



Inorganic food additive nanomaterials alter the allergenicity of milk proteins

Wut H. Phue, Ke Xu, Saji George*

Department of Food Science & Agricultural Chemistry, McGill University, 21111, Lakeshore Road, Sainte-Anne-de-Bellevue, QC, H9X 3V9, Canada

ARTICLE INFO

Handling Editor: Dr. Jose Luis Domingo

Keywords:

Milk allergy
Inorganic food additive materials
Allergenicity
E551
E171

ABSTRACT

While inorganic nanomaterials are copiously incorporated in food products, their impact on the allergenicity of food proteins is largely unknown. This study analyzed the effect of widely used food additive nanomaterials (silica and titania) on the antigenicity and allergenicity of milk proteins (β -lactoglobulin and casein) and skimmed milk. Changes in the antigenicity of milk proteins in the presence of dietary nanomaterials were identified using an indirect-ELISA assay, while the change in allergenicity was studied using mast cell (LAD2) sensitized using allergic human sera. Results showed an enhancement in the allergenicity of milk proteins/skimmed milk interacted with particles (both silica and titania). Similarly, mast cell degranulation (a proxy for allergenicity) was higher when exposed to particle interacted skim milk where nanomaterials of titania showed the highest effect, and this tendency was retained even after subjecting to simulated gut digestion. Particles induced alterations in the structure of milk proteins, as evidenced by our studies, are reasoned to expose epitopes that increase allergenicity of milk proteins.

1. Introduction

Food allergy is defined as ‘an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food’ (Boyce et al., 2011). The increasing occurrence of life-threatening episodes and lack of effective treatment options make food allergies a growing public health concern worldwide. Bovine milk is a major source of animal proteins for human nutrition. Unfortunately, however, milk allergy is one of the most common food allergies in young children, affecting 2–3% of the general population (Castillo and Cassola, 2017). Research has shown that most of the sensitive children overcome food allergies by the age of 3; however, the reaction still can develop or appear in adulthood (Pelto et al., 1999; Bengtsson et al., 1996). The adverse outcome from milk allergy span from cutaneous reactions, respiratory episodes, gastrointestinal (GI) distress to life threatening anaphylactic shock (Fiocchi et al., 2010; Castillo and Cassola, 2017; Turnbull et al., 2015).

There are around 20 proteins reported to induce IgE-mediated allergy in cow milk, and 10 of them are reported in the official Allergen Nomenclature Database from the World Health Organization and International Union of Immunological Societies (WHO/IUIS) (Madsen et al., 2014). Structure of protein is a critical determinant of its

allergenicity. Studies have shown that extremes of pH and temperature conditions encountered during food processing could alter conformational and linear epitopes in allergenic proteins whereby changing their antigenicity and allergenicity (Madsen et al., 2014; Mine and Zhang, 2002; Burks et al., 1992). For instance, Maleki et al. reported that the allergenicity of peanut increased 90-fold higher after roasting which they related with protein modifications caused by the Maillard reaction (Maleki et al., 2000). Allergenicity to proteins could increase, decrease or remain unchanged in response to food processing conditions given the possibilities of destruction of epitope sequences, formation of new epitopes, increased accessibility of epitopes facilitated by change in structural conformational of proteins depending on the type and extent of food processing conditions (Castillo and Cassola, 2017).

Inorganic food additives materials such as silicon dioxide (E551) and titanium dioxide (E171) have been used by food industries as anticaking agents and white pigment, respectively (Athinarayanan et al., 2015). As reported earlier, 33% of E551 (food-grade SiO_2) and 36% of E171 (food-grade TiO_2) exist in nanometric size range (<100 nm in diameter) (Dekkers et al., 2011; Weir et al., 2012). Recent studies from our group have shown that proteins of relevance to milk allergy could be enriched when particles are dispersed in milk (Phue et al., 2021) and the interaction of proteins with dietary particles could alter the structure and

* Corresponding author.

E-mail address: saji.george@mcgill.ca (S. George).

<https://doi.org/10.1016/j.fct.2022.112874>

Received 9 November 2021; Received in revised form 28 January 2022; Accepted 15 February 2022

Available online 17 February 2022

0278-6915/© 2022 Elsevier Ltd. All rights reserved.

function of proteins (Phue et al. 2020, 2021). However, the consequence of particle-milk protein interaction on antigenicity and allergenicity of milk proteins have not been addressed. Since interactions of particles with proteins altered protein structures, it was reasonable to hypothesize that interactions of dietary particles with prominent food proteins could alter its antigenic and allergic properties. We tested this hypothesis by studying the alterations of the IgG binding (using ELISA) and allergenicity (using mast cell degranulation assay) of milk and individual milk proteins (β -lactoglobulin and casein) in the presence of food-grade particles of silica and titania.

2. Materials and methods

2.1. Materials

Food-grade silicon dioxide particles (FG-SiO₂-NP) in nano-size (AEROSIL 200F) and food additives silicon dioxide particles, E551 (SIPERNAT 22) were obtained from Evonik Corporation (NJ, United States). Food grade titanium dioxide nanoparticles (FG-TiO₂-NP, cat # A020-II) were purchased from CNMI industrial corporation, China, and food additives titanium dioxide particles (E171, cat # 13463-67-7) were obtained from Minerals-Water, UK. All particles were used as received. Casein (cat # 5890) was purchased from Sigma Aldrich (St. Louis, MO, USA), and β -lactoglobulin (cat #S0131) was obtained from Davisco Foods International Inc. (Eden Prairie, MN, USA). Quebon skimmed milk (Agropur Dairy Cooperative, QC, Canada) was purchased from a local grocery store. For enzyme-linked immunosorbent assay (ELISA), primary antibody for casein (Anti-casein rabbit antibody-cat # ab166596), primary antibody for β -lactoglobulin (Anti-LGB rabbit antibody-cat # ab112893) and secondary anti-rabbit antibody (cat # 6721) were purchased from Abcam (Cambridge, UK). Unless stated otherwise, all other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). Stock buffer of 0.1 M of phosphate buffer (pH 6.5) was prepared using deionized water obtained from a Milli-Q water system (Millipore Sigma, Massachusetts, USA) and was stored at 4 °C before making the working concentration (20 mM) prior to each experiment.

2.2. Characterization of pristine dietary particles and milk interacted dietary particles

Silica and titania particles obtained initially in powder form were dispersed in distilled Milli-Q water (deionized water) at 10 mg mL⁻¹ concentration (stock solution) for further studies. Skimmed milk with minimal fat content was used as a model food matrix for studying the transformation of milk allergenicity in the presence of dietary particles. Skimmed milk stored at 4 °C was centrifuged (17,000 g for 10 min) to remove any debris, and the supernatant was collected for further use. Aliquots (100 μ L) of dietary particles were added to 900 μ L of milk (final concentration 10 mg mL⁻¹) and incubated for 1 h at 37 °C. Control particle samples were prepared by adding the same proportions of 20 mM sodium phosphate buffer (pH 6.5) (no proteins) to the particle solution. Particles were then pelleted out by centrifugation (20,000 g) for 15 min in a temperature-controlled centrifuge (Sigma 3-30 KS, Sigma Zentrifugen, Germany) at 4 °C, unbound milk proteins in the supernatant were discarded. Particle pellets were resuspended in 1 mL of 20 mM sodium phosphate buffer (pH 6.5) and the resulting suspension was centrifuged at 20,000 g for 15 min, and the supernatant was discarded to avoid loosely bound proteins forming the 'soft corona' on particle surface (Phue et al., 2021). This step was repeated twice to retain hard corona formed on the particles. Particles thus obtained were used freshly for further studies.

