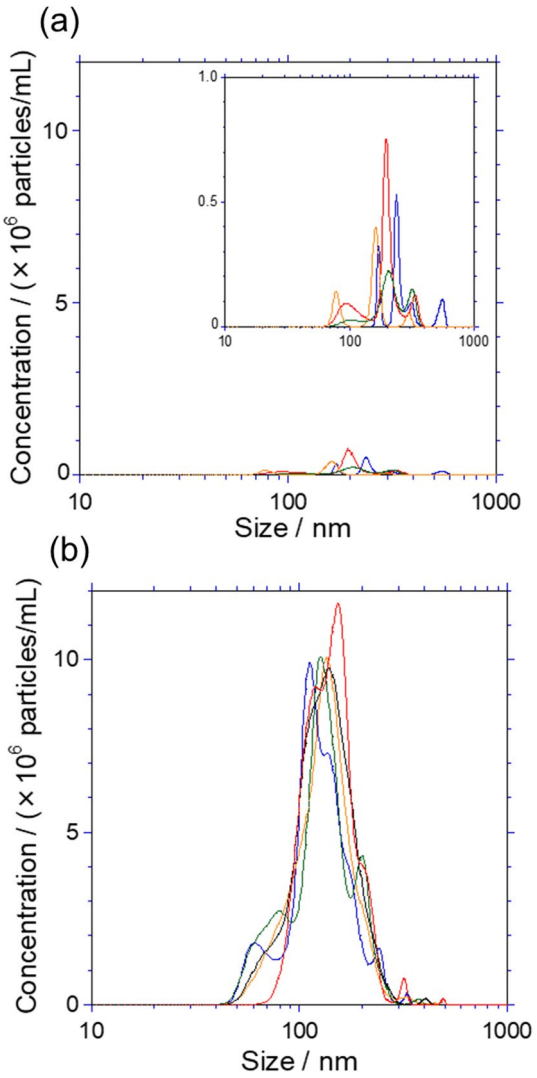

Fig. 1 Size distribution of exosomes extracted from **a** a culture supernatant of MDA-MB-231 cell and **b** a raw milk. The inserted figure in **a** shows a magnified image of the size distribution. Each line drawing in different color indicates the different samples. (Number of samples: 5)

exosomes could be an inexpensive alternative transport carrier to liposomes [12, 13].

The incubation method is widely used for introducing substances into exosomes. This method utilizes the hydrophobic interaction between the introduced substance and the phospholipid bilayer of exosomes, and does not require special equipment or complicated operations because all that is required is to mix the introduced substance and exosomes. On the other hand, previous studies have focused on the introduction of the target substance, and the amount introduced into exosomes has been low. In a study of paclitaxel introduction into milk-derived exosomes, the introduction efficiency using the electroporation and sonication methods was 5.3% and 28.29%, respectively, while the incubation method was low at 1.44% [14]. However, the electroporation and the sonication methods potentially change the exosomes' characteristics including the size and the zeta potential. Although there might be some relationship between the loading capacity and the characteristics changes, the possibility of flip-flop and phase transition of the exosomes' lipid membrane cannot be denied. In order to improve the amount of transfection, it is necessary to investigate the relationship between the nature of the transfected substance and the amount of transfection.

Therefore, in this study, we focused on the partition coefficient of the transfected substances and aimed to investigate the relationship with the efficiency of exosome transfection. Antioxidants (astaxanthin, α-tocopherol, curcumin, chlorogenic acid, β-carotene, rutin, and resveratrol (Table S1)) with different *n*-octanol/water partition coefficients, which is an index of hydrophobicity of the introduced substances, were introduced into milk-derived exosomes using incubation method. The amount of introduction was evaluated according to the distribution coefficient. Furthermore, we aimed to evaluate exosomes as transport carriers by measuring the long-term storage stability and antioxidant capacity of exosomes with introduced antioxidants.

Experimental

Cell culture

For cell culture, Dulbecco's Modified Eagle Medium (DMEM) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with heat-inactivated 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific K. K., Tokyo, Japan) was used.

