### Title

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### Journal

Biomedical Journal, 44(6)

### ISSN

2319-4170



### Authors

Peng, Hsin-Hsin
Huang, Pei-Rong
Young, John D
et al.



### Publication Date

2021-12-01

### DOI

10.1016/j.bj.2020.05.008

Peer reviewed

Available online at www.sciencedirect.com

ScienceDirect

Biomedical Journal

journal homepage: www.elsevier.com/locate/bj


Original Article

# Physical attributes of salivary calcium particles and their interaction with gingival epithelium


*Hsin-Hsin Peng* [a,b,c], *Pei-Rong Huang* [d], *John D. Young* [a,b,e,f,**], *David M. Ojcius* [a,b,e,g,*]

[a] *Center for Molecular and Clinical Immunology, Chang Gung University, Taoyuan, Taiwan*
[b] *Laboratory of Nanomaterials, Chang Gung University, Gueishan, Taoyuan, Taiwan*
[c] *Laboratory Animal Center, Chang Gung Memorial Hospital at LinKou, Taoyuan, Taiwan*
[d] *R & D Center, Cell Doctor International Co., Taipei, Taiwan*
[e] *Chang Gung Immunology Consortium, Chang Gung Memorial Hospital at LinKou, Taoyuan, Taiwan*
[f] *Biochemical Engineering Research Center, Ming Chi University of Technology, New Taipei, Taiwan*
[g] *Department of Biomedical Sciences, University of the Pacific, Arthur Dugoni School of Dentistry, San Francisco, CA, USA*

ARTICLE INFO

*Article history:*
Received 5 November 2019
Accepted 10 May 2020
Available online 23 May 2020

*Keywords:*
Calcium
Gingival epithelium
Particle internalization
Saliva
Salivary particles
Innate immunity

ABSTRACT

*Background:* The formation of dental plaque and its involvement in the pathogenesis of periodontitis is a topic of intense interest given the high prevalence of periodontitis in humans. Even though calcium-based particles play an active role in both dental plaque formation and periodontitis, few publications describe the physical-chemical properties of these particles.

*Methods:* Saliva samples were collected from healthy volunteers. From these samples, saliva-derived particles were isolated and stained for calcium using calcein or Fluo-4. The salivary particles were also subjected to characterization by flow cytometry and immunoblotting. Internalization of calcein-labeled salivary particles by gingival epithelial cells was visualized by confocal microscopy.

*Results:* We found that calcium-based salivary particles from healthy volunteers varied greatly in size but were enriched in particles of sizes at or greater than 1.5 μm. Immunoblotting analysis of the salivary particles identified several proteins including albumin, fetuin-A, and statherin, which have been found in calcium phosphate particles from other tissues or are known to modulate calcium homeostasis in saliva. In addition, calcium particles were internalized by both gingival epithelial cells and monocyte-derived macrophages.

*Conclusion:* Salivary calcium particles were enriched in the micrometer range, internalized by gingival epithelial cells, and contain albumin, fetuin-A and statherin, regulators of particle formation. These characteristics of the calcium-based salivary particles and their biological activities provide a basis for further studies to understand the molecular basis for pathogenesis of periodontitis.

* *Corresponding author*. Center for Molecular and Clinical Immunology, Chang Gung University, 259, Wenhua 1st Rd., Gueishan, Taoyuan 33302, Taiwan.
** *Corresponding author*. Laboratory of Nanomaterials, Chang Gung University, 259, Wenhua 1st Rd., Gueishan, Taoyuan 33302, Taiwan.
E-mail addresses: jdyoung@mail.cgu.edu.tw (J.D. Young), dojcius@pacific.edu (D.M. Ojcius).
Peer review under responsibility of Chang Gung University.
https://doi.org/10.1016/j.bj.2020.05.008

## At a glance commentary

### *Scientific background on the subject*

Calcium particles are abundant in saliva and are thought to play an important role in calcium homeostasis. But the particle sizes, their composition, and their interaction with host tissue had never been characterized.

### *What this study adds to the field*

The size of salivary calcium particles is mainly in the micrometer range. The particles can be internalized by host cells, and therefore could also alter host physiology. The particles contain albumin, fetuin-A and statherin; albumin and fetuin-A regulate particle formation, while statherin is involved in calcium homeostasis.

