



Effects of pH and concentrations of colostrum whey and caseinate on fabrication of nanoparticles and evaluation of their techno-functionalities and in vitro digestibility

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Abstract

Intermolecular interactions between colostrum whey and sodium caseinate were studied in aqueous medium under different pH conditions (3–7) using dynamic light scattering (DLS) technique. The surface charge density of casein was modified through pH adjustment to increase the interaction between casein and whey protein. The stable nanoparticles (NPs) with mean hydrodynamic diameter of <125 nm was prepared by mixing sodium caseinate and colostrum whey protein at pH 6.5. The effects of pH (3–7), ionic strength (50–300 mM of NaCl₂) was studied on the stability of particles. The differential scanning calorimetry showed that the thermal stability of NPs was higher (128.3 °C) compared to their native proteins. NPs in liquid form at 25 °C showed remarkable stability up to 1 month. The FTIR assay confirmed that the secondary structures of the whey proteins remained unaltered. Furthermore, the Scanning Electron Microscopy (SEM) displayed the spherical shape of the nanoparticles in exquisite detail. The in vitro study of NPs indicated that the recovered Igs content was higher in NPs (IgG 68%, IgA 14.5%, and IgM 19.5%) as compared to control (IgG 42.7%, IgA 9.7%, and IgM 14.4%). Colostrum whey-based NPs can be used as a dietary supplement to deliver nutrients and growth factors to the body and can have various health benefits, such as improving immunity, promoting wound healing, and enhancing athletic performance.

Keywords Casein · Colostrum · Digestibility · Nanoparticles · Whey

Introduction

The bovine colostrum (first milk in early lactations of 3–7 days after parturition) whey contains high concentration of immunoglobulins (Igs), lactoferrin, alpha-lactalbumin (α -La), beta-lactoglobulin (β -Lg), and bovine serum albumin (BSA) as compared to mature milk [1]. Colostrum is vital for the development of the immune system of neonate due to its bioactive protein components [2]. Only a small

fraction of colostrum is required to build and improve the immune system, and the rest can be processed in drug formulations containing gastrointestinal functions, food supplements, infant formula, and cosmetics [3]. β -Lg is the major component of whey protein and contains an isoelectric point (pI) of 5.2, molecular weight (Mw) of 18.4 kDa, thermal denaturation temperature (T_m) of 70 °C and its native state, 4 of its five cysteine residues involved in disulfide bondings. The α -La, the second major whey protein has pI of 4.8, Mw of 14.2 kDa, T_m of 61 °C, and its structure is stabilized by four intermolecular disulfide bonds and calcium [4]. BSA contains 583 amino acids, cross-linked with 17 cystine residues in single chain, and has a Mw of 66.4 kDa and T_m of 74 °C.

Casein is a phosphoprotein with pI of 4.6, Mw of 20–25 kDa and contains a wide range of benefits such as stabilizers, and carriers of bioactive compounds. There are four different types of casein such as α_{S1} -, α_{S2} -, β - and κ -casein which can be identified due to solubility in Ca²⁺ solutions

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[5]. The β and α_s -caseins are calcium insoluble and found inside the micellar structure, however the k-casein is calcium soluble and generally found on the micellar surface. The absence of Ca^{2+} leads to a release of soluble caseins, while high concentrations of calcium cause the aggregation of casein [5].

The use of milk proteins to produce nanoparticles (NPs) has been investigated in many previous studies such as β -Lg [6–8], Lactoferrin [9], Whey [10], and casein [8], [11], [12]. The protein components of colostrum have not yet been explored for their potential to develop NPs, with advantage of colostrum bioactive proteins such as Igs and Lactoferrin. The pH, ionic strength of the solution as well as surface charge density of the whey protein molecules have significant effect on the formation of intramolecular or intermolecular (e.g., with casein) complexes [13–15]. But these interactions between colostrum whey and caseinate have also not been fully explored for the development of complexes. The aim of this study was, thus, to explore the effect of pH and concentrations of colostrum whey and caseinate to produce the NPs through coacervation process in the presence of Ca^{+2} . This study is novel in terms of fabricating the NPs with colostrum whey and caseinate. The NPs formation of colostrum proteins can improve their digestibility and stability in different environments. These NPs were prepared to preserve the bioactive proteins of colostrum (Igs) and can also be utilized to deliver some other bioactive components like tryptophan or serotonin to enhance the nutraceutical efficiency of colostrum protein [16].

Materials and methods

Materials

Colostrum (Protein 13.5%, Fat 6.8%, Lactose 2.2%, and Ash 1.2%) of Holstein Friesian cow's first lactation (first lactation contains highest concentration of bioactive proteins as observed in our previous study [17]) was generously donated by David Enterprises, Bangkok, Thailand, and all the required chemicals were purchased from CTi & Science Co. Ltd., Thailand. The above-mentioned supplier also supplied sodium caseinate with 95.5% dry matter, 1.2% sodium, and 94.2% protein content. Thailand. Sodium caseinate containing around 95.5% dry matter, 1.2% sodium and 94.2% protein content was also supplied by the above-mentioned supplier. The ELISA kits (Pink One) containing, standard Igs, wash buffer, detection antibody and stop solution to estimate the immunoglobulins (IgG, IgA, and IgM) were procured from LABISKOMA Seoul, Korea. Colostrum whey (moisture content 5.8%, protein 80%, lactose 0.8%, and ash 1.5%) were isolated from skim colostrum by

lowering the pH to 4.6 with 0.1 N HCL solution and then freeze drying using a laboratory scale freeze dryer (Model 55–4 Labogene, Denmark).