Human breast cancer cell lines, MDA-MB-231, were seeded in 25 cm^2 flasks (Thermo Fisher Scientific K. K.) and cultured in a CO_2 incubator (MCO-170AICUV-PJ, PHC Holdings Corporation, Tokyo, Japan) at 37 °C and 5% CO_2. The cells were checked with an inverted microscope (ECLIPSE Ts2; Nikon Corporation, Tokyo, Japan), trypsinizing and replating when the cells are sub-confluent, 60–80%, as shown in Figure S1.

Exosome extraction

For cell-derived exosome extraction, the cell culture supernatant was centrifuged at 3000 × *g* for 15 min at 4 °C to remove cell debris and apoptotic bodies, and then, filtered through a 0.22 μm filter (Millex-GS Syringe Filter, Merck KGaA, Darmstadt, Germany) to remove microvesicles. This solution was transferred to an ultracentrifuge bottle (334105A, Eppendorf Himac Technologies Co., Ltd., Hitachinaka, Japan) and ultracentrifuged at 110,000 × *g* for

80 min at 4 °C (CS100GXL, Eppendorf Himac Technologies Co., Ltd.). After removing the supernatant, 1 mL of PBS was added to the ultracentrifugation bottle and the inner wall of the bottle was washed 10 times with a pipette. Nine mL of PBS was added 9 separate times to collect the non-specifically adsorbed exosomes on the wall. The recollected solution was ultracentrifuged at 110,000 × *g* for 80 min at 4 °C. After the supernatant was removed, the exosomes pellet was suspended with 200 µL of PBS and transferred to a protein low suction tube and stored at − 80 °C before use.

For milk-derived exosome extraction, 40 mL of commercially available raw milk (Kyushu Nyugyo, Oita, Japan, pasteurized for 2 s at 130 °C) was dispensed into 50 mL centrifugation tube and incubated for 30 min at 37 °C. 400 µL of acetic acid (FUJIFILM Wako Pure Chemical Corporation) was added into the milk and incubated for 10 min at 37 °C, and then, centrifuged at 10,000 × *g* for 10 min at 4 °C. The whey was transferred to another 50 mL centrifugation tube and centrifuged again at 10,000 × *g* for 10 min at 4 °C. The whey was filtered through a pore size of 0.22-µm filter and ultracentrifuged at 110,000 × *g* for 80 min at 4 °C. After removing the supernatant, 1 mL of PBS was added to another ultracentrifugation bottle and the inner wall was washed 10 times with a pipette. 9 mL of PBS was added 9 separate times to collect the non-specifically adsorbed exosomes on the wall. The recollected solution was ultracentrifuged at 110,000 × *g* for 80 min at 4 °C. After the supernatant was removed, the exosomes pellet was suspended with 200 µL of PBS and transferred to a protein low suction tube and stored at − 80 °C before use.

Characterization of exosomes

AFM observations were performed by a scanning probe microscope (Dimension Icon, Bruker Japan K.K., Kanagawa, Japan) with a silicon probe (PPP-NCH, NANOSENSORS™, Neuchatel, Switzerland) (Tip radius of curvature < 10 nm, Resonance frequency: 330 kHz). The scan rate was set to 2 Hz in tapping mode. The mica was attached on the metal plate by a double-sided tape and the new layer was cleaved by scotch tape for the sample deposition. In an inclined petri dish, 500 µL of 3-aminopropyltriethoxysilane (Tokyo Chemical Industry Co., Ltd.) was dropped at the bottom and exposed the mica to the vapor for 1 h to chemically modify the mica surface. Then, 2 µL of the sample was dropped onto the mica. The sample was dried by mild air blow using an air gun and kept in a drying desiccator overnight, and then, provided as an observation sample.

Nano Sight (Nano Sight NS300, Malvern Panalytical Ltd., Malvern, United Kingdom) was used to measure the concentration and the size of exosomes. The operation was performed by following the supplier's instruction, and the camera level and detection threshold was set as 13 and 11, respectively. Protein concentration included in the exosomes was determined by TaKaRa BCA Protein Assay Kit (T9300A, Takara Bio Inc., Shiga, Japan) using the plate reader.