Pristine and milk interacted particles suspended in 1 mL of 20 mM sodium phosphate buffer (pH 6.5) to achieve 1 mg mL⁻¹ concentration were characterized for their size (and shape), and surface charge using transmittance electron microscopy (TEM), and dynamic light scattering technique (DLS), respectively. For TEM, 5 μ L of particles suspension

(100 μ g mL⁻¹) was dropped onto 200-mesh Cu TEM grids with carbon film and was air-dried at room temperature for an hour. TEM images were acquired at 120 kV accelerating voltage using Tecnai G2 F20 200 kV Cryo-STEM (Field Electron and Ion Company (FEI), Oregon, USA). The hydrodynamic diameter and zeta potential of the particles were determined using DLS (Nanobook OMNI Instrument, USA). For this, working concentrations of particles were prepared at 50 μ g mL⁻¹ in PBS buffer (pH 6.5). The scattering angle was kept at 90° with a holder temperature of 25 °C, and monodispersion medium viscosity of 0.89 mPa s. The measurements were run at an applied voltage of 100 V.

2.3. Quantification of protein adsorption on dietary particles

After the final wash of milk interacted particles, 100 μ L of 20 mM sodium phosphate buffer was added to achieve a particles concentration of 10 mg mL⁻¹. To quantify the protein in the hard corona, strongly adsorbed protein layer that is retained after washing of milk interacted dietary particles (Milani et al., 2012), 25 μ L of washed particle suspensions were mixed with 25 μ L of protein eluting buffer (containing 2% sodium dodecyl-sulfate (SDS) in 20 mM sodium phosphate buffer) and was incubated for 1 h at room temperature (Srinivasan et al., 2020). Particles were separated from the solution by centrifugation at 20,000 g for 15 min, and the protein eluted to the supernatant was used for protein quantification as detailed elsewhere (Srinivasan et al., 2020). For this, 10 μ L of supernatant were mixed with 100 μ L of Pierce 660 (Thermo Fischer Scientific, MA, U.S.A) containing 50 mM ionic detergent compatible reagent (IDCR) (Thermo Fischer Scientific, MA, U.S.A). The resulting mix was incubated for 5 min at room temperature before measuring the absorbance at 660 nm using a microplate reader (SpectraMax i3x, Multi-mode Microplate Reader, Molecular Devices, USA). Protein concentration was calculated using a standard curve obtained using bovine serum albumin.

SDS-PAGE of eluted proteins from milk interacted particles was conducted to determine prominent proteins associated with surface corona. Twenty microliters of the milk interacted particles samples were mixed with sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue. Subsequently, 5% β -mercaptoethanol was added to one set of particles pelleted, vortexed, and boiled for 5 min. Particles were removed from sample buffer by centrifugation at 20,000 g for 15 min; protein-containing sample solution was collected and transferred into a fresh tube. Fifteen microliters of each sample were loaded to a sample well of a gradient (4–20%) SDS-polyacrylamide gel. Proteins were separated in the gel using 100 V for 1.5 h in a running buffer containing 2.5 mM Tris, 19.2 mM glycine and 0.01% SDS. The gel was then stained in standard Coomassie blue-methanol-acetic acid solution for 30 min at room temperature. These gels were subsequently washed with de-staining solution (40% methanol, 10% acetic acid and 50% water) for 30 min at RT to visualize the protein bands.

2.4. Analysis of alteration in protein secondary structure by attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy

The ATR-FTIR spectra of β -lactoglobulin and casein before and after interacting with dietary particles were used to assess changes in the secondary structure of proteins upon binding with dietary particles. For this, equal volumes of the proteins (10 mg mL⁻¹) and dietary particles (10 mg mL⁻¹) were mixed in a clean glass tube and were incubated at 37 °C for 1 h. Pure protein samples were prepared by adding the same proportions of DI water to the protein solution. Two microliters of sample were dropped on the ATR probe and left to dry for 15 min. FTIR spectra were collected in the wavelength range of 4000–400 cm⁻¹ at a resolution of 4 cm⁻¹ and 128 scans in Nicolet Summit ATR-FTIR spectrometer (Thermo Fisher Scientific, MA, USA). Each spectrum was corrected for background noise. FTIR peaks corresponding to particles were subtracted from the spectrum of the protein-particle complex. Fourier

self-deconvolution was used to resolve overlapping bands in the FTIR spectrum in the range of amide I 1700–1600 cm^{-1} (Yang et al., 2015) by OMNIC software.

2.5. Quantitative analysis of free thiol group by Ellman's assay

Disulphide bonds are determinants of protein structure, stability and formation of conformational IgE epitopes (Pekar et al., 2018). Therefore, we determined the disruption of disulfide bonds when proteins were interacted with dietary particles as detailed previously (Riener et al., 2002). Briefly, 200 μL of 1 mg mL^{-1} proteins (pure or particle interacted) and 50 μL of 2 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were added to 100 μL of tris buffer (1M) in an Eppendorf tube. The solution was topped up to 1 mL with DI water. The absorbance of the resulting solution was measured using SpectraMax i3x multi-mode microplate reader at 412 nm. Different concentrations of acetylcysteine were used for preparing the standard curve, and protein denatured by 6 M guanidinium chloride was used as a positive control.

2.6. Assessing changes in the quaternary structure of proteins in presence of dietary particles

The changes in the quaternary structure of proteins interacted with particles were determined using changes in the intrinsic fluorescence of tryptophan, tyrosine, and phenylalanine. The emission intensity of protein suspension (0.1 mM in 20 mM phosphate buffer-pH 6.5) was compared with those containing various concentration of dietary particles (0 mM–100 mM). The quenching studies were repeated at different temperatures 20 °C, 25 °C, 30 °C and 35 °C to identify the type of interaction between particles and proteins. The binding affinity of each particle to proteins was calculated using the Stern-Volmer equation as shown below,

$$F_0/F = 1 + K_{sv} \cdot [Q] = 1 + K_q \cdot \tau^0 [Q]$$

Where F_0 and F represent the steady-state fluorescence intensities in the absence and presence of silica particles, respectively, K_q is the biomolecular quenching constant, and τ^0 is the fluorescence lifetime in the absence of particles. The value of τ^0 is a constant: 1.28 ns for β -lactoglobulin (Gorji et al., 2015) and 0.982 ns for casein (Panja et al., 2018). K_{sv} is the Stern-Volmer quenching constant, and $[Q]$ is the concentration of the particles.

2.7. Protein identification of in vitro digested milk samples by one-dimension gel electrophoresis

Milk in the presence of different dietary particles was passed through the *in vitro* digestion system. The simulated *in vitro* digestion was conducted, and digestion fluid (SSF, SGF and SIF) were prepared as reported earlier (Brodtkorb et al., 2019). Briefly, 100 μL of the dietary particles (SiO_2 and TiO_2) (10 mg mL^{-1}) were mixed with milk and dilute with simulated salivary fluid (SSF) in a 1:1 (v/v) ratio at pH 7 (oral phase). The solutions were kept in this oral phase for 2 min at 37 °C in continuous orbital shaking condition. Subsequently, the oral phase solution was mixed with simulated gastric fluid (SGF), that contained gastric enzymes, pepsin and gastric lipase in a 1:1 ratio (v/v) at pH3 (gastric phase). The gastric phase solutions were incubated at 37 °C for 2 h under agitation following which the resulting gastric chyme was mixed with simulated intestinal fluid (SIF) which contained bile salts and pancreatin, in a 1:1 (v/v) ratio at pH7. The resulting suspension was incubated at 37 °C for another 2 h under agitation. Activities of enzymes (amylase, pepsin, gastric lipase and pancreatin) present in the resulting solution were terminated by boiling the suspension for 10 min. The resulting solutions were used for antigenicity and allergenicity testing (as detailed below). Parallely, particles-protein complex present in this mix were separated by centrifugation at 20,000 g for 15 min. Particle pellet thus

obtained was suspended in 50 μL of PBS buffer (pH 6.5) and was mixed with 100 μL of SDS sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% (v/v) glycerol, 0.01% bromophenol blue and 5% β -mercaptoethanol to elute proteins adsorbed onto particles. The suspension was vortexed, boiled for 5 min and centrifuged (20,000 g for 15 min) to remove particles. The resulting solution containing denatured protein -was transferred to a fresh Eppendorf tube. Twenty microliters of each sample were loaded to each sample well of a 4–20% SDS-polyacrylamide gel for electrophoresis as detailed earlier.

