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Establishment of the collection, storage and preservation methods and their influence on stability of human salivary exosome

***Abstract*—**The functions displayed by exosomes derived from saliva and other body fluids have been established. This paper studied the stability of human salivary exosome beginning from the collection mode, storage, and its preservation methods. Unstimulated saliva samples were collected from healthy subjects. Protease inhibitor was added into each samples and stored under different temperatures and at varying periods of time. The exosomes were isolated by ultracentrifugation and confirmed by using Western Blot. Exosome morphology was inspected by Scanning Electron Microscope (SEM) and the protein concentration was determined using the Protein (Bradford) Assay. The exosome particle size distribution and concentration were calculated using Nanoparticle Tracking Analysis (NTA). The protein assay showed no significant differences in the exosome protein concentration values for all conditions. Western Blot analysis also showed no differences in the presence of exosome and all the samples were positive for protein CD63. SEM analysis showed the fine shape of exosome which is round, in vesicle form with the size ranging between 10 nm and 100 nm. NTA determined the individual mean and the clumping exosome size was 203 nm. Human salivary exosomes remained intact in the absence of protease inhibitor and in different storage temperatures.

Keywords*—*salivary exosome, human saliva, Nanoparticle-Tracking Analysis, exosome storage, protease inhibitor

1 INTRODUCTION

Human saliva has many essential functions which are not only useful for the oral system, but also for other body systems. In the past few years, numerous research articles have been published describing several salivary components and their distribution, confirming the biochemical composition and physiology of the proteins existing in the human salivary fluid [1][2]. The presence of exosome in human's whole saliva has been demonstrated before [3]. Exosomes are small vesicles (30 – 120 nm), secreted by all types of cultured cells and found in abundance in body fluids, including blood, urine, ascites fluid, amniotic fluid and cultured medium of cell cultures [4]. Due to the established functions showed by exosomes derived from other body fluids, human salivary exosome is now marked for further research as saliva can be a non-invasive source of the exosomes. Previous studies had successfully given results of the structural-mechanical characterization of nanoparticles for exosomes in human saliva [5] and also managed to characterize the structural and transcriptome contents of salivary exosome [6]. The collection, storage, preservation and normalization of human urinary exosomes for biomarker discovery had already been established [7].

Exosome research is an expanding field and the development of the isolation methods have rapidly progressed as well. The advances in isolation techniques were summarized in detail by a number of review papers [8][9][10]. The established isolation techniques are ultracentrifugation-based techniques [11], size-based techniques [12][13], exosome precipitation [8][14], immunoaffinity capture-based techniques [15][11][13], and microfluidic techniques [16][17][18].

The increasing efficiency in the technological progress of exosome isolation techniques has widened the choice of techniques to suit the researchers. Every technique has its own advantages and disadvantages. The ultracentrifugation method was chosen for this research as it is cost effective and with low contamination risks along with large sample

capacity that can yield substantial amounts of exosomes. This technique requires minor technical expertise and saves time as it requires little or no sample pretreatments at all [14]. In estimation, about 56% of all exosome isolation techniques employed by users were accounted in exosome research [15]. Thus ultracentrifugation-based techniques have become the ideal option of isolation method for exosome isolations among researchers.

Previously, there had been a lack of suitable techniques for quantifying the size and quantity of the isolated exosome. Early method of exosome quantification is mostly on determining its protein concentration by using protein assay. However, recently, the Nanoparticle Tracking Analysis (NTA) technique has been derived and this advance technology is able to thoroughly quantify exosome samples in size, number of particles, concentration, real life time visualization and individual particle tracking [19]. Hence, the aim of this study was to clarify the collection, storage, and preservation methods for salivary exosomes, which can be a solid foundation for further use in research and biomarker discovery.

2 METHODOLOGY

2.1 Human saliva collection

(a) Pre-collection

Human saliva samples were collected in accordance with the recommendations in the International Conference on Harmonization - Guidelines for Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki (European Medicines Agency, 2002). The protocol was approved by the Human Research Ethics Committee, Universiti Sains Malaysia (JEPeM) (FWA Reg. No: 00007781; IRB Reg. No: 00004494) (Appendix 1).

(b) During collection

Subjects were asked to refrain from eating, drinking, or using oral hygiene products for at least 1 hour prior to collection and were asked to rinse their mouth with water, after which, about 5 ml of unstimulated saliva was collected from each subject.

(c) Post-collection

About 30 ml of the saliva sample was centrifuged at 6000xg for 20 minutes [1]. The supernatant was then removed and allocated in six different tubes.

(d) Sample handling and storages

Six tubes of samples were divided into two groups; with the presence and absence of protease inhibitor. Protease inhibitor in the ratio 1:1 was added to three of the tubes with samples and the remaining three were kept as samples without protease inhibitor (controls).

To study the effective methods for the storage and preservation of human salivary exosome, six tubes of samples were subjected to three different protocols (a pair of samples consists of 1 tube of sample with protease inhibitor and 1 tube of sample without protease inhibitor): a) stored at 4°C for 1 hour; b) stored at -20°C for 1 week; and c) stored at -80°C for 1 month. After the storage period, all samples underwent the exosome collection process (Fig. 1). These methods were adapted and modified from a previous method for urinary exosome isolation [7].