Dental plaque is composed of numerous bacterial species, glycoproteins, mucus and other components [1,2]. Subsequent mineralization of the dental plaque results in formation of calculus, which is firmly attached to the surface of the tooth [3]. Along with other known major contributors to periodontal disease such as oral microorganisms and their pro-inflammatory products, dental calculus stimulates inflammation of the periodontium, which in its advanced state may further develop into periodontitis [4,5], one of the most common inflammatory diseases in humans.

Metagenomic studies show that dysbiosis of dental plaque is correlated with periodontitis development [6,7]. Moreover, *in vivo* mice models show that keystone periodontal pathogens, such as *Porphyromonas gingivalis*, trigger dysbiosis in microbial communities and thus facilitate biofilm formation and mineralization [8,9]. Nevertheless, the mechanisms of calculus formation still remain to be elucidated.

Saliva is a major environmental determinant of the oral cavity. It is a protein- and electrolyte-enriched body fluid actively secreted by the major and minor salivary glands [10]. Saliva is critical for dental health not only by supporting remineralization, buffering, and antimicrobial action, and enhancing resistance to plaque formation in the oral environment, but also by providing lubrication and thereby maintenance of the mucosal integrity of oral and upper gastrointestinal surfaces [10–12].

Saliva is saturated with calcium and phosphate ions which is needed to prevent corrosion of the enamel surface of teeth [13]; but the presence of these ions can also lead to spontaneous formation of calcium phosphate particles when ions exceed homeostatic concentrations. The formation of calcium phosphate particles and their deposition have been reported during pathological calcification in diseases including atherosclerosis [14–16]. Within the oral cavity, calcium phosphate particles exist in saliva and gingival crevicular fluid (GCF), and their abundance appears to correlate with oral diseases such as periodontitis; and calcium phosphate is suspected to be an etiological factor in ectopic calcification-related diseases that involve dental calculus, dental pulp stones, and saliva gland stones [17,18]. Conversely, there is an inverse correlation between periodontitis and dental caries, and formation of plaque with higher calcium concentration is associated with low caries incidence, as periodontitis-affected subjects have more intact teeth compared with subjects who are free of disease [19,20]. Sewon et al. proposed that periodontitis-affected subjects may have an increased intraoral mineralization capacity as their saliva may contain factors that favor mineralization. These findings demonstrate that the calcium content in saliva plays a direct and perhaps dominant role in the homeostasis of the oral environment and therefore modulation of oral pathology. On the other hand, the specific contribution of salivary particulate content to disease is poorly understood.

In this study, we characterized particles isolated from the saliva of healthy human volunteers. We found that the profile of salivary particles was heterogeneous, with a broad distribution in particle size. Nonetheless, the particles contain protein markers such as albumin and fetuin-A that make them similar to other biologically derived mineralo-organic particles [15,21–33]. In addition, calcium phosphate particles can be internalized by gingival epithelial cells and macrophages. These findings, together with the knowledge of the pro-inflammatory responses induced by other biological mineralo-organic particles [27,34], may shed light on how salivary particles participate in oral inflammation and homeostasis.

## Materials and methods

### *Saliva collection and preparation of salivary particles*

Saliva specimens were obtained from healthy volunteer donors with no known significant health problems or oral diseases and conditions, in accordance with the guidelines approved by the Institutional Review Board. Informed consents were obtained from the donors enrolled in the study. The donors were requested not to eat, drink or perform oral hygiene before the collection procedure. Saliva was collected by direct expectoration into a container. Saliva process was modified from procedures previously described elsewhere [35]. For isolation of salivary particles, saliva was mixed with Dulbecco's phosphate-buffered saline (DPBS) at the volume ratio of 1 (saliva):3 (DPBS) and centrifuged at 100×*g* for 1 min. Saliva solution was filtered through 30-μm and then 5-μm filters. In some experiments, calcein (Merck) or cell-impermeable Fluo-4 (Thermo Fisher Scientific) was added to the filtered mixture at 20 mg/ml for 10 min or 1 mM for 60 min at room temperature, respectively, for calcium-sensitive staining. The dye-treated particle suspension was then subjected to centrifugation at 16,000×*g* for 10 min. After centrifugation, the supernatant was carefully removed and discarded. The pellet containing the particles was resuspended in DMEM; the particle suspension in DMEM was then used in the particle internalization experiment. The centrifugation/resuspension cycle was repeated three times to remove free dye.