2.8. Testing for alteration in the antigenicity of milk proteins interacted with particles

Nine hundred microliters of skimmed milk and milk proteins (1 mg mL^{-1} of β -lactoglobulin and casein) were mixed with 100 μL of dietary particles (10 mg mL^{-1}). The control sample was prepared by adding the same proportions of 20 mM sodium phosphate buffer (pH 6.5) (without particle) to the protein solution.

Change in the antigenicity of milk proteins when interacted with dietary particles were analyzed using ELISA assay. ELISA plate was prepared by coating 100 μL milk proteins or milk proteins interacted with particles (50 ng mL^{-1}) onto the wells of microliter plate (Nunc Maxisorp 96-well ELISA plate) in coating buffer (0.1 M Na_2HPO_4 (pH 9.5) in DI water) for overnight at 4 °C. The uncoated samples were discarded, and free binding sites of the wells were blocked with blocking buffer containing 2% BSA in 1 $\times$ TBS (50 mM Tris-HCl and 150 mM NaCl in deionized (DI) water) for 1 h at 37 °C. These wells were then incubated with primary antibody (pAb) at 1:5000 dilution for β -lactoglobulin pAb and 1:2000 for casein pAb in blocking buffer for 1 h at room temperature (RT). The wells were washed to remove unbinding antibodies with washing buffer (1X TBS added with 0.05% tween-20) for four times and the plate was further incubated for 1 h at RT with secondary antibody at a 1:10000 dilution in blocking buffer. Substrate solution (5 mM of tetramethylbenzidine-TMB) was added after a final washing step (4 $\times$) with washing buffer and incubated for 5 min at RT for the enzymatic color development. The reaction was stopped by adding 100 μL of 2 M sulphuric acid. The absorbance was measured at 450 nm using the microplate reader, and IgG binding sites were calculated using a standard curve of control β -lactoglobulin and casein proteins.

2.9. Human mast cell degranulation assay to assess allergenicity of milk in presence and absence of dietary particles

Human Laboratory of Allergic Diseases 2 (LAD2) mast cells (kindly supplied by Dr. A. S. Kirshenbaum, National Institutes of Health, Bethesda, MD, USA) were cultured in StemPro-34 serum-free medium (Gibco #10640) with STEM PRO-34 nutrient supplement (Gibco #10641–025), 1% (v/v) penicillin-streptomycin (Cat # 450-201-EL, Wisent, Quebec, Canada), 1% (v/v) L-glutamin-200 mM (Gibco # 25030–081), 0.2% (v/v) primocin-50 mg (cat # ant-pm-05, Invivogen, California, USA), and 100 ng mL^{-1} rhSCF (cat # 78062, STEMCELL Technologies, Vancouver, Canada) at 37 °C and 5% CO_2 . Hemi-depletions were performed weekly as instructed by the supplier. The release of β -hexosaminidase was detected according to a reported method (Wang et al., 2020). Briefly, LAD2 cells (1×10^5 cells/well) were seeded on a 96-well plate in a complete cell culture medium for overnight and then cells were sensitized by diluted sera (1:50, v/v in complete cell culture medium) obtained from milk allergy patients (supplied by PlasmaLab International, Everett, WA, USA.). Specific IgE levels for f2 allergen of patient's sera were 20.886 kU/L, 19.267 kU/L, 55.582 kU/L, 74.7 kU/L, and 41.9 kU/L, respectively. After incubating for 24 h, 80 μL of cell supernatant were carefully removed by multichannel pipette. Allergic activation was induced by incubating with 100 μL of milk in the presence of dietary particles (milk protein concentration-100 ng mL^{-1}) for 3 h in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 20 mM HEPES at

pH-7.4). Positive controls were prepared by adding 100 μL of 0.1% Triton X-100 in Tyrode's buffer to the sensitized cells. The supernatant from the LAD2 cells incubated with particles interacted proteins/milk (50 μL) was incubated with 50 μL of *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide (substrate for β -hexosaminidase) dissolved in 0.1 M citrate buffer (pH 4.5) for 90 min at 37 °C. The enzyme reaction was terminated by adding 100 μL 0.1 M glycine-carbonate buffer (1 M glycine and 1 M sodium carbonate, pH-10). The absorbance of the hydrolyzed substrate was measured using SpectraMax i3x multi-mode microplate reader at 405 nm. The cell degranulation results are expressed as the percentage of β -hexosaminidase released relative to the positive control (Triton X-100) sample. These experiments were repeated with milk samples subjected to *in vitro* digestion in the absence and presence of dietary particles.

2.10. Statistical analysis

Experiments were performed in triplicates and replicated at least three times. The data collected in this study are expressed as the mean value $\pm$ standard deviation (SD). Statistical comparisons were made by Duncan (one-way ANOVA) and paired *t*-test, and *p*-value ≤ 0.05 was considered a significant difference.

3. Results

3.1. Characterization of the particles

The physicochemical properties of pristine dietary particles and protein interacted particles were evaluated by TEM and DLS, and the

results are shown in Fig. 1. TEM was carried out to verify the presence of surface corona milk interacted particles and to determine the particles' size, shape, and agglomeration status. The presence of a semi-transparent layer, characterized by a lower electron density, suggestive of surface corona formation on particles was evident on particles interacted with milk (red arrow in Fig. 1B). FG-SiO₂-NP was constituted by spherically shaped particles of ~ 20 nm diameter and showed agglomeration (Fig. 1B), while E551 showed aggregation (inter-connected particles) (Fig. 1D). The overall hydrodynamic size of FG-SiO₂-NP and E551 were 539.62 (pdi 0.29) nm and 35635.85 (0.53) nm, respectively (tabular data, Fig. 1J). Pristine titania nanoparticles (Fig. 1F) and E171 (Fig. 1H) were pseudo-spherical in shape and their primary sizes were ~ 120 nm and 50–300 nm, respectively. The hydrodynamic size of FG-TiO₂-NP and E171 were 448.59 nm (pdi 0.19) and 335.98 nm (0.24), respectively (tabular data, Fig. 1J). Milk interacted silica and titania particles showed surface corona on the outer surface of the aggregated or agglomerated particles and in the interstitial spaces between the particles (see arrow in Fig. 1C, E, G and I). The thickness of corona layers ranged from 200 to 700 nm (tabular data, Fig. 1J). Except for FG-SiO₂-NPs, the hydrodynamic sizes of particles generally reduced after interacting with milk or milk proteins. Zeta potential measured using DLS for all tested particles showed a net negative surface charge, and generally, the surface charge of all tested particles increased after acquiring surface corona.

3.2. Quantification of protein corona formation

Proteins adsorbed onto the particles were quantified with colorimetric assay and SDS-PAGE, and the results are shown in Fig. 2A, and B.