2.2 Exosome Isolation

Then, all the saliva supernatants from three different protocols were ultracentrifuged at 110,000xg for 2 hours at 4°C. Following ultracentrifugation, the aqueous layer was removed and the pellet containing the exosomes was dissolved in 1 ml PBS for each tube and kept at a temperature of -20°C until further use (Fig. 1) [1].

2.3 Scanning Electron Microscope

Morphology of exosome was inspected under FEI Quanta 450 Scanning Electron Microscope (SEM) (FEI, Oregon, USA). The -20°C isolated exosome sample with protein inhibitor was randomly chosen for viewing under SEM. Method of slide preparation was based on the method of Yamada *et al.,* (2012) with a slight modification to suit the sample type. One hundred microliter of isolated exosome were diluted in 100 µl of Phosphate Buffered Saline (PBS) (ratio 1:1) and re-suspended in the tube. Five microliters of the sample were dropped on a round glass slide and slowly spread. The slide was then let to dry in an air dryer incubator for 24 hours [24]. The slide was then coated with gold-coating and was viewed under a High Voltage (HV) of 5.00 kV and a Working Distance (WD) of 9.5 mm. The slide was viewed under 30000x, 50000x, 60000x and 120000x magnifications respectively. The image obtained was measured to find out the range in sizes of exosomes.

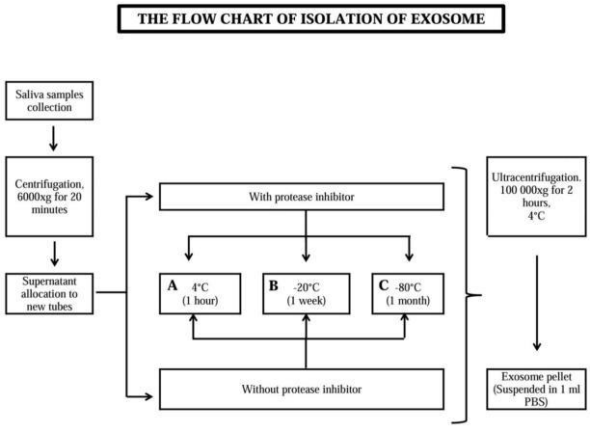

Figure 1: Flowchart of human salivary exosome isolation method. After the samples were centrifuged and the supernatant was isolated into 6 different tubes, protease inhibitors were added to three of them and the rest were kept as samples without protease inhibitor. Then each pair of samples with and without protease inhibitor were kept under three different protocols. A) Protocol 1: Storage at 4°C for 1 hour; B) Protocol 2: Storage at -20°C for 1 week; and C) Protocol 3: Storage at -80°C for 1 month.

2.4 Bradford Protein Assay

Protein concentration of the human salivary exosome was determined using Coomassie Plus^{TM} (Bradford) Assay Kit (ThermoScientific, Illinois, USA). A standard curve was plotted using the average blank-corrected 595nm measurement for each Bovine Serum Albumin (BSA) standard against its concentration in µg/ml. All the samples were tested using the standard microplate protocol provided by the manufacturer and the standard curve was used to determine the protein concentration of each sample.

2.5 SDS-PAGE and Western Blot

The exosome was confirmed by SDS-PAGE and western blot by using exosome protein markers. Ten microliters of each sample were loaded into the wells of 12% acrylamide/bis-acrylamide and electrophoresis was done at 30 mA (1 gel) for 90 minutes (Volts: 200 V). The gels were then stained by Coomassie Brilliant Blue Solution (Bio-Rad, California, USA).

Duplicated gels were then transferred onto nitrocellulose membrane and blocked with 5% non-fat dry milk in Phosphate Buffer Saline with Tween-20 (PBST) for 1 hour at room temperature. Membranes were then incubated with anti-CD63 antibody (ThermoScientific, Illinois, USA) and conjugated with horse-peroxidase secondary antibody (ThermoScientific, Illinois, USA) The antibody-antigen reactions were visualized by using Enhanced Chemiluminescence (ECL) Plus Western blotting detection (ThermoScientific, Illinois, USA) and viewed under Fluorochem2 Image Analyzer. PageRuler Prestained Protein Ladder (ThermoScientific, Illinois, USA) was used to check the molecular weight of the targeted protein. Anti-β actin antibody (ThermoScientific, Illinois, USA) was used as the housekeeping gene as it is one of the common genes used as loading control. Quantification of protein bands densitometry was carried out using ImageJ 1.50i software by Wayne Rasband (National Institutes of Health, USA). ImageJ is an open source image processing program for multidimensional image data with a focus on scientific imaging. The bands intensity values were converted to peak area unit of pixel.