### *Culture and staining of macrophages and gingival epithelial cells*

The culture of the human monocytic cell line THP-1 (American Type Culture Collection; ATCC) was maintained in Roswell Park Memorial Institute (RPMI) 1640 containing 10% FBS and primed with 200 nM phorbol myristate acetate (PMA; ENZO Life Science) for 24 h before the start of experimentation. The culture of gingival epithelial cells (GEC) was previously described [36] and was maintained in defined keratinocyte-SFM containing growth supplement, bovine pituitary extract, and 10% FBS, and split at 1:4 every three days with media replacement on the day before splitting the confluent monolayer. To prepare for visualization of particle internalization by THP-1 cells and GEC, THP-1 cells or GEC were stained with CellMask Orange (Thermo Fisher Scientific) at 1,000 ppm for 15 min or 2,000 ppm for 60 min, respectively, at 37 °C. After staining, the dye-containing culture supernatant was removed, and the cells were rinsed with DPBS three times.

### *Fluorescence microscopy*

Calcein-labeled salivary particles were observed and photographed by DM6 B upright microscope (Leica Microsystems). For salivary particle internalization study, calcein-stained salivary particles resuspended in DMEM were added to the culture of THP-1 cells or GEC stained with CellMask Orange at volumetric ratio of 1:10. Internalization of calcein-labeled salivary particles by cells in culture was monitored by a multiphoton laser-scanning microscope (Zeiss LSM 510 META NLO; Carl Zeiss) while maintained at 37 °C with a heating unit (XL S and TempModule S; Carl Zeiss).

### *Flow cytometry*

Salivary particles were labeled with calcein as described above and subjected to flow cytometry using the Attune NxT flow cytometer (Thermo Fisher). The Attune NxT Software V2.6 was used for analysis. Commercially obtained 1.5 μm polybeads (Polysciences, Inc.) were used as size reference. For each sample, the particle population was first gated for calcium presence (Ca+) and absence (Ca-). Each of the two separate gated populations were further separated into two populations of smaller (<1.5 μm) and larger (≥1.5 μm) particles. Comparison of the gated populations was made on the basis of their sum total as 100%.

### *Immunoblotting*

For detection of particle-binding proteins, the salivary particle suspension was subjected to centrifugation at 16,000×*g* for 10 min. The resulting pellets were resuspended in sodium dodecyl sulfate (SDS) loading buffer (50% glycerol, 10% SDS, 12.5% β-mercaptoethanol, 313 mM Tris–HCl, 0.05% bromophenol blue; pH 6.8) with 50 mM EDTA. Particle samples resuspended in loading buffer were then heated at 95 °C for 5 min, followed by centrifugation at 16,000×*g* for 5 min. Post centrifugation, the supernatant of the samples was loaded