Encapsulation of FITC-Dextran and antioxidants into exosomes

Exosomes with a total protein concentration of 20 µg/mL and 10 µL of 3.5 mg/mL FITC-Dextran (F0918; Tokyo Kasei Kogyo) were used for the encapsulation experiments by electroporation. Electroporation buffer containing OptiPrep™ (Cosmo Bio Co., Ltd., Tokyo, Japan) was prepared following the supplier's instruction. 70 µL of the exosome solution was mixed with 1 µL of the FITC-Dextran and transferred to a 1-mm electrode gap cuvette (EC-001, Nepa Gene Co., Ltd, Chiba, Japan). Electroporation was executed using a function generator (FG120, Yokogawa Electric Corporation, Tokyo, Japan) through a power amplifier (HSA4101, NF Corporation, Yokohama, Japan). Only a single pulse with 50-ms pulse width was applied under a different electric field from 500 to 1500 V/cm. The electroporated samples were ultrafiltered to remove the FITC-Dextran which was not encapsulated into the exosomes by an ultrafiltration (UFC510096, Merck KGaA, Darmstadt, Germany). After ultrafiltration, the concentrated solution was transferred to a 96-well microplate (675086, Greiner Bio-One International GmbH, Tokyo, Japan), and the fluorescence intensities were measured by a plate reader (T9300A, Tecan, Männedorf, Switzerland) at an excitation wavelength of 488 nm and a fluorescence wavelength of 530 nm.

Antioxidants, astaxanthin, α-tocopherol, chlorogenic acid, β-carotene, rutin, and resveratrol were all purchased from FUJIFILM Wako Pure Chemical Corporation. 10 mg of the antioxidants were dissolved in 10 mL of ethanol-acetonitrile (1:1, *v*/*v*) solution. 50 µL of the antioxidant solution was mixed with 500 µL of milk-derived exosomes prepared at a protein concentration of 20 µg/mL and incubated at 37 °C for 60 min. The solution was transferred to an ultracentrifuge bottle and 10 mL of PBS was added, and then, ultracentrifuged at 135,000 × *g*, 4 °C for 90 min to remove unencapsulated antioxidants. After the supernatant was removed, the ultracentrifuged exosomes were extracted by washing with 500 µL of 1×PBS and the absorbance and the fluorescence for the quantification of encapsulated antioxidants into exosomes were measured. For absorbance measurements, 476 nm, 453 nm, and 430 nm were used for astaxanthin, β-carotene, and curcumin quantification, respectively, using the plate reader. Chlorogenic acid, rutin, and resveratrol encapsulated into exosomes was quantified by the absorbance at wavelengths of 267 nm, 350 nm, and 350 nm, respectively, using a spectrophotometer (V-560, JASCO Corporation, Tokyo, Japan). α-Tocopherol was quantified by

a spectrofluorometer (FP-8300, JASCO Corporation) at excitation wavelength of 290 nm and fluorescence wavelength of 322 nm. To evaluate the antioxidant capacity, Hydroxyl Radical Antioxidant Capacity (HORAC) assay (Oxford Biomedical Research, Inc., Rochester Hills, MI) was performed by following the supplier's instruction.

MTT assay for cancer cells

MDA-MB-231 cells were used for a cancer cell antiproliferation experiment of β-carotene-encapsulated exosomes by the MTT assay. The cells were seeded in 25 cm^2 flasks and cultured in a CO_2 incubator at 37 °C with a CO_2 concentration of 5%, in DMEM medium with 10% FBS. 100 µL of the medium, 50 µL of PBS and freshly prepared β-carotene-encapsulated exosomes were added in the cell solution of 5.0×10^3 cells/well, and then, incubated for 72 h. MTT assay was performed by following the supplier's instruction.