2.6 Nanoparticle Tracking Analysis

The number of human salivary exosome particles per volume, the mean size of exosome and the concentration of the exosomes yield was calculated using NanoSight NS300 (Malvern, England, UK) with the NTA Version: NTA 3.0. Exosome samples were diluted to 100x in PBS for viewing under the camera. About 0.3 ml of samples were injected into the sample chamber and directly viewed. The images of the exosomes were captured in 10 seconds movie form, and the particles tracked were calculated and graphed.

The results obtained were in “finite track-length adjusted” (FTLA) size per concentration graph and averaged FTLA size per concentration graph with standard error of the mean. The 10 seconds movie form of exosomes were presented in image capture form for the 1st second, 3rd second, 5th second and 8th second to show the real live visualization done by the NTA.

3 RESULTS

3.1 Confirmation of human salivary exosomes

This research confirmed the exosomes isolated based on the exosomes morphology, size range, and the presence of the protein markers. Exosomes morphology was analyzed by SEM. SEM image viewed under 30,000x magnifications (Fig. 2) showed the exosomes presented in clumps. When the magnifications were increased to 50,000x magnifications (Fig. 3), the fine shape of exosomes which were round, in vesicle form could be observed. Meanwhile, a single exosome could be observed under 120000x magnifications (Fig. 4).

Table I: Result list obtained from NTA Result

Nanoparticle Tracking Analysis	
Statistics	**Merged Data Results (nm)**
Mean	204.20
Mode	117.20
SD	92.90
D10	96.80
D50	187.50
D90	317.60
Statistics	**Mean ± Standard Error (nm)**
Mean	203.00 ± 11.00
Mode	123.50 ± 8.10
SD	90.80 ± 7.00
D10	96.60 ± 9.20
D50	189.40 ± 13.30
D90	325.20 ± 18.70
Concentration	7.99^{8} ± 5.29^{7} particles/ml 40.50 ± 2.70 particles/frame 50.20 ± 1.90 centers/frame

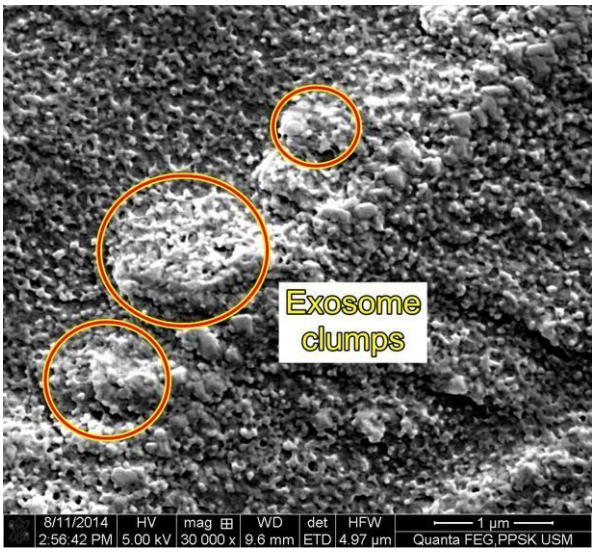

Figure 2: The image of human salivary exosome under 30000x magnification of SEM. The exosomes appeared to be in clumps.

Exosomes size range was analyzed by SEM and NTA. From SEM analysis, Fig. 3 showed measurement of five exosomes with 55.37 nm, 53.18 nm, 67.02nm, 67,09 nm and 43.71 nm diameters. Fig. 4 shows a single exosome detected with the size of 87.72 nm. The few randomly chosen exosomes measured sizes were within the exosome size range as reported by previous researches. This result was supported by another exosomes size range analysis, the NTA. The size distribution profile data (Fig. 5) were shown over-plotted in Graph A, meanwhile, the mean and the standard error of the mean were shown in Graph B, with the size of the exosomes peaks annotated. The average FTLA sizes per concentration result from detection graphs (Fig. 5) were merged and represented in summary result list (Table I). The modal particle sizes were described as in few keys. The SD is the measure of the width (spread) of the size distribution profile.

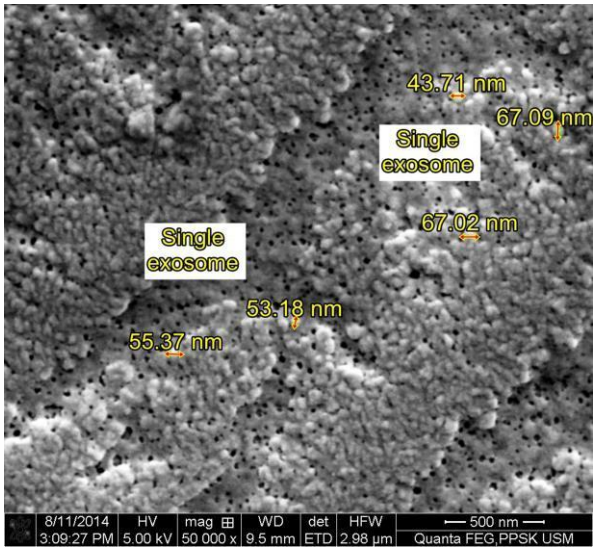

Figure 3: The image of human salivary exosome under 50000x magnification of SEM. Few randomly chosen exosome vesicles were measured and their sizes are stated as labeled.