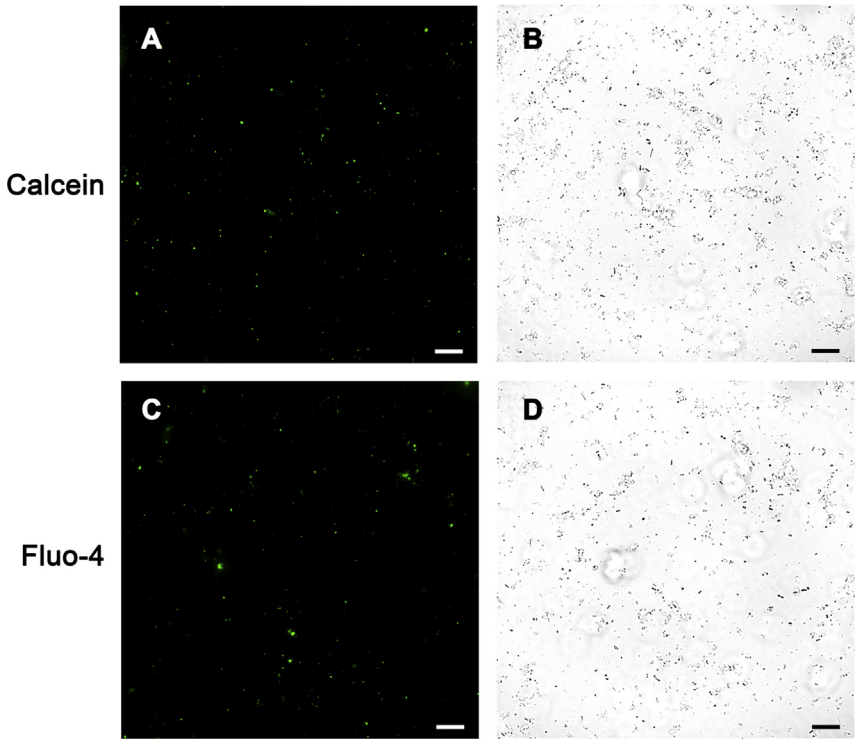


Fig. 1 **Calcium-containing particles are detected in saliva.** Calcein (A) or Fluo-4 (C) was added to the filtered saliva as described in *Materials and Methods*. Brightfield images in panel B and panel D were taken at the same field as panel A and panel C, respectively. Images are representative of three independent experiments. Scale bar: 10 μm.

onto a SDS-PAGE gel for electrophoresis and transferred to PVDF membranes. Mouse anti-human albumin, mouse anti-human fetuin-A, and goat anti-human statherin antibodies (Santa Cruz Biotechnology) were used for immunoblotting detection. Goat anti-mouse IgG and donkey anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

### *Statistical analysis*

Statistical analysis was performed using SigmaStat v3.0. Wilcoxon signed-rank test with normality check for comparisons of two groups was used; sample pairs passing normality check were analyzed by paired t-test.

## Results

### *Calcium-containing particles are detected in saliva*

To determine whether calcium phosphate or calcium-based particles can be detected in saliva, we collected saliva-derived particles by centrifugation and filtration. The collected particles were then stained by FITC-labeled calcein [Fig. 1A and B (brightfield)] or Fluo-4 [Fig. 1C and D (brightfield)] [37], a stain for $Ca^{2+}$. Labeled particles and aggregates could be detected in saliva samples. The particles appeared to have varied shapes and wide-ranging size distributions, indicative of diversity in the physical characteristics of saliva-derived particles.

### *Distribution of particle sizes in saliva*

Flow cytometry analysis was performed on samples of salivary particles to determine the calcium content and size distribution of particles found in saliva. Particle association with calcium could be determined by staining with calcein, whose fluorescence was used as the basis for separating calcium-rich particles from non-calcium particles [Fig. 2A]. The concurrent signal detection in the YL1 channel was used to exclude background auto-fluorescence from the observed events. These calcium-rich particles from saliva varied greatly, and their sizes spanned the entire forward-scatter scale [Fig. 2B]. Of the six samples tested, between 32% and 99% of total particles contained calcium [Table 1]. These calcium-positive particles consisted of particles measuring smaller than 1.5 μm (between 7% and 40% of total particles) and at or greater than 1.5 μm (between 21% and 68% of total particles). On average, close to half of all salivary particles [Table 1] were calcium-positive particles of micrometer scale, which were known to have significant roles in inflammatory responses. Comparison of the two size groups