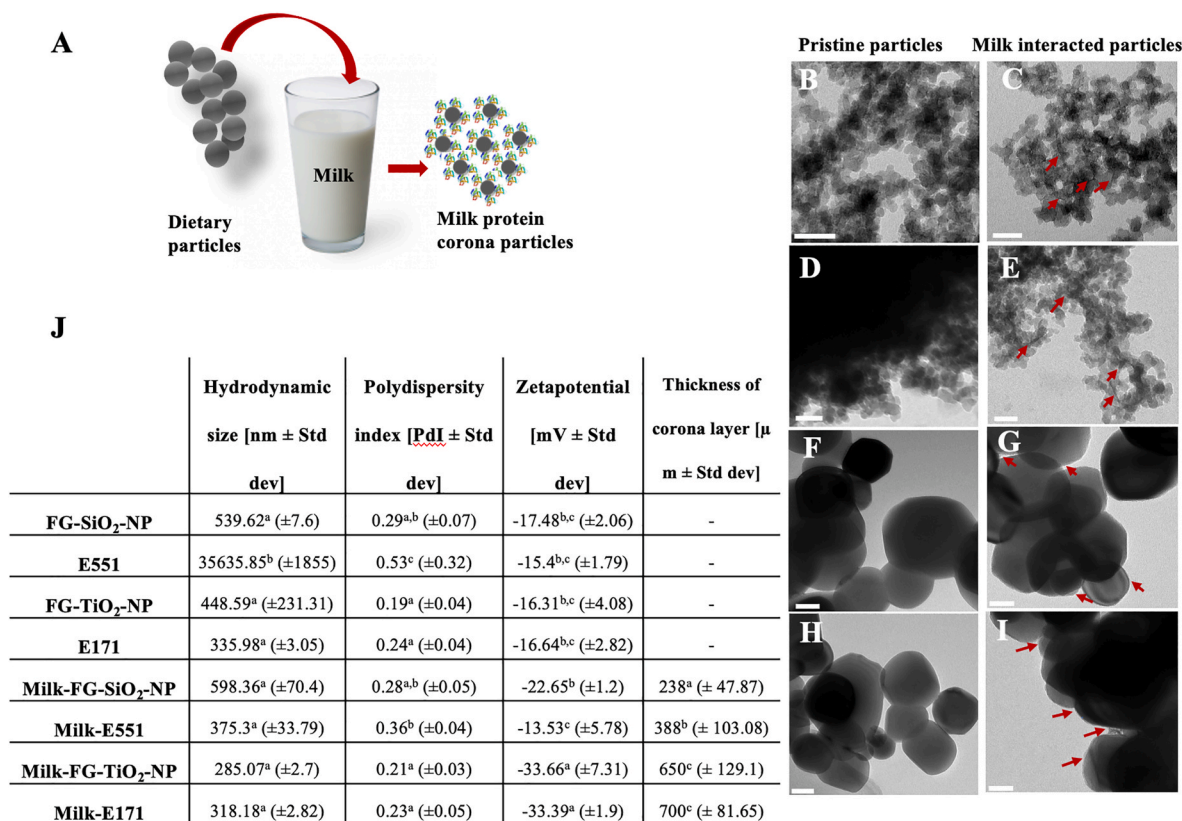


Fig. 1. Characterization of pristine and milk interacted dietary particles by TEM and DLS. The images were obtained by dropping 5 μL of particles suspension ($100 \mu\text{g mL}^{-1}$) onto 200-mesh Cu TEM grids with carbon film and using TEM (Tecnai G2 F20 200 kV Cryp-STEM). Scale bar: 50 nm.

(A) Schematic of the protein corona formation, (B) FG-SiO₂-NP, (C) Milk-FG-SiO₂-NP, (D) E551, (E) Milk-E551, (F) FG-TiO₂-NP, (G) Milk-FG-TiO₂-NP, (H) E171, (I) Milk-E171, and (J) hydrodynamic diameter, polydispersity index and, surface charge of dietary particles in the presence and absence of milk in PBS are determined using DLS, and thickness of corona layer onto the dietary particles. Means with different small letters in the same column are significantly different to each other (Duncan, *P* < 0.05).

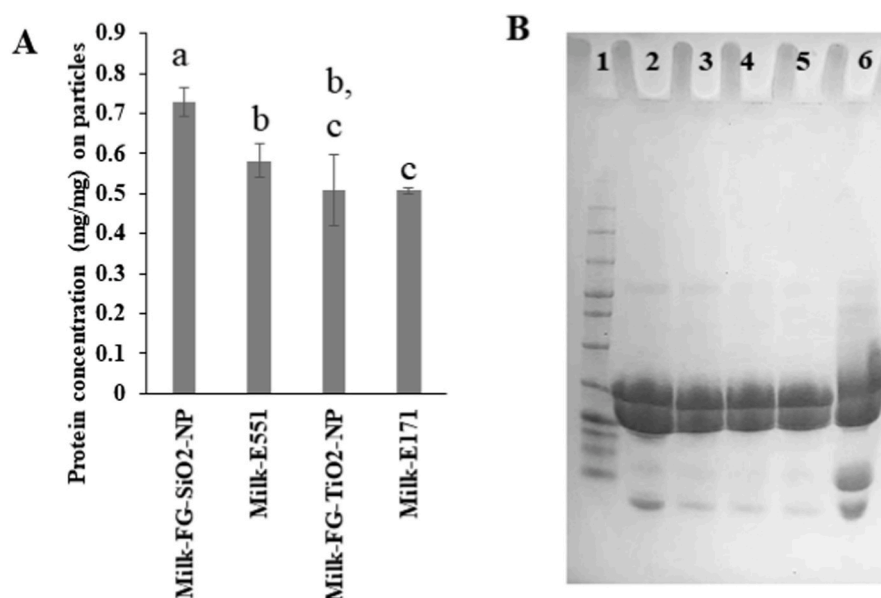


Fig. 2. Quantification of protein adsorption on dietary particles. (A) Particles suspended and incubated in protein suspensions were retrieved using centrifugation. The protein corona concentration was quantified by Pierce 660 containing 50 mM ionic detergent compatible reagent (IDCR). Average values are plotted in the graph with different small letters indicate significant differences (Duncan, $p < 0.05$). (B) SDS-PAGE pattern of proteins retrieved from milk interacted particles. Lane (1) Marker, (2) milk-FG-SiO₂-NP, (3) milk-E551, (4) milk-FG-TiO₂-NP, (5) milk-E171, and (6) milk.

* a = Polymeric immunoglobulin receptor, b = BSA, c = Casein Fraction (α -casein, β -casein, κ -casein), d = β -Lactoglobulin, e = α -Lactalbumin.

FG-SiO₂-NP had the highest amount of proteins adsorbed on its surface followed by E551, FG-TiO₂-NP and E171. Total protein estimation from particle surface concurred with the gel electrophoresis results where protein bands from FG-SiO₂-NP were darker and thicker (Fig. 2B). Predominant milk proteins recovered from the hard-corona formed on particles (both titania and silica), as revealed in SDS-PAGE, were bands

corresponding to β -lactoglobulin and casein (Fig. 2B, lane 6). Based on this observation, we chose these proteins for antigenicity and allergenicity studies.