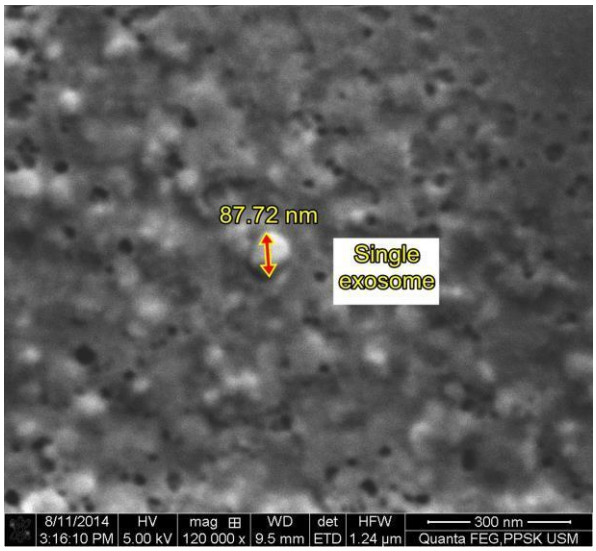

Figure 4: The image of human salivary exosome under 120000x magnification of SEM. A single vesicle of exosome was captured, and the size was measured and stated as labeled.

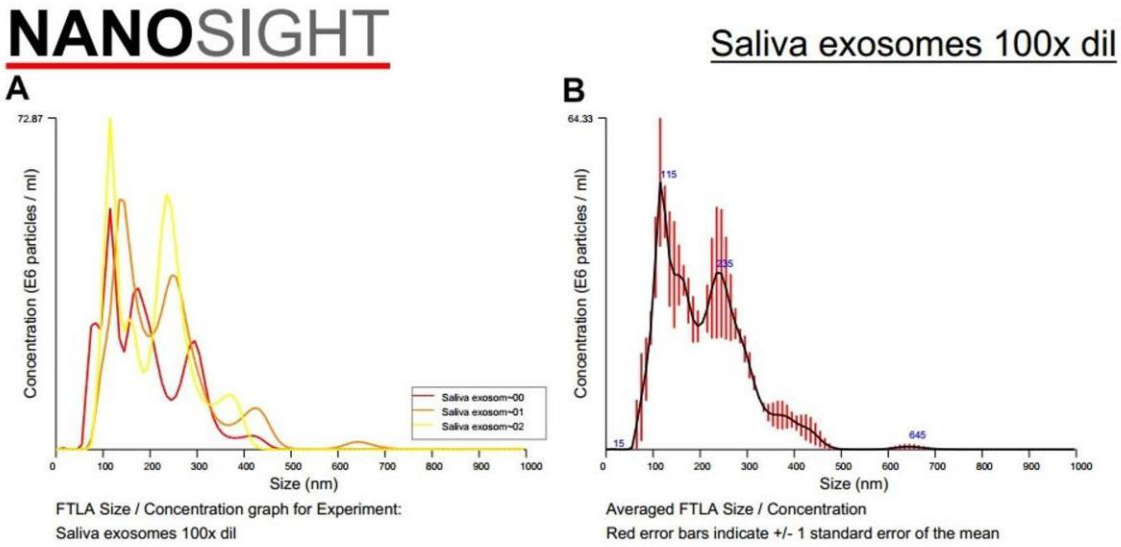

Figure 5: Nanoparticle Tracking Analysis (NTA) on the human salivary exosomes under 100x dilutions. Graph A shows the FTLA size per concentration graph, taken as triplicates and Graph B showed the averaged FTLA size per concentration with standard error of the mean.

The D10, D50 and D90 values indicated percent under size, as shown the in Table I, 50% of the exosomes 189.4 nm or smaller, giving another indication of the spread of particles sizes within the sample. The mean size of exosomes was 203.0 nm with 11.00 nm of standard error.

The exosomes protein markers were detected from SDS-PAGE and Western blot analysis. SDS-PAGE gel (Fig. 6) showed protein separation bands for all samples and the Western blot result (Fig. 7) showed the detection of CD63 (70 kDa) in all samples. The β-actin, the internal control protein marker was also detected. Band intensity or thickness is indicative of the proteins relative abundance, therefore, from the results analyzed by ImageJ software, 4°C sample without protease inhibitor (6204.326 pixels) showed strongest bands intensity compared to other samples. However, for this research, the purpose of Western blot was to check on the presence of human salivary exosome protein markers only, while actual quantification of protein concentrations was based on Bradford protein assay results.

Human salivary exosomes concentration was also measured by Bradford protein assay and NTA. Protein assay chart (Fig. 8) showed the values for all samples concentrations in µg/ml. Different from protein assay, NTA analysis measured exosomes concentration by visualization and detection of exosome particles per volume. The -20°C sample with protease inhibitor was chosen for measurement. The concentration calculated from NTA was 7.99 x 10^8 particles/ml and about 40.5 particles were captured per frame (Table I). The images of the exosomes were also captured from the 10 seconds real-time movie of exosomes visualization (Fig. 9). These results were concurrent with the findings that had been reported on the morphology and visualization from other researches [3][25].