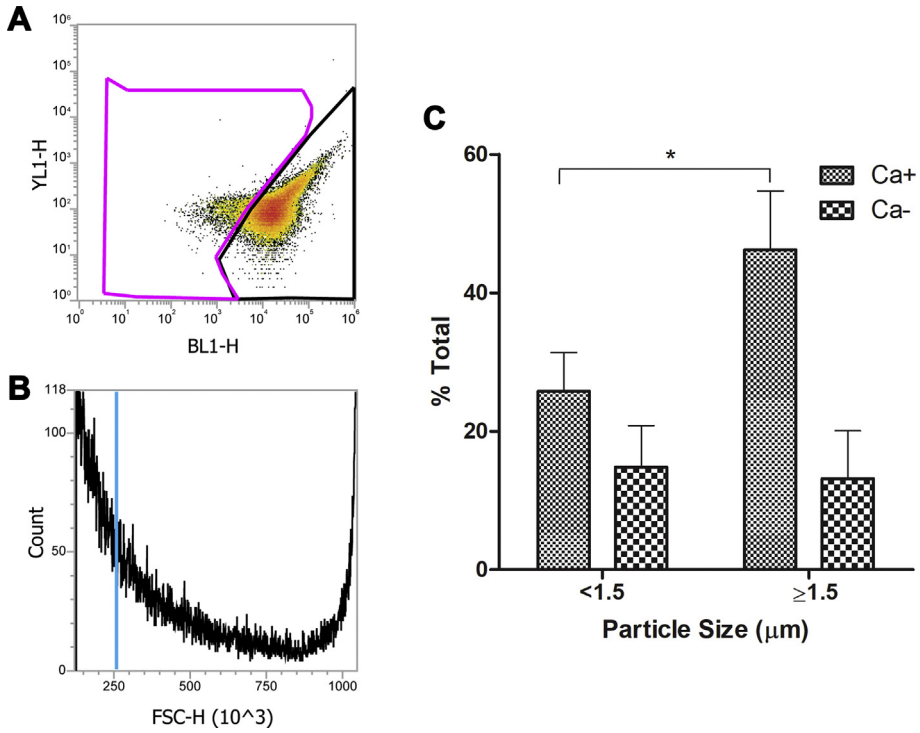


Fig. 2 **Distribution of particle sizes in saliva.** Particles in saliva were analyzed by flow cytometry. (A) Calcium-positive particles were identified by calcein staining (BL1 channel), and the extent of fluorescence bleeding into the YL1 channel was utilized to exclude unstained events. Black-bordered region: calcein-positive events; pink-bordered region: calcein-negative events. (B) The size distribution of the calcein-stained particles was depicted in a histogram. The blue line representing standard beads rated at 1.5 μm was included in the analysis as a reference of particle size. (C) Samples were analyzed to determine the prevalence of micron-to-sub-micron (particle diameter < 1.5 μm) and micron-sized particles (particle diameter at or greater than 1.5 μm) among the calcium-positive (Ca+) salivary particles, versus non-calcium (Ca-) salivary particles that were not stained by calcein. The statistical significance of the differences is highlighted (*: $p < 0.05$).

**Table 1 Proportion and size of calcium-positive particles in saliva.**

| Sample | Ca-positive particles[a] | Ca-positive particles < 1.5 μm[a] | Ca-positive particles ≥1.5 μm[a] |
|---|---|---|---|
| 1 | 99 | 31 | 68 |
| 2 | 93 | 36 | 57 |
| 3 | 97 | 30 | 67 |
| 4 | 34 | 7 | 26 |
| 5 | 77 | 40 | 37 |
| 6 | 32 | 11 | 21 |
| Mean ± SEM | 72 ± 13 | 26 ± 6 | 46 ± 8 |

[a] % total.

within calcium-rich particles showed statistically significant enrichment in the micrometer range as opposed to submicron particles [Fig. 2C]. In brief, saliva-derived particles are diverse in their calcium content over a broad spectrum of particle size distribution but are enriched in micrometer-sized calcium-positive particles.

### *Internalization of salivary particles by human cells*

FITC-calcein-labeled salivary particles were co-cultured with PMA-primed THP-1 cells to allow visualization of the salivary particle internalization by macrophages. By 0.5 h after addition of the particles to the cells, the particles could be seen being internalized by THP-1 cells [Fig. 3]. Similarly, FITC-calcein signals were also detected within the cytoplasm of GEC after particle-cell co-culture for 1 h, consistent with a process of particle phagocytosis by these cells [Fig. 3]. Taken together, these results demonstrated active internalization of calcium-positive salivary particles by macrophages and gingival epithelial cells, both of which are present in the gingival epithelium of the oral cavity.