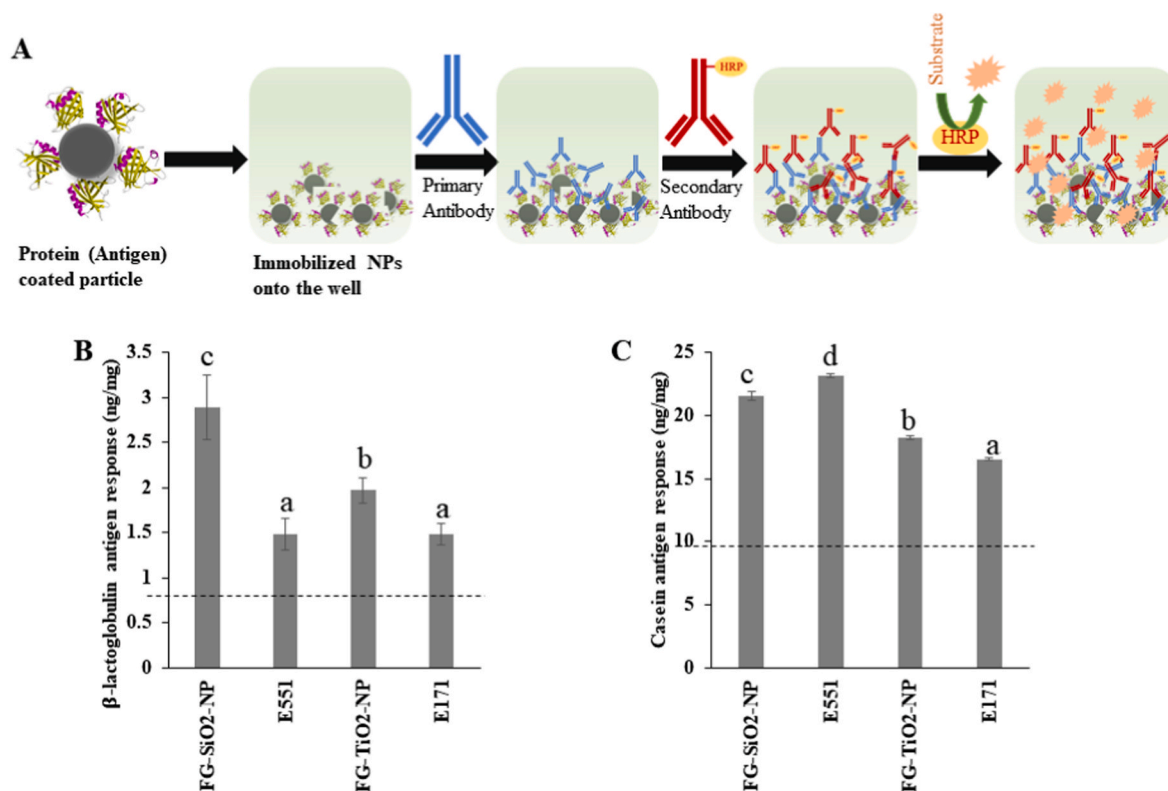


Fig. 3. Quantification of the antigen response in the presence of inorganic dietary particles by indirect enzyme-linked immunosorbent assay (ELISA). (A) Schematics of the indirect-ELISA for quantifying antigenicity of the proteins. ELISA plates were coated with antigen (50 ng mL⁻¹) (B) the rabbit anti- β -lactoglobulin in 1:5000 dilution was added to the plate, (C) the rabbit anti-casein in 1:2000 dilution was added to the plate, followed by adding the HRP linked anti-rabbit antibody (1:10000) and HRP substrate 3,3',5,5'-Tetramethylbenzidine (TMB). The colorimetric signal was measured at 450 nm by micro plate reader. Average values are plotted in the graph with different small letters indicate significant differences (Duncan, $p < 0.05$).

*The dotted line represents the antigenicity of control protein.

3.3. Antigenicity of milk proteins increased when interacted with dietary particles

Fig. 3 A shows the workflow schematic for identifying the antigenicity of protein after interacting with dietary particles. Changes in antigenicity of β -lactoglobulin and casein in the presence of dietary particles are evident from Figs. 3B, C, SI 1A and B. Generally, antigenicity of β -lactoglobulin increased in the presence of dietary particles in comparison of non-interacted protein (Fig. 3B). The antigenicity towards β -lactoglobulin increased in milk samples interacted with FG-SiO₂-NP and E171 while it remained unchanged for E551 and decreased in the case of FG-TiO₂-NP (SI 1A). The effect of particles on the antigenicity of casein in the presence of dietary particles is shown in Fig. 3C. Casein antigenicity increased in the presence of all tested dietary particles, and it was highest in the presence of FG-SiO₂-NP (Fig. 3C). In addition, the casein antigenicity was not significantly different from milk sample containing FG-TiO₂-NP (SI 1B).

3.4. Structural transformation of proteins after interacting with dietary particles

The ATR-FTIR spectra of the proteins in the presence and absence of the particles are presented in Fig. 4A and B. The amide I region of the infrared spectra of proteins is sensitive to their secondary structure (Dousseau and Pezolet, 1990). E551 interacted β -lactoglobulin showed the highest level of secondary structure disruption followed by FG-SiO₂-NP, E171 and FG-TiO₂-NP when compared with pure β -lactoglobulin sample (Fig. 4A). Notably, the α -helix structure of FG-TiO₂-NP

interacted β -lactoglobulin increased compared to the control. The secondary structure of E551 interacted casein also showed changes-the severity of which followed E551 > FG-SiO₂-NP > FG-TiO₂-NP > E171 > pure casein protein (Fig. 4B).

3.5. Quantitative analysis of free thiol group in the presence of dietary particles

Free thiol content was estimated using Ellman's assay to study the effect of unfolding of protein in the presence of dietary particles. As shown in Fig 4 C and D, the presence of FG-TiO₂-NP and E171 in both proteins (β -lactoglobulin and casein) resulted in significantly higher free thiol content compared to control proteins, while FG-SiO₂-NP and E551 had similar free thiol concentrations as the native protein.

3.6. Binding affinity of β -lactoglobulin and casein to dietary particles

The fluorescence intensity measured at 25, 30, 35 and 40 °C suggested changes in the stability of tertiary structure of proteins (β -lactoglobulin and casein) when interacted with dietary particles (Fig. 4E). The type of fluorescence quenching of when proteins bind to particles was distinguished using Stern-Volmer diagrams. The Stern-Volmer plots were not entirely linear when the quencher concentration was higher (>15 mM), especially in the case of titania particles. This is due to the inner filter effect or concentration quenching (Koka, 2011). However, as determined from the linear response in lower particle concentrations, titania particles induced dynamic quenching. Generally, the bimolecular quenching rate (K_q) of β -lactoglobulin and casein were higher when they

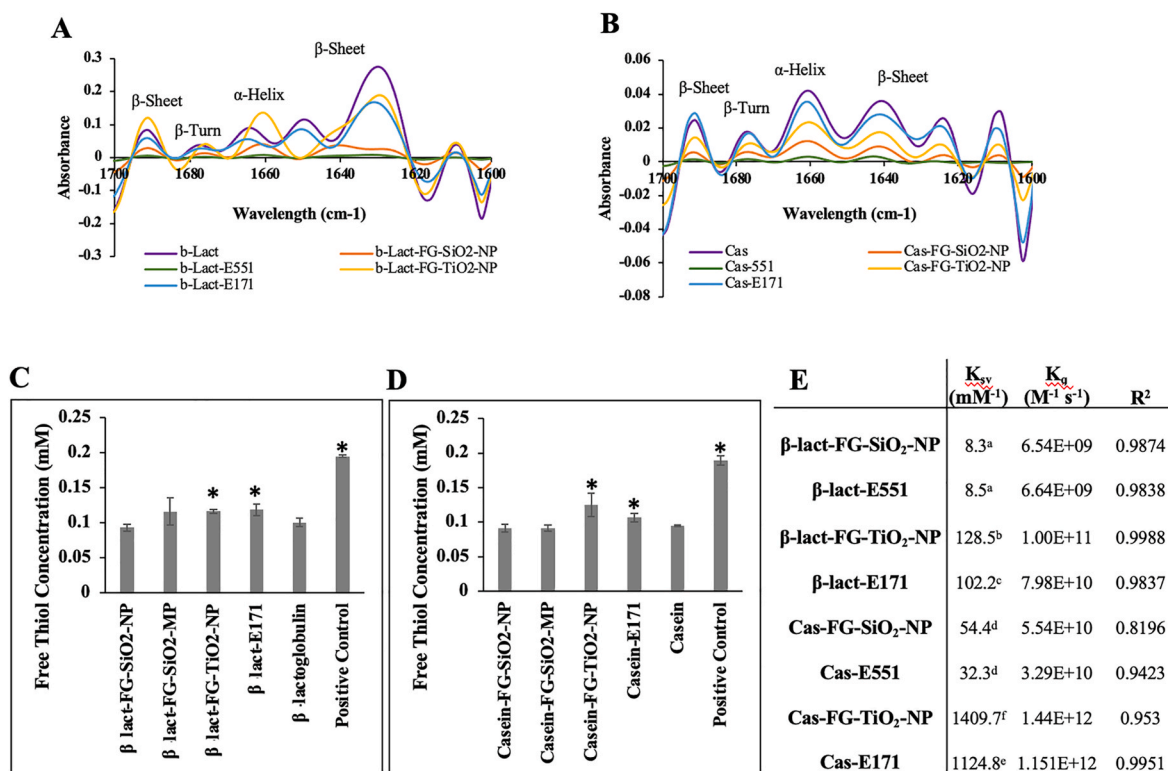


Fig. 4. Characterization of protein conformational changing by FTIR and fluorescence quenching. (A) FTIR analysis of the secondary structure of β -lactoglobulin in the presence of inorganic dietary particles. (B) FTIR analysis of the secondary structure of casein in the presence of inorganic dietary particles. Samples (protein + particle) suspended in water were dropped and dried on the ATR probe before taking the spectra for a wavenumber range of 4000–400 cm⁻¹ using ATR-FTIR. The spectra were generated by Fourier self-deconvolution of wavenumber of amide I band between 1700 and 1600 cm⁻¹. (C) Free thiol content of β -lactoglobulin in the presence of dietary particles. (D) Free thiol content of casein in the presence of dietary particles. * Indicate statistical significance (*t*-test) in comparison to control protein, $p \leq 0.05$, $N = 3$. (E) Table summarizes binding affinity of β -lactoglobulin and casein: Stern-Volmer constant (K_{sv}) and bimolecular quenching rate parameter (K_q) of β -lactoglobulin and casein in the presence of inorganic dietary particles calculated from Stern-Volmer plot. Means with different small letters in the same column are significantly different to each other (Duncan, $P < 0.05$).