3.2 Establishment of human salivary exosomes storage condition

The results showed that there was no effect of protease inhibitor on the human salivary exosomes. There was also no effect of different temperature storage on the human salivary exosomes. Details of the effect of both conditions on the samples are as follows.

***(a)** Effect of protease inhibitor*

The sample result for protein assay (Fig. 8) showed no significant difference ($p > 0.05$) on the concentration value, regardless of the presence of protease inhibitors. The SDS-PAGE analysis of Coomassie blue solution (Fig. 6) and Western blot analysis of CD63 (Fig. 7) showed that there was no significant difference in the intensity of the signals or bands, regardless of the protease inhibitors addition. The intensity of the bands was also correlated with the protein concentration of exosome samples. The expression of CD63 in all samples showed the exosomes were intact, with or without the addition of protease inhibitor.

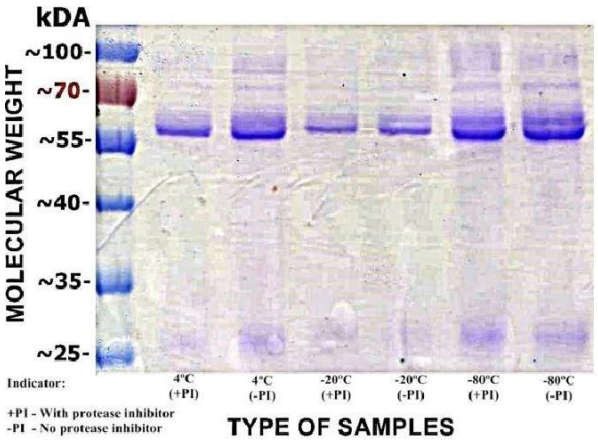

Figure 6: The image of the SDS-PAGE for all types of samples. The result shows the protein separation bands appeared almost the same intensity for all samples regardless with or without protease inhibitor for all conditions with the strongest band detected at 55-70 kDa of molecular weight.

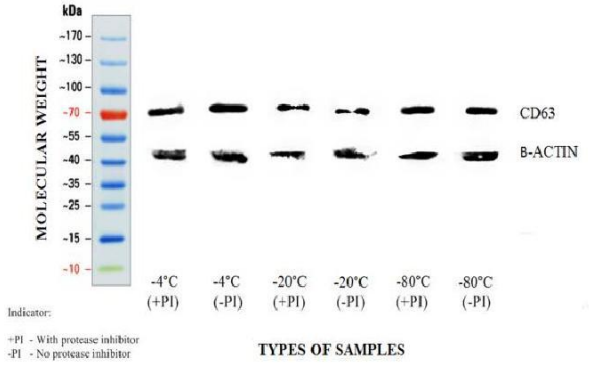

Figure 7: The CD63 expression by western blot result image. All samples showed expression of CD63 at 70 kDa and the β-actin antibody was used as internal control.