Results and discussion

Characterization of milk-derived exosomes

To confirm and quantify the exosomes extracted from raw milk, the size, the concentration, and the total protein concentration were measured and compared with the cell-derived exosomes. As shown in Fig. 1a and Table 1, the size distribution of cell-derived exosomes had a peak at 110–120 nm and the mean size was 189 ± 5 nm. On the other hand, the size distribution of milk-derived exosomes peaked at 100–110 nm with a mean diameter of 139 ± 1 nm (Fig. 1b). AFM images shown in Figure S2 also support the above size data. The exosome concentration, in which the most significant differences were observed, were $(2.25 \pm 0.07) \times 10^7$ particles/mL and $(1.27 \pm 0.03) \times 10^9$ particles/mL for the cell-derived and the milk-derived exosomes, respectively. The total protein concentration including membrane protein was 0.508 ± 0.001 µg/mL and 35.17 ± 0.09 µg/mL for the cell-derived and the milk-derived exosomes, respectively. The total protein contained in a single exosome can be estimated as $(2.26 \pm 0.07) \times 10^{-7}$ µg/exosome and $(2.77 \pm 0.06) \times 10^{-7}$ µg/exosome for the cell-derived and the milk-derived exosomes, respectively, if we assume that all the exosome contains the equal amount of the protein. This result suggested that all the proteins except that contained within exosomes in the raw milk such as casein and albumin could be removed by the pretreatment and the higher extraction of the milk-derived exosomes compared with the cell-derived exosomes. Munagala et al. extracted 335 mg of proteins contained in exosomes from 1 L of raw milk, which is about 10 times amount of our study [10]. It has been reported that the removal rate of proteins contained in raw milk is relatively low by centrifugation alone [15, 16]. Although they used only $13{,}000 \times g$ ultracentrifugation for the extraction, the pH of the raw milk sample was lowered to the isoelectric point of casein, which is a main component of milk protein, and removed the proteins by ultracentrifugation at $10{,}000 \times g$ in this study. Therefore, exosomes solution containing fewer contaminants of mill-derived proteins could be obtained in our method compared to the other study [10].

Encapsulation of substances into exosomes

Electroporation is one of the major techniques to encapsulate foreign substances into exosomes, liposomes, and cells. The optimum electroporation condition for encapsulation of foreign substances into milk-derived exosomes was investigated to compare with the following incubation method. Figure 2 shows the results of encapsulating FITC-Dextran (molecular weight: 10,000) into exosomes by electroporation under several electric field condition, but no clear increase of the encapsulation was observed under these electric field condition from 500 to 1500 V/cm. The electric field of 1000 V/cm was widely used for this purpose [17, 18], but the encapsulation efficiency was low and the reproducibility was poor. It has been reported that the application of electric field causes irreversible membrane rupture of exosomes, which may reduce the encapsulation efficiency and the reproducibility [19]. The paper also reported that the concentration of exosomes in the sample solution affected the encapsulation efficiency in the electroporation method, and that a high encapsulation efficiency was achieved at exosome concentrations of 0.25–1.0 mg/mL. In this study, the exosome concentration was relatively low, ~ 20 µg/mL, and thus, the low encapsulation efficiency could be reasonable using electroporation. Therefore, in this study, we decided not to use electroporation method for encapsulating the antioxidants into the exosomes.

Table 1 Characterized data of cell- and milk-derived exosomes

Sample	Mean diameter/nm	Concentration/($\times 10^5$ particles/mL)	Total protein concentration/(µg/mL)
Cell-derived exosomes	189 ± 5	2.25 ± 0.07	0.508 ± 0.001
Milk-derived exosomes	139 ± 1	127 ± 3	35.17 ± 0.09

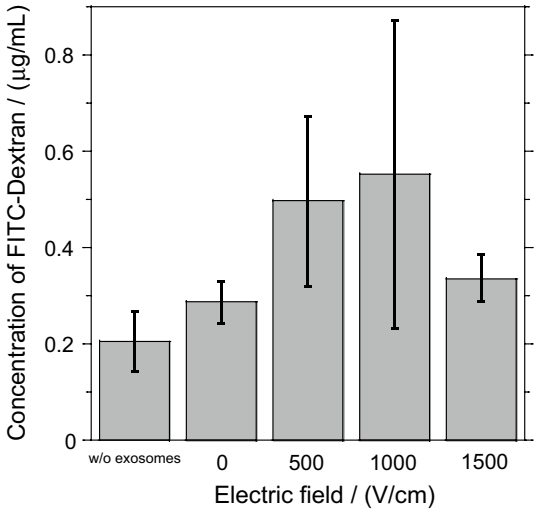

Fig. 2 Encapsulation of FITC-Dextran (MW: 10,000) into exosomes by electroporation method. The error bars show the standard deviation of 4 different samples