### *Identification of proteins in salivary particles*

Particles harvested from the saliva of three individuals were subjected to SDS-PAGE and immunoblotting analysis for detection of particle-associated proteins. Both albumin and fetuin-A were previously observed in calcium particles from other tissues [15,22–25,33,38], and they were also found to be associated with salivary particles as shown by immunoblotting [Fig. 4]. Statherin, a known salivary protein, was not previously reported in calcium particles from other tissues but was detected in salivary particles. Both fetuin-A and albumin are predominant proteins found in serum-derived mineralo-organic particles, while it has been suggested that statherin may function as a transporter of calcium and phosphate during secretion in the salivary glands. The association of the known particle-associated factors with the salivary particles, concurrent with the presence of calcium-based minerals, therefore indicates a similarity that may exist between saliva-derived particles and other biologically derived mineral particles, while the presence of statherin

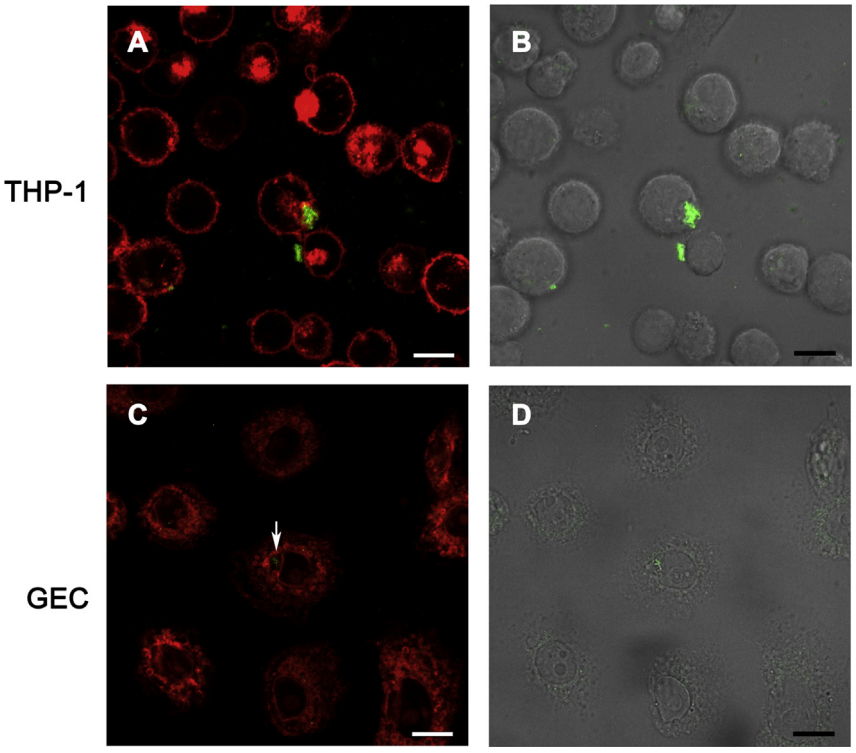


Fig. 3 **Internalization of salivary particles by macrophages and GEC.** The THP-1 macrophages (A) and GEC cells (C) were stained with CellMask Orange (red) to highlight their plasma membrane in co-cultures with calcein-stained calcium-positive salivary particles (green). The brightfield view of the same images are shown in panels (B) and (D), respectively. The internalized particle in (C) is indicated (white arrow). Data are representative of three independent experiments. Scale bar: 10 μm.

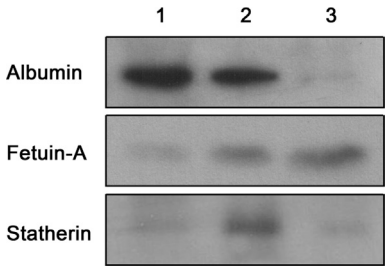


Fig. 4 **Protein identification in salivary particles.** Salivary particles were subjected to SDS-PAGE and immunoblotting analysis. The presence of particle-bound albumin, fetuin-A, and statherin in samples obtained from three different individuals was probed (lanes 1 to 3) with specific antibodies.

reflects the unique role that the salivary particles may play in the oral cavity.