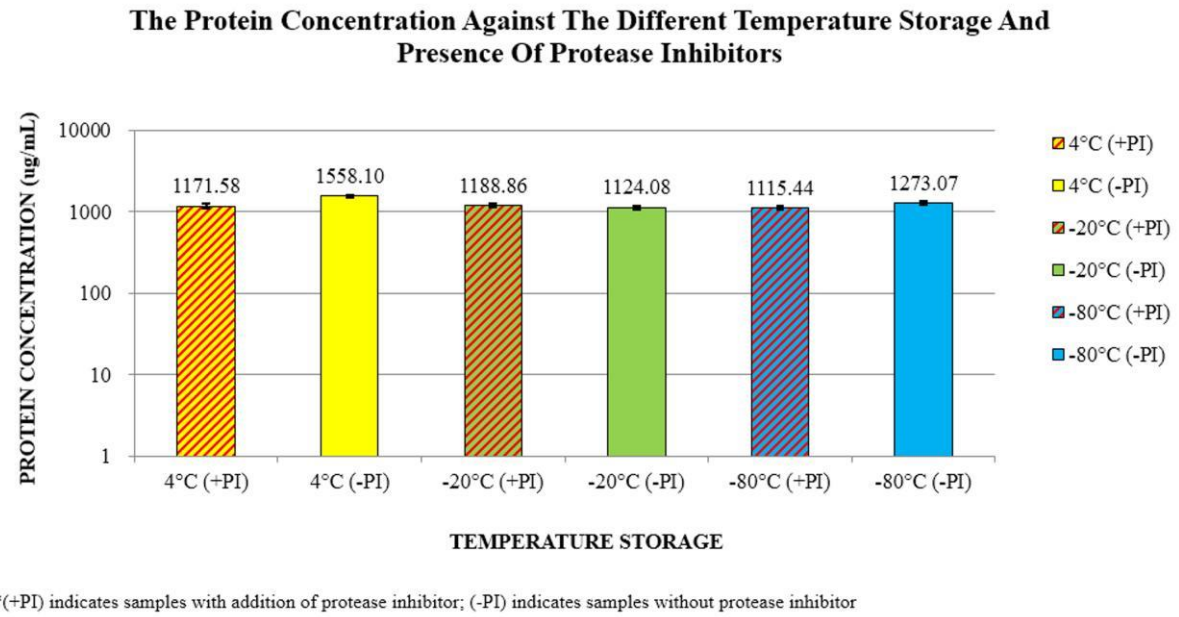

Figure 8: The chart of the protein concentration against the different temperature and presence of protease inhibitors. The results show no significant difference. (Data represent the mean value ± SEM; EXCEL 2016: Statistical Inference for Two-variable Regression Analysis, $p > 0.05$).

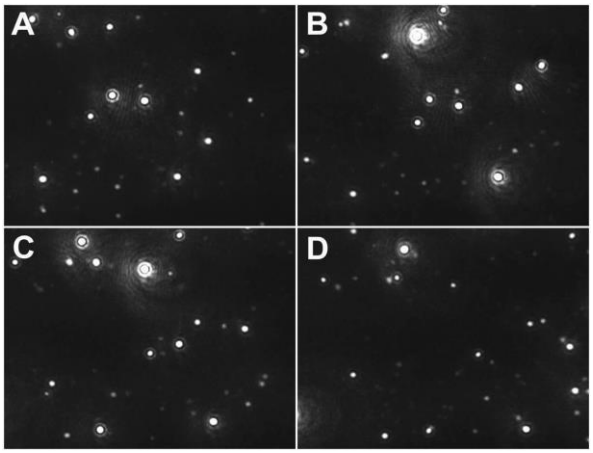

Figure 9: The images of 10 seconds real-time movie of exosomes captured from NTA. A) Image was captured at the 1st second. B) Image was captured at the 3rd second. C) Image was captured at the 5th second. D) Image was captured at the 8th second. Exosomes appeared to be shiny and in vesicle form.

(b) *Effect of different storage condition*

The protein concentration for all exosomes samples (Fig. 8) from protein assay showed no significant difference in the concentration value regardless of the storage temperature ($p > 0.05$). SDS-PAGE analysis of target protein (Fig. 6) and Western blot analysis of CD63 (Fig. 7) showed that there was no significant difference in the intensity of the signals or bands, regardless of the different temperature storage. All exosomes samples expressed CD63, hence showing that exosomes were still intact and not affected by the temperature storage.

4 DISCUSSION

SEM image viewed under 30,000x magnifications (Fig. 2) showed crowded exosomes. This may be due to the high concentration of exosomes in the sample suspension. Even without fixation method, the exosomes were still distinct enough for observation and viewing. This, therefore, supports that the exosome is a stable component. The image obtained from SEM supported the presence of exosomes by showing the fine shape of exosome which was round, with a size range in between 10 nm-100 nm (Fig. 2, Fig. 3 and Fig. 4).

The NTA results showed that the sizes calculated were quite inconsistent with the variation in the sizes that were captured (Fig. 5 and Table I). The published range of exosomes size was within 30-120 nm [5][4]. The mean size obtained from NTA was 203 nm, showing a larger particle captured. The standard deviation was 90.8 ± 7.0 nm, which also showed the huge difference in sizes range. Since NTA was not able to manually separate particles, the larger size detected was probably due to the clumping of exosomes themselves. Therefore, even under the 100x dilutions, the exosomes concentration calculated was high and if the clumping of exosomes was put into consideration, the actual concentration of exosomes could be higher where exosomes are in a single form. Notwithstanding, this showed that human salivary exosomes could be yielded even with low amount of human saliva.

As observed, the clumping of exosomes as seen under SEM and showed by NTA results still manifested even though the steps to prevent it from occurring had been carried out before all the tests were done. The same exosomes clumping matter had been encountered by other previous researches as well. The clumping of exosomes is primarily due to the shearing forces of the exosomes during the preparation process [26] and exosome aggregation [27]. NTA managed to give us the concentration of the exosomes isolated, however, due to the clumping of the exosomes, we believe it was more reliable to use exosomes protein concentration from protein assay result (Section 3.3.2) for treatment of cells *in vitro*.

All samples were detected to express CD63, the exosomes protein marker by SDS-PAGE and Western blot analysis. All samples showed significant bands on the membrane. This finding not only confirmed the presence of exosomes in the isolated human saliva via protein markers, but also proved that the exosomes were intact and in good quality for further tests.

Protease inhibitors are protein molecules that inhibit the function of enzyme protease from performing proteolysis, which is the catabolism of protein by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. Due to the ability to inhibit the proteolysis, protease inhibitor can act as a preservative of biological fluid and samples from the degradation of proteins. These results showed that the addition of protease inhibitor into the saliva samples yielded no significant difference ($p > 0.05$) on the exosomes protein concentration, the intensity of signal on the SDS-PAGE membranes and CD63 detection on Western blot. These findings contradicted the results published by a research on urinary exosome which showing the necessity in using protease inhibitor [7]. The urinary exosomes samples without protease inhibitors had no signal or decreased signal compared to the samples with protease inhibitor.