were interacted with titania particles compare to silica particles (Fig. 4E). For instance, the K_q values for β -lactoglobulin and casein when interacted with silica particles (FG-SiO₂-NP and E551) in comparison with titania particles (FG-TiO₂-NP and E171) were ~ 16 and 30 times lower, respectively. In comparison to FG-SiO₂-NP and E551, the quenching constant and quenching rate were higher in β -lactoglobulin adsorbed onto E551 while higher in casein adsorbed onto FG-SiO₂-NP. The quenching constant and quenching rates were higher in FG-TiO₂-NP compared to FG-TiO₂-NP and E171 (Fig. 4E).

3.7. Quantitative analysis of mast cell degranulation

Food antigens bind to specific IgE on the surface of mast cells, trigger degranulation leading to allergy symptoms. As shown in Fig. 5, dietary particles interacted with milk samples caused a significant increase in LAD2 degranulation, indicated by the release of β -hexosaminidase. The presence of food additive titanium dioxide (E171) showed the highest level of LAD2 degranulation, followed by FG-TiO₂-NPs. In order to understand the protein stability during gastric digestion in the presence of dietary particles, the allergenicity of milk in the presence of dietary particles was tested after passing through the simulated gut digestion system. Contrary to our expectations, the β -hexosaminidase release was still higher for milk samples subjected to *in vitro* digestion in the

presence of FG-TiO₂-NP and E171 particles. Notably, however, the degranulation was slightly decreased in the presence of FG-SiO₂-NP and E551 particles.

4. Discussion

E551 and E171 are common inorganic food additives materials used in food products, including dairy products. Milk is one of the “big eight” food allergens where casein and β -lactoglobulin are identified as major food allergens in milk (Wang et al., 2020). Understanding the consequence of particle interaction on allergenicity of milk is pivotal for understanding the relevance of food additive nanoparticles in shaping health and disease outcomes of food components. Our studies demonstrated that dietary nanoparticles alter the structure and increase the allergenicity of milk proteins—an observation that has far-fetched implications given the rising incidences of food allergy.

TEM examination of particles interacted with milk showed the presence of surface corona as a translucent layer of thickness that varied from 200 to 700 μm (Fig. 1J). As shown in our previous studies, the surface corona of milk interacted particles is constituted by protein, lipids and carbohydrates as major classes of biomolecules (Phue et al., 2021). Generally, OH groups present on particle surfaces has high surface energy and mediate agglomeration of particles in the absence of

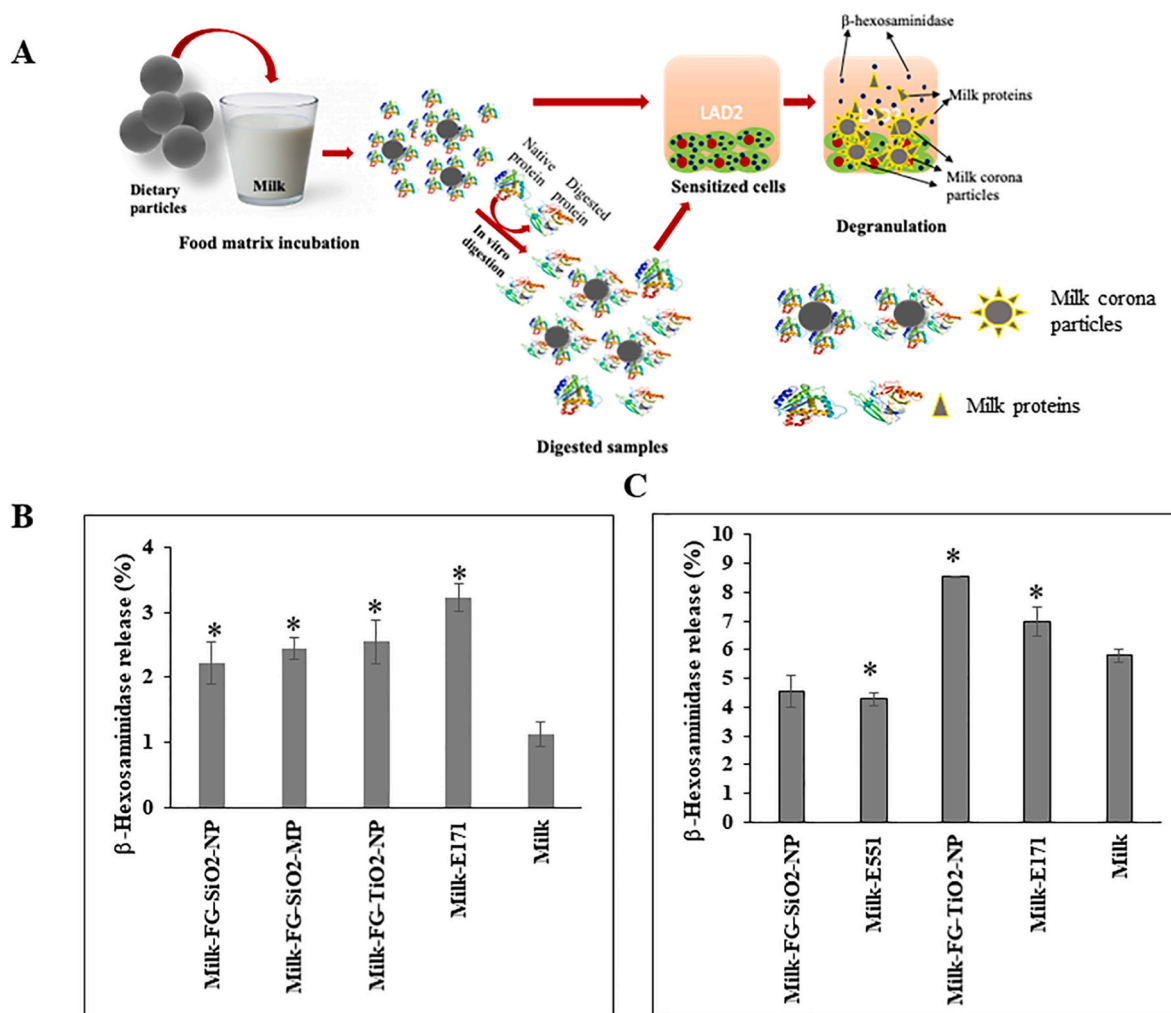


Fig. 5. Allergenicity of milk in the presence of dietary particles on mast cells. (A) Schematics of the degranulation assay to evaluate milk allergenicity in the presence of dietary particles and *in vitro* digested samples. (B) The release of β -hexosaminidase was measured by LAD2 degranulation assay after triggering with milk. Milk in the presence of dietary particles was diluted by Tyrode's buffer (100 ng mL⁻¹) and exposed to the sensitized LAD2 cell to trigger the cell degranulation. (C) The release of β -hexosaminidase was measured by LAD2 degranulation assay after exposing to *in vitro* digested milk in the presence and absence of dietary particles. * Indicate statistical significance (*t*-test) in comparison to control protein, $p \leq 0.05$, $N = 3$.

matrix molecules. When suspended in complex media such as milk, these OH groups interact with functional groups on biomolecules through hydrogen bonding to mediate their binding onto surface (Barhoum et al., 2015; Kongsinlark et al., 2012). These biomolecule-particle interactions facilitate the retention of biomolecules on the surface and provide a net negative surface charge (Fig. 1, tabular data). The electrostatic and steric hindrance (from adsorbed biomolecules) keeps particles separated when suspended in complex media (Chanteau et al., 2009), which could explain the reduced hydrodynamic size of milk interacted particles (Fig. 1, tabular data). As observed under TEM, the primary size of E551 is similar to FG-SiO₂-NP. However, E551 is more agglomerated and had less interstitial space in comparison to FG-SiO₂-NP. Aggregation of primary particles that leads to reduced interstitial space (that provide anchoring points for biomolecules) could be the reason for the lower level of surface adsorbed proteins in E551 in comparison to FG-SiO₂-NPs (Fig. 2). However, both silica particles (E551 and FG-SiO₂-NPs) were much smaller than titania particles (E171 and FG-TiO₂-NPs). Evidently, the lesser surface area to volume ratio could explain the lower amount of surface corona proteins in titania particles compared to silica particles (Fig. 2).