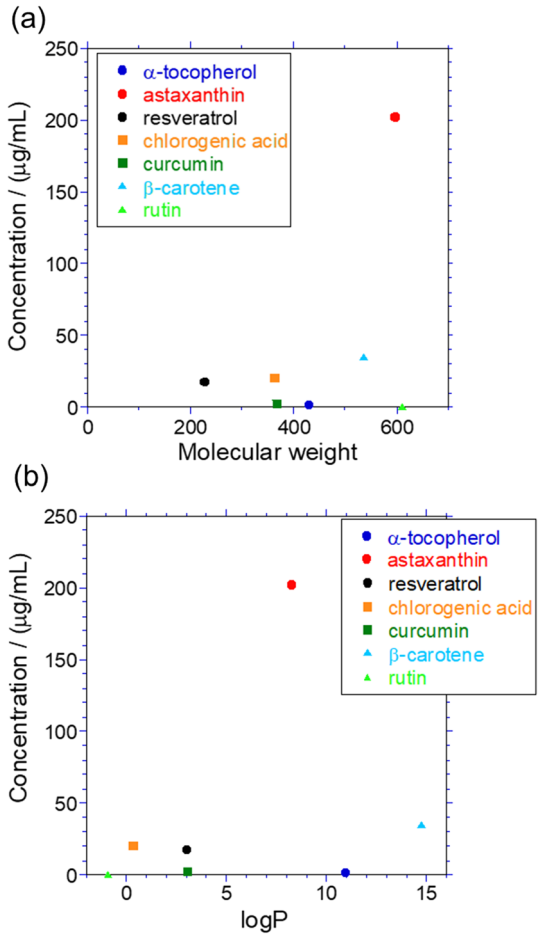

Fig. 3 Encapsulation of antioxidants into the milk-derived exosomes. The encapsulated concentration of antioxidants against **a** the molecular weights and **b** the distribution coefficients, *P*. The error bars show the standard deviation of 4 different samples, but they were enough small to overlap the dots

The antioxidants were encapsulated into the milk-derived exosomes by a simple incubation method. As shown in Fig. 3, astaxanthin, α-tocopherol, curcumin, chlorogenic acid, β-carotene, rutin, and resveratrol were encapsulated into exosomes as 202.4 ± 0.5 μg/mL, 1 ± 1 μg/mL, 2.2 ± 0.1 μg/mL, 19.8 ± 0.1 μg/mL, 35.0 ± 0.3 μg/mL, 0.13 ± 0.01 μg/mL, and 17.59 ± 0.02 μg/mL, respectively. The encapsulated concentration was also plotted as a function of important indicators for the incubation method, the molecular weight and the octanol/water partition coefficient, in Fig. 3a, b, respectively. In phospholipid bilayer penetration, substances of smaller molecular weights and larger diffusion coefficients are known to easily penetrate the membrane in the incubation method [20]. However, the molecular weights of the antioxidants and the encapsulated concentration showed not much of a correlation as shown in Fig. 3a. Although this relationship was different from the previous studies, octanol/water distribution coefficients might have a greater impact on the penetration process [21]. Figure 3b shows encapsulation of β-carotene and astaxanthin, which have high distribution coefficients, but α-tocopherol did not show expected encapsulation depending on its high distribution coefficient. This may be attributed to the hydroxy group on the chroman ring of α-tocopherol. It has been reported that the hydroxy group on the chroman ring of α-tocopherol strongly interacts with the phosphate moiety of phospholipids and the terminal alkyl chain interacts with the alkyl chain of phospholipids in the cell membrane, localizing on the surface of exosome, thereby removing reactive oxygen species in the cell membrane and inhibiting lipid peroxidation [22–24]. In exosomes, α-tocopherol is also thought to localize on the surface of the phospholipid bilayer, not penetrate into the exosome. Therefore, it is likely that α-tocopherol showed lower encapsulation efficiency than β-carotene and astaxanthin, which can penetrate into the interior of the exosome membrane. Astaxanthin also has a terminal hydrophilic group, but its long alkyl chain is oriented in parallel with the alkyl chain of the phospholipid bilayer, and the terminal hydroxy group and ketone can form hydrogen bonds with the phosphate moiety of the phospholipid bilayer, which may account for the high encapsulation efficiency [25]. β-Carotene and Astaxanthin have similar chemical structures except the end phenolic hydroxyl groups but showed significant difference of the encapsulation efficiency. One of the possible reasons is that Astaxanthin entry into the membrane seems more difficult than β-carotene due to the terminal hydroxy group and ketone, but once Astaxanthin enter in the membrane, hydrogen bonding with the phosphate moiety anchored strongly and prevent the escape from the membrane. Resveratrol and