Therefore, they suggested that protease inhibitors are necessary to prevent the degradation of urinary exosomes protein [7]. However, these results showed that human salivary exosome is stable and do not need any preservation; this suggests that human saliva may contain important substances which prevent the degradation of exosomes that need further investigation.

These findings showed there was no effect of different temperature storages on the exosomes protein concentration, the intensity of signal on the SDS-PAGE membranes and CD63 detection on Western blot results for all samples. These findings were supported by another finding that was done on plasma exosomes [28]. The result on plasma exosomes tested also showed no significant influence on the exosomal miRNA despite the different storage conditions, except for short-term storage at 4°C [28]. However, there is differences between salivary exosomes and urinary exosomes in term of stability. The results obtained from urinary exosomes showed a major loss of proteins due to the freezing at -20°C from the Bicinchoninic Acid (BCA) protein assay, gel-electrophoresis and Coomassie-Blue staining tests results. Yet, only by freezing at -80°C, all the specific urinary exosome-associated protein samples were well preserved [7]. In contrast, human salivary exosomes in this study showed stability at each temperature (4°C, -20°C and -80°C) tested.

The mechanism that controls exosomes stability has not yet been studied in detail. However, from a previous study by on proteomic analysis of exosomes from human saliva suggested that the proteins contained in exosomes may play a significant role in regulating their stability [3]. One of the proteins that may influence the stability of the exosomes is heat shock protein (Hsp) 70. Hsp70 has been proven to be present in human salivary exosomes and has been listed as one of the exosomal biomarker [3]. In general, heat shock protein functions as chaperone for other cellular proteins in assisting the folding and the establishment of protein shapes and prevent protein aggregation as well as helping in stabilize unfolded proteins [29]. A research that studied on Hsp70 presence in exosomes from three different body fluids (amniotic fluid, saliva and urine) also supported this finding [30]. Compared to salivary exosomes, only three out of 12 sucrose density fractions of urinary exosomes were detected by Western blot analysis, meanwhile, Hsp70 was detected in all 12 fractions of salivary exosomes. The higher level of Hsp70 detected was suggested to be the reason for the higher stability of salivary exosome compared to urinary exosomes. The most recent study done on exosome's recovery yield and stability by various storage condition and from all the proteins investigated; only Hsp70 shows the minimum loss even for the long storage at room temperature [31].

Overall, with the discovery of varied exosomes functions so far, it has raised the interest in the clinical applications of exosomes due to their ability in prognosis, therapy, biomarkers and many more functions that still under continuous studies. As suggested by researches, exosomes play a significant role in the intercellular communication, other than being the most promising candidate for a natural drug delivery [33][34]. From here, human salivary exosomes need more proactive effort in unrevealing its true potential. As it is majorly involved with oral system, human salivary exosomes can be one of the components that is involved in periodontal regeneration that could contribute to tissue engineering. The undiscovered contents of human salivary exosomes such as its proteomic and genomic materials could hold onto the key for potential biomarkers for chronic diseases detection as well. However, by having an established method in storage that can maintain its stability, further analytical studies can be taken from here.

5 CONCLUSION

Exosomes from human saliva had been successfully isolated and their presence was confirmed from its morphology, correct size and protein markers. The results show no significant difference in the quantity and quality of the exosomes either with or without the addition of protease inhibitors or under different storage temperatures. Hence, proving that human salivary exosome is a stable and consistent biological fluid sample.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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REFERENCES

[1] Shahidan WNS. MicroRNA analysis in saliva [Doctoral (Academic) thesis]. Japan: The University of Tokushima Graduate School; 2011.

[2] Schenkels LCPM, Veerman ECI, Amerongen AVN. Biochemical composition of human saliva in relation to other mucosal fluids. *Critical Reviews in Oral Biology and Medicine*; 1995;6:pp. 161-75.

[3] Ogawa Y, Miura Y, Harazono A, et al. Proteomic analysis of two types of exosomes in human whole saliva. *Biological and Pharmaceutical Bulletin*; 2011;34(1):pp. 13-23.

[4] Keller S, Sanderson MP, Stoeck A, et al. Exosomes: From biogenesis and secretion to biological function. *Immunology Letters*; 2006;107(2):pp. 103-8.

[5] Sharma S, Rasool HI, Palanisamy V, et al. Structural-mechanical characterization of nano-particles-exosomes in human saliva, using correlative AFM, FESEM and force spectroscopy. *ACS Nano*; 2010;40:pp. 1-11.

[6] Palanisamy V, Sharma S, Deshpande A, et al. Nanostructural and Transcriptomic Analyses of Human Saliva Derived Exosome. *PLoS ONE*; 2010;5 (1):pp. 1-11. Epub 5 January 2010.

[7] Zhou H, Yuen PST, Pisitkun T, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. *Kidney International*; 2006;69(9):pp. 1461-76.