Previous studies from our group showed that the adsorption of proteins onto the surface of particles could disrupt the structural conformation of the protein (Phue et al. 2019, 2020). Particle mediated alteration in the tertiary and secondary structure was found to reduce the activity of enzymes relevant to human nutrition (Phue et al. 2019, 2020, 2021). Therefore, it was rational to hypothesize that particle-protein interactions could alter allergenicity to proteins implied in prevalent food allergy. Generally, the IgG-binding activity of β -lactoglobulin and casein increased after interacting with particles suggesting that binding proteins to particle surface-enhanced antibody accessibility to epitopes (Fig 3B and C). FG-SiO₂-NPs showed higher antigenicity than E551 (food grade silica particles), which could be explained by the higher level of protein adsorption by FG-SiO₂-NPs, as discussed above. However, the antigenicity of β -lactoglobulin and casein in the milk sample presented of E551 and FG-TiO₂-NP did not have the same impact as pure proteins. Presence of other biomolecules such as lipids, carbohydrates, and metabolites in the surface corona of milk interacted particles might have also influenced the antigenicity of β -lactoglobulin, and casein adsorbed on particles interacted with milk.

Alterations in protein conformation during unfolding can affect the IgE reactivity and influence the allergenicity of the proteins (Besler et al., 2001). Therefore, we analyzed the changes in proteins' secondary and tertiary structure through FTIR, fluorescence quenching, and free thiol content. Fluorescence quenching suggestive of changes in tertiary structure was more evident when proteins were interacted with titania particles in comparison silica particles. Similarly, the availabilities of free thiol groups in β -lactoglobulin and casein were also relatively higher when these proteins were interacted with titania. Interestingly, however, the secondary structures of proteins were not affected noticeably by interacting with titania as much as with silica particles. These data suggest that interactions of proteins with titania particles induce changes in tertiary or quaternary structure but silica-induced changes at secondary structure of proteins. Notably, however, the increase in antigenicity of proteins was higher when interacted with silica particles than with titania particles. This discrepancy might be due to the lower amount of proteins adsorbed onto titania particles in comparison to silica particles (on an equal mass basis of particles used) which might offset any enhancement in the antigenicity of proteins adsorbed on titania particles.

Mast cell degranulation assay is primarily used as a proxy to measure the ability of an antigen to trigger the release of inflammatory mediators such as histamine and β -hexosaminidase when primed mast cell encounter antigen (Thomas et al., 2007). Although histamine is the major mediator for allergenic responses, it is less desirable for *in vitro* investigations as its half-life in the extracellular fluid is short (1 min) (Huang et al., 2016). For this reason, the release of β -hexosaminidase is

widely used to perform the cell degranulation assay, which constitutes late-phase activation and a longer half-life (8–24 h). The mast cell stimulated by human milk allergy patient sera activates the Fc ϵ RI signalling (the high-affinity IgE receptor) and triggers the cell degranulation and allergic mediators by antigen (Hussain et al., 2012). Our results showed that the level of β -hexosaminidase release increased in the presence of dietary particles (Fig. 5B). As observed for antigenicity, the increase in allergenicity could also be ascribed to the increasing availability of linear epitope as a result of conformational changes induced by protein-particle interactions. While these studies showed the implications of dietary nanoparticles in exacerbating the adverse allergic response towards milk proteins, we also questioned the relevance of this observation when subjected to digestive enzymes. As such, the immunogenic potential of protein is very much dependent on its stability when exposed to digestive environment (Thomas et al., 2007). Therefore, we examined the allergenicity of particle-interacted proteins that were subjected to *in vitro* digestion system simulating protein digestion in human gastro-intestinal system (Thomas et al., 2007). Notably, the allergenicity of titania interacted milk increased compared to milk alone after subjecting to *in vitro* digestion. As evident from protein binding affinity studies, titania particles strongly interact with milk proteins, and that strong interaction is envisaged as the reason for increased the stability of the proteins during the digestion steps. In addition, the digestive enzymes adsorbed on the particles, and incomplete digestion of milk protein in the presence of dietary particles, as evident from SDS-PAGE (Fig S12) could have triggered stronger allergen response in sensitized cells when exposed to *in vitro* digested mix of particles and milk.

Our studies demonstrated that particle mediated alterations in protein structure could enhance allergenicity of milk proteins. Although, many studies have addressed the influence of different food processing conditions on the food allergies, the role of nanoparticles present in widely used food additives in shaping immunogenic potential of food proteins have been overlooked. Apart from inducing alterations in protein structure, interaction of allergenic proteins could also enhance the bioavailability of allergenic epitopes. The increased bioavailability result from the resistance of proteins adsorbed onto particles to complete digestion and possible particle enabled trespass of intact allergen surface adsorbed proteins across epithelial layer (as revealed by recent studies from our group). Thus, while these studies suggest the role of dietary particle in enhancing allergenicity of food proteins more studies using animal models are warranted for gaining deeper understanding how dietary nanoparticles could alter the biology of food allergy. Nonetheless, our findings have far-fetched implications for managing severity of food allergy and in making dietary choices given the rising incidences of food allergies globally and its association with consumption of highly processed food often containing inorganic nanoparticles of silica and titania.

CRedit authorship contribution statement

Wut H. Phue: Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Ke Xu:** Investigation, Formal analysis, Methodology. **Saji George:** Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We acknowledge funding from NSERC RGPIN-2017-04727 and Canada Research Chair/George/X-coded/248475 for supporting this

research work. We thank Dr. Hojatollah Vali and Kelly Sears for technical support with TEM imaging. We would like to express our gratitude to Dr. A. S. Kirshenbaum, National Institutes of Health, for giving LAD2 cell line to study degranulation assay.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2022.112874>.