## Discussion

In this study, we characterized the size of the particles in saliva and identified proteins associated with these particles. These particles were calcium-based, with close to half of collected salivary particles from different individuals consisting of calcium-containing components. These particles exist in diverse shapes over a wide spectrum of sizes, and they recruit salivary proteins to the particle complex. Of these particle-recruited factors, albumin and fetuin-A have established roles in mineral particle formation and inhibition that may have a significant contribution in facilitating mineral particle development in the saliva, while statherin is specific for saliva.

Salivary particles were present in a continuing spread of sizes. This wide particle size distribution may represent assembly of particle complexes at various stages of aggregation, or they may represent multiple sources of particles. The role of saliva in interacting with salivary particles and modulating their aggregation may also be considered, as recent evidence suggests that, depending on the chemical properties of their surfaces, nanoparticles introduced into saliva could agglomerate with increased binding to mucins in saliva [39].

As shown in this study, saliva-derived particles could be internalized by both gingival epithelial cells and monocyte-derived macrophages. Other studies demonstrated that serum-derived, calcium-based mineralo-organic particles in the micrometer range interact with fibroblasts and immune cells including macrophages and NP [26,27,34]. It is conceivable that salivary particles may undergo similar processes in stimulating pro-inflammatory activation of gingival tissue and thereby priming it for development of periodontitis. On the other hand, saliva is not pro-inflammatory and therefore there appear to be salivary factors capable of modulating the particle-induced pro-inflammatory activity. Further study on hallmarks of inflammatory responses elicited by salivary particles, including cytokine secretion, may provide insight on this concept by delineating the exact involvement of salivary particles in inflammatory responses during periodontal events.

The association of fetuin-A with the salivary particles suggests the potential for regulation of particle properties in the saliva. Together with albumin, fetuin-A has been recently shown to modulate mineral particle formation and growth in body fluids [15,21–23]. Other studies reported the presence of fetuin-A in saliva and suggested other roles in the oral cavity. Thus, fetuin-A is known to be actively involved in orthodontically induced inflammatory root resorption (OIIRR), an unavoidable consequence of orthodontic movement [40]. Increasing levels of fetuin-A in saliva and GCF were also observed in patients with dental calculus [41]. Furthermore, fetuin-A was identified in the etiology of sialolithiasis, or salivary calculi [42]. Fetuin-A may modulate calcium particle formation and actively participate in the process of plaque growth and development into calculus. We also found that statherin, a calcium transporter that maintains soluble calcium levels in saliva by preventing precipitation of calcium phosphate [13], is associated with calcium-based salivary particles. Statherin plays a role not only in antimicrobial defense but also in inhibiting precipitation of calcium phosphate in the salivary glands and mouth [13,43]. Its association with the salivary particles therefore suggests that calcium-based particles participate in both calcium homeostasis and pathogenesis of dental caries.

Although we specifically investigated the association of fetuin-A, albumin, and statherin in salivary particles, the likelihood is high that there are other significant particle-associated factors that remain to be identified. A proteomic approach that surveys the complete profile of particle-associated proteins may provide further insight in the action of the salivary particles on the mechanisms of action [25].

## Conclusions

The characterization of salivary calcium particles marks the first steps in understanding their role in the pathogenesis of periodontitis. It is likely that particle-associated factors including albumin, fetuin-A, and statherin, together with other as-yet-unidentified salivary components, participate in the regulation of oral calcium homeostasis and development of saliva-derived particles. Elucidation of the formation of the salivary particles and their interaction with gingival epithelium should lead to a better understanding of periodontitis development and could suggest design of therapeutic options.

## Conflict of interest

The authors state that they do not have a conflict of interest.

## Acknowledgements

We thank Yu-Ju Liu for assistance throughout this study. This study was supported by funds from Primordia Institute of New Sciences and Medicine and by grants from Chang Gung

University (QZRPD146), Ming Chi University of Technology (0XB0), and Chang Gung Memorial Hospital (CMRPG3F0773 and CMRPG3I0341). We thank the Core Instrument Center and Microscopy Center of Chang Gung University for technical support. We also thank Yu-Jr Lin of the Research Service Center for Health Information, Chang Gung University, for providing statistical consultation in support of our study.

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