[8] Li P, Kaslan M, Lee SH, et al. Progress in Exosome Isolation Techniques. *Theranostics* 2017;7(3):pp. 789-804.

[9] Lässer C, Eldh M, Lötvall J. Isolation and characterization of RNA-containing exosomes. *Journal of Visualized Experiments*; 2012(59):pp.

[10] Greening DW, Xu R, Ji H, et al. A Protocol for Exosome Isolation and Characterization: Evaluation of Ultracentrifugation, Density-Gradient Separation, and Immunoaffinity Capture Methods. In: Posch A, editor. Proteomic Profiling: Methods and Protocols. New York, NY: Springer New York; 2015. p. 179-209.

[11] Théry C, Amigorena S, Raposo G, et al. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. Current Protocols in Cell Biology: John Wiley & Sons, Inc.; 2006.

[12] Cheruvanky A, Zhou H, Pisitkun T, et al. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *American Journal of Physiology - Renal Physiology*; 2007;292(5):pp. F1657-F61.

[13] Wang K, Zhang S, Weber J, et al. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Research*; 2010;38(20):pp. 7248-59.

[14] Zeringer E, Barta T, Li M, et al. Strategies for Isolation of Exosomes. *Cold Spring Harbor Protocols*; 2015:pp. 319-24.

[15] Zarovni N, Corrado A, Guazzi P, et al. Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches. *Methods*; 2015;87:pp. 46-58.

[16] Lee K, Shao H, Weissleder R, et al. Acoustic Purification of Extracellular Microvesicles. *ACS Nano*; 2015;9(3):pp. 2321-7.

[17] He M, Crow J, Roth M, et al. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology *Lab on a Chip*; 2014;14(19):pp. 3773-80.

[18] Wang Z, Wu H-j, Fine D, et al. Ciliated micropillars for the microfluidic-based isolation of nanoscale lipid vesicles. *Lab on a chip*; 2013;13(15):pp. 2879-82.

[19] Malloy A. Count, size and visualize nanoparticles. *Materials Today*; 2011;14(4):pp. 170-3.

[20] Heintze U, Birkhed D, Björn H. Secretion Rate and Buffer Effect of Resting and Stimulated Whole Saliva as a Function of Age and Sex. *Swedish Dental Journal*; 1983;7(6):pp. 227-38.

[21] Ekström J, Khosravani N, Castagnola M, et al. Saliva and the Control of Its Secretion. In: Ekberg O, editor. Dysphagia: Diagnosis and Treatment. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 19-47.

[22] Lagerlof F, Dawes C. The Volume of Saliva in the Mouth Before and After Swallowing. *Journal of Dental Research*; 1984;63(5):pp. 618-21.

[23] Díaz-Martín V, Manzano-Román R, Valero L, et al. An insight into the proteome of the saliva of the argasid tick Ornithodoros moubata reveals important differences in saliva protein composition between the sexes. *Journal of Proteomics*; 2013;80:pp. 216-35.

[24] Yamada T, Inoshima Y, Matsuda T, et al. Comparison of methods for isolating exosome from bovine milk. *Journal of Veterinary Medical Science*; 2012;74(11):pp. 1523-5.

[25] Wu Y, Deng W, Klinke DJ. Exosomes: Improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *Analyst*; 2015;140:pp. 6631-42.

[26] Schneider A, Simons M. Exosomes: Vesicular carriers for intercellular communication in neurodegenerative disorders. *Cell Tissue Research*; 2013;352:pp. 33-47.

[27] Hood JL, Scott MJ, Wickline SA. Maximizing exosome colloidal stability following electroporation. *Analytical Biochemistry*; 2014;448:pp. 41-9.

[28] Ge Q, Zhou Y, Lu J, et al. miRNA in plasma exosome is stable under different storage conditions. *Molecules*; 2014;19(2):pp. 1568-75.

[29] Buchner J, Walter S. Molecular chaperones–cellular machines for protein folding. *Angewandte Chemie*; 2002;41(7):pp. 1098-113.

[30] Keller S, Ridinger J, Rupp A-K, et al. Body fluid derived exosomes as a novel template for clinical diagnostics. *Journal of Translational Medicine*; 2011;9(1):pp. 86.

[31] Cheng Y, Zeng Q, Han Q, et al. Effect of pH, temperature and freezing-thawing on quantity changes and cellular uptake of exosomes. *Protein & Cell*; 2018:pp.

[32] Lee M, Ban J, Im W, et al. Influence of storage condition on exosome recovery. *Biotechnology and Bioprocess Engineering*; 2016;21:pp. 299-304.

[33] Simpson RJ, Lim JWE, Moritz RL, et al. Exosomes: proteomic insights and diagnostic potential. *Expert Reviews Proteomics*; 2009;6(3):pp. 267-83.

[34] Lai RC, Yeo RWY, Tan KH, et al. Exosomes for drug delivery - A novel application for the mesenchymal stem cell. *Biotechnology Advances*; 2012;31:pp. 534-51.