References

- Athinarayanan, Jegan, Alshatwi, Ali A., S Periasamy, Vaiyapuri, Al-Warthan, Abdulrahman A., 2015. 'Identification of nanoscale ingredients in commercial food products and their induction of mitochondrially mediated cytotoxic effects on human mesenchymal stem cells. *J. Food Sci.* 80, N459–N464.
- Barhoum, Ahmed, Van Lokeren, Luk, Rahier, Hubert, Dufresne, Alain, Van Assche, Guy, 2015. 'Roles of in situ surface modification in controlling the growth and crystallization of CaCO₃ nanoparticles, and their dispersion in polymeric materials. *J. Mater. Sci.* 50, 7908–7918.
- Bengtsson, U., Nilsson-Balknäs, U., Hanson, L.A., Ahlstedt, S., 1996. 'Double blind, placebo controlled food reactions do not correlate to IgE allergy in the diagnosis of staple food related gastrointestinal symptoms. *Gut* 39, 130–135.
- Besler, Matthias, Steinhart, Hans, Paschke, Angelika, 2001. 'Stability of food allergens and allergenicity of processed foods. *J. Chromatogr. B Biomed. Sci. Appl.* 756, 207–228.
- Boyce, Joshua A., Amal, Assa'ad, Burks, A Wesley, Stacie, M., Jones, Hugh, Sampson, A., Wood, Robert A., Marshall, Plaut, Cooper, Susan F., Fenton, Matthew J., Hasan Arshad, S., 2011. 'Guidelines for the diagnosis and management of food allergy in the United States: summary of the NIAID-sponsored expert panel report. *J. Am. Acad. Dermatol.* 64, 175–192.
- Brodtkorb, André, Egger, Lotti, Alving, Marie, Alvito, Paula, Assunção, Ricardo, Simon, Ballance, Bohn, Torsten, Bourlieu-Lacanal, Claire, Boutrou, Rachel, Carrière, Frédéric, 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.* 14, 991–1014.
- Burks, A Wesley, Williams, Larry W., Thresher, Wayne, Connaughton, Cathie, Cockrell, Gael, Helm, Ricki M., 1992. 'Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. *J. Allergy Clin. Immunol.* 90, 889–897.
- Castillo, Daniela S., Cassola, Alejandro, 2017. 'Novel sensitive monoclonal antibody based competitive enzyme-linked immunosorbent assay for the detection of raw and processed bovine beta-casein. *PLoS One* 12, e0182447.
- Chanteau, B., Fresnais, J., Berret, J.-F., 2009. 'Electrosteric enhanced stability of functional sub-10 nm cerium and iron oxide particles in cell culture medium. *Langmuir* 25, 9064–9070.
- Dekkers, Susan, Krystek, Petra, Peters, Ruud JB., Lankveld, Daniëlle PK., Bokkers, Bas GH., Paula, H van, Hoeven-Arentzen, Bouwmeester, Hans, Oomen, Agnes G., 2011. 'Presence and risks of nanosilica in food products. *Nanotoxicology* 5, 393–405.
- Dousseau, Françoise, Pezolet, Michel, 1990. 'Determination of the secondary structure content of proteins in aqueous solutions from their amide I and amide II infrared bands. Comparison between classical and partial least-squares methods. *Biochemistry* 29, 8771–8779.
- Fiocchi, Alessandro, Holger, J., Schünemann, Jan, Brozek, Restani, Patrizia, Beyer, Kirsten, Troncone, Riccardo, Martelli, Alberto, Terracciano, Luigi, Bahna, Sami L., Rancé, Fabienne, 2010. 'Diagnosis and rationale for action against cow's milk allergy (DRACMA): a summary report. *J. Allergy Clin. Immunol.* 126, 1119–1128 e12.
- Gorji, Elham, Ghorbani, Rocchi, Elisa, Schleining, Gerhard, Bender-Bojalil, Denisse, Furtmüller, Paul G., Piazza, Laura, J Iturri, Jagoba, José, L., Toca-Herrera, 2015. 'Characterization of resveratrol–milk protein interaction. *J. Food Eng.* 167, 217–225.
- Huang, Lufen, Jiang, Pi, Wu, Jianlin, Zhou, Hua, Cai, Jiye, Li, Ting, Liu, Liang, 2016. 'A rapid and sensitive assay based on particle analysis for cell degranulation detection in basophils and mast cells. *Pharmacol. Res.* 111, 374–383.
- Hussain, Salik, Jeroen, A.J., Vanoirbeek, Peter, H.M., Hoet, 2012. 'Interactions of nanomaterials with the immune system. *Wiley Interdiscipl. Rev.: Nanomed. Nanobiotechnol.* 4, 169–183.
- Koka, Vivian Dzigbodi, 2011. A Study of the Effects of Titanium Dioxide Nanoparticles on the Fluorescent Intensity of Fluorescent Compounds in the Presence of Known Quenchers.
- Kongsinlark, Anong, Rempel, Garry L., Prasassarakich, Pattarapan, 2012. 'Synthesis of monodispersed polyisoprene–silica nanoparticles via differential microemulsion polymerization and mechanical properties of polyisoprene nanocomposite. *Chem. Eng. J.* 193, 215–226.
- Madsen, Jeanette Lund, Kroghsbo, Stine, Madsen, Charlotte Bernhard, Pozdnyakova, Irina, Barkholt, Vibeke, Katrine Lindholm Bøgh, 2014. The impact of structural integrity and route of administration on the antibody specificity against three cow's milk allergens—a study in Brown Norway rats. *Clin. Transl. Allergy* 4, 1–11.
- Maleki, Soheila J., Chung, Si-Yin, Champagne, Elaine T., Raufman, Jean-Pierre, 2000. 'The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* 106, 763–768.
- Milani, Silvia, Baldelli Bombelli, Francesca, Andrzej, S Pitek, Dawson, Kenneth A., Radler, Joachim, 2012. 'Reversible versus irreversible binding of transferrin to polystyrene nanoparticles: soft and hard corona. *ACS Nano* 6, 2532–2541.
- Mine, Yoshinori, Zhang, Jie Wei, 2002. 'Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *J. Agric. Food Chem.* 50, 2679–2683.
- Panja, Sudipta, Khatua, Deb Kumar, Halder, Mintu, 2018. 'Investigations on the effect of fatty acid additives on casein micelles: role of ethylenic unsaturation on the interaction and structural diversity. *ACS Omega* 3, 821–830.
- Pekar, Judith, Ret, Davide, Untersmayr, Eva, 2018. 'Stability of allergens. *Mol. Immunol.* 100, 14–20.
- Pelto, L., Impivaara, O., Salminen, S., Poussa, T., Seppänen, S., Lilius, E.M., 1999. 'Milk hypersensitivity in young adults. *Eur. J. Clin. Nutr.* 53, 620–624.
- Phue, Wut H., Liu, Mengxi, Xu, Ke, Srinivasan, Divya, Ismail, Ashraf, George, Saji, 2019. 'A comparative analysis of different grades of silica particles and temperature variants of food-grade silica nanoparticles for their physicochemical properties and effect on trypsin. *J. Agric. Food Chem.* 67, 12264–12272.
- Phue, Wut Hmone, Bahadi, Mazen, James, J., Dynes, Wang, Jian, Kuppli, Venkata, Ismail, Ashraf A., Hameed, Aneela, George, Saji, 2021. 'Protein-biomolecule interactions play a major role in shaping corona proteome: studies on milk interacted dietary particles. *Nanoscale*.
- Phue, Wut Hmone, Srinivasan, Divya, Hameed, Aneela, Yaylayan, Varoujan, George, Saji, 2020. Food grade silica nanoparticles cause non-competitive type inhibition of human salivary α -amylase because of surface interaction. *Nano Select.*
- Riener, Christian K., Kada, Gerald, Gruber, Hermann J., 2002. Quick measurement of protein sulphydryls with Ellman's reagent and with 4, 4'-dithiodipyridine. *Anal. Bioanal. Chem.* 373, 266–276.
- Srinivasan, Divya, Hmone Phue, Wut, Xu, Ke, George, Saji, 2020. 'The Type of Dietary Nanoparticles Influences Salivary Protein Corona Composition. *NanoImpact*, p. 100238.
- Thomas, Karluss, Herouet-Guicheney, Corinne, Ladics, Gregory, Gary, Bannon, Cockburn, Andrew, Crevel, Rene, Fitzpatrick, Julie, Mills, Clare, Privalle, Laura, Vieths, Stefan, 2007. 'Evaluating the effect of food processing on the potential human allergenicity of novel proteins: international workshop report. *Food Chem. Toxicol.* 45, 1116–1122.
- Turnbull, J.L., Adams, H.N., Gorard, D.A., 2015. The diagnosis and management of food allergy and food intolerances. *Aliment. Pharmacol. Ther.* 41, 3–25.
- Wang, Chong, Xie, Qiang, Wang, Yanbo, Fu, Linglin, 2020. 'Effect of ultrasound treatment on allergenicity reduction of milk casein via colloid formation. *J. Agric. Food Chem.* 68, 4678–4686.
- Weir, Alex, Paul, Westerhoff, Fabricius, Lars, Hristovski, Kiril, Von Goetz, Natalie, 2012. 'Titanium dioxide nanoparticles in food and personal care products. *Environ. Sci. Technol.* 46, 2242–2250.
- Yang, Huayan, Yang, Shouning, Kong, Jilie, Dong, Aichun, Yu, Shaoning, 2015. Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nat. Protoc.* 10, 382–396.