curcumin have also been reported to localize on the surface on phospholipid bilayers, suggesting low encapsulation [26, 27]. These antioxidants are highly hydrophobic due to their conjugated double bonds and interact with the alkyl chains of phospholipids, but hydrogen bonds between the phenolic group and the phosphate moiety might prevent from entering and escaping from the hydrophobic region of phospholipid bilayer [28, 29]. When they present in the membrane, they prevent peroxidation of phospholipids by removing reactive oxygen species, similar to α-tocopherol [30, 31]. These results suggested that not only the distribution coefficient of the antioxidants but also the terminal functional group and hydrophobic alkyl chain length might have a significant impact on exosome encapsulation.

Antioxidant capacity assay of antioxidant-encapsulated exosomes

To measure the antioxidant capacity encapsulated in the exosomes, HORAC assay was performed for β-carotene as shown in Fig. 4. The β-carotene sample showed lower fluorescence intensity at higher concentrations in both aqueous and organic solvents, and the β-carotene-introduced exosomes in aqueous solvents also showed lower fluorescence intensity at higher concentrations. This may be because the excitation wavelength of fluorescein used in this experiment, 480 nm, overlaps with the absorbance wavelength of β-carotene, resulting in lower fluorescence intensity in the samples containing higher concentrations of β-carotene. However, the results in Fig. 4 did not confirm the maintenance of fluorescence intensity shown by the removal of hydroxyl radicals of antioxidants, as in the case of the standard sample, gallic acid. This might be due to the stable encapsulation of β-carotene inside the exosomes. The time course of the first derivative of the fluorescence intensity is shown in Fig. 4b and found that all β-carotene samples except the 400 μM β-carotene in aqueous solution showed larger minima than those in the same concentration of gallic acid or solvent. This result suggested that β-carotene encapsulated into the exosomes exhibits long-term antioxidant properties rather than short-term antioxidant capacity as does gallic acid.

Evaluation of storage stability of antioxidant-encapsulated exosomes

To evaluate the stability of the antioxidant-introduced exosomes, the concentration and the size of the exosomes were measured every week for 1 month using Nano Sight. Figure S3 shows the change over time of the β-carotene-encapsulated milk-derived exosome concentration and the size. The exosomes concentration slightly decreased over time and the mean particle size increased over time.

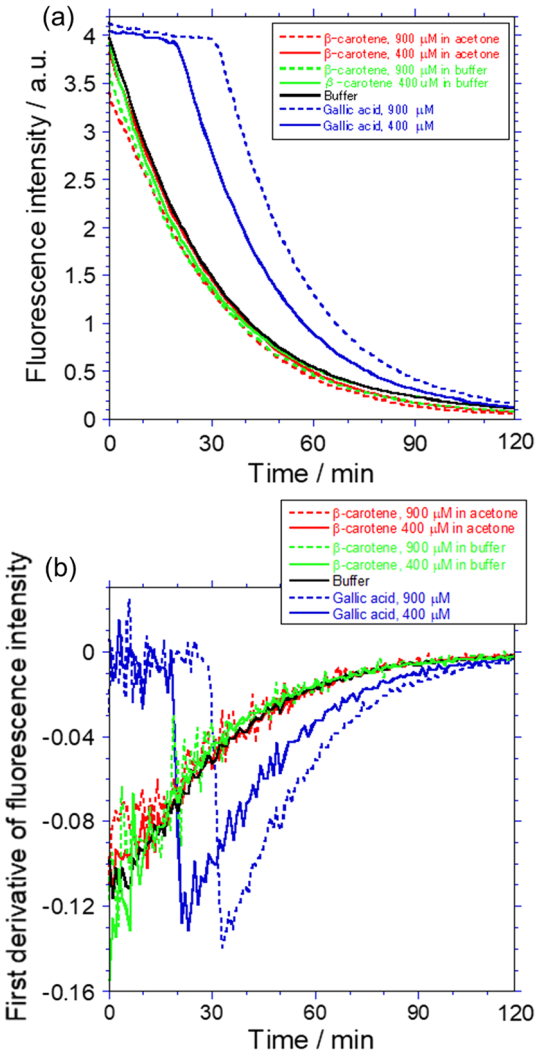

Fig. 4 Time course of **a** fluorescence intensities and **b** the first derivatives in the HORAC assay

Since it has been reported that storage of exosomes at 4 °C results in aggregation of exosomes, this result was understandable [32–34]. The clear increase and the following decrease of the size observed after 4 weeks might be assumed to large agglomerates formation and the size over 1 μm was not consider as the exosome concentration and size in this Nano Sight measurement. The sample No. 1 and 3 might contain large agglomerates more than the others and showed relatively small average size, ~ 60 nm, at the initial date. Although the absolute sizes of the 5 samples were different, all samples traced the similar size changes and showed the reproducible results. Exosome aggregation could prevent smooth adsorption and distribution of antioxidant-encapsulated exosomes in the body, long-term dispersibility is one of the important parameters in the future application of functional foods.

UV absorption of the β-carotene-encapsulated exosome was measured every week for one month to evaluate the stability of the encapsulated substance. Since the phospholipid bilayer of exosomes derived from live cells, reactive oxygen species are present in the membrane, and β-carotene has been reported to quench reactive oxygen species contained in the phospholipid bilayer [35, 36]. Therefore, a decrease of β-carotene concentration encapsulated in exosome over time was observed. Long-term storage methods such as pre-quench of extracted exosomes before antioxidants encapsulation should be developed.

Anticancer activity of the antioxidant-encapsulated exosomes

As shown in Fig. 5, the viability of the cells with exosomes and β-carotene-transfected exosomes was 81.5 ± 0.5% and 76.4 ± 0.3%, respectively. A decrease in cell viability was observed in both samples, however, no significant difference was observed between the exosome and β-carotene-encapsulated exosome ($p = 0.00345$). A reasonable understanding is that the antioxidant is not a drug but a nutrition and 2 to 3 β-carotene per an exosome is encapsulated in this condition, so the concentration might not be enough to show the effect. This study used the concentration of β-carotene around 50 μg/mL, while the clear effects were found in the concentration range from 10 to 40 mg/mL [37, 38]. Therefore, a method of larger amount of antioxidants loading into exosomes or higher concentration of exosomes should be developed for the future application of cancer cell treatments.

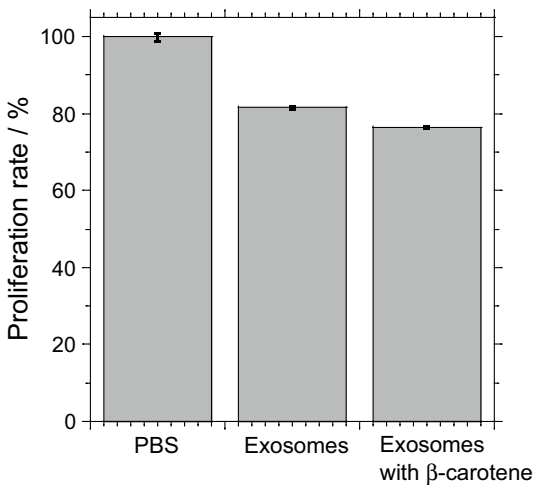

Fig. 5 Cell proliferation assay (MTT assay) by β-carotene-encapsulated exosomes ($n = 5$)

Conclusion

In this study, raw milk-derived exosomes were characterized and used as a cargo of antioxidants. The exosome extracted from raw milk had 50–150 nm in diameter and fairly high concentration compared with cell-derived exosomes. The antioxidants encapsulation efficiency was evaluated by the molecular weight and the octanol/water partition coefficient. The antioxidants capacity and the long-term stability tests were also conducted. The antioxidants with a high distribution coefficient were easily encapsulated into exosomes except α-tocopherol. The β-carotene-encapsulated exosome did not show obvious antioxidant capacity, but it showed long-term antioxidant capacity compared to the bare antioxidant. In stability studies, they kept the initial quality for 3 weeks, but after 4 weeks, exosome aggregation was observed. These results implied the potential application of antioxidant-encapsulated exosomes as an application of new supplemental drugs or functional foods.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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