Preparation of colostrum whey-sodium caseinate complexes

Na-caseinate (moisture content 4.5%) solutions were prepared by dissolving the sodium caseinate (0.1–0.5% w/v) in distilled water (75 mL) through overnight magnetic stirring at room temperature to complete the rehydration. The colostrum whey solutions (0.25–1% w/v in 40 mL of distilled water) were also prepared by overnight magnetic stirring at room temperature. L-arginine (50 mg) was added to each solution of whey and Na-caseinate. After rehydration, whey solution was slowly added into the Na-caseinate solution under magnetic stirring (at 150 rpm). The CaCl_2 (200 mM) was also added to each sample and kept on stirring for 2 h before adjusting the pH. The NPs were filtered using a 0.2 μm syringe filter (Millipore, USA) before further analysis and freeze-dried using a laboratory scale freeze dryer (Model 55–4 Labogene, Denmark) at -52°C for 24 h. The effect of CaCl_2 (50–300 mM) concentrations on ζ -potential, stability, and mean particle diameter of NPs was determined.

Characterization of fabricated NPs

The size of NPs was measured using the dynamic light scattering (DLS) technique; electrophoretic mobilities were measured using the laser doppler velocimetry (LDV) technique; and the zeta potential was determined through phase analysis light scattering (M3-PALS) on ZetaSizer (Nano Zs, Malvern Instruments Ltd.UK). The particle sizing cell was set to automatic mode, and the analysis was performed at 25°C every 20 s for ten replications. The mean diameter was estimated from the correlation function of the light intensity of particles and presented as a Z-average [18].

Fourier transform infrared spectroscopy

Fourier Transform Infrared spectroscopy was performed to observe the structural changes in protein constituents [17]. The freeze-dried powder sample (2 mg) was pressed into pellets and placed on the optical crystal cell. The spectra were obtained at room temperature within the frequency range of 4000 to 500 cm^{-1} using a Nicolet iS50 FT-IR Spectrometer (Thermo Scientific, USA).

Thermal stability analysis using differential scanning calorimetry

The NPs (1–2 mg) were hermetically sealed in an aluminum pan and tested from 40 to 150 °C using a differential scanning calorimeter (DSC) (Model-214, NETZSCH, Germany). The heating and nitrogen purge rates were 10 °C per minute and fifty milliliters per minute, respectively. The maximum deflection temperature of samples was observed as denaturation temperature. Each experiment was conducted in triplicates and an empty pan was used as reference [17].

Scanning electron microscopy

The powder samples were placed on double-sided carbon tape attached to specimen stub while the loosely attached sample was blown away by nitrogen. A thin layer of gold was applied for 45 s on the sample loaded specimen stub, using a sputter coating machine (Ion Sputter, HITACHI, E-1010, Japan) at a sputter current of 23 mA. The morphology of NPs was visualized using a scanning electron microscope (SEM) (HITACHI S-3400 N, Japan) at an accelerating voltage of 15 Kv [19].

In vitro release study of immunoglobulins (Igs)

Pepsin (120 mg) was dissolved in 30 mL of 0.1 M HCl, and gastric saline was prepared by dissolving 0.11 g KCl, 0.025 g CaCl₂, 0.06 g NaHCO₃ and 0.31 g NaCl in 100 mL of water, and pH was adjusted to 2.5 by adding 0.1 M HCl. The simulated small intestinal fluid (SSIF) was made by dissolving the NaCl (0.54 g), KCl (0.065 g), CaCl₂ (0.033 g), pancreatin (200 mg), lipase (5 mg), trypsin (6.5 mg) and bile extract (1.2 g) in 100 mL of NaHCO₃ (0.1 M), and pH was set to 7.0 by adding 0.1 M NaOH [20], [21]. For gastric digestion, the sample (5 mL) was added to saline (27 mL) and pepsin (2 mL). The pH of the sample was controlled to 2.5 and incubated at 37 °C for 1.5 h. The pH was adjusted to 5.3 using sodium bicarbonate (0.9 M) solution to stop the gastric digestion. The 10 mL of digested samples was mixed with SSIF (3.0 mL) for intestinal digestion, and pH was adjusted to 7.0. The intestinal digestion took 5 h at 37 °C, and the supernatant was collected after centrifugation of sample for 5 min (at 4000 g). The Igs content were determined using ELISA and bio accessibility was calculated using following equation [17].

$$\text{Bio accessibility} = \frac{\text{Total Igs in the sample} - \text{Amount of Igs digested}}{\text{Total Amount of Igs in the samples}} \times 100$$

Storage stability analysis of NPs

The storage stability analysis of NPs was conducted in liquid state at room temperature (25 °C) for 28 days after the fabrication. The samples were taken at different time intervals (days 1, 3, 7, 14, 21 and 28) and analyzed on ZetaSizer according to the above-mentioned method (Sect. 2.3) to observe the changes in particle charge and size.

Statistical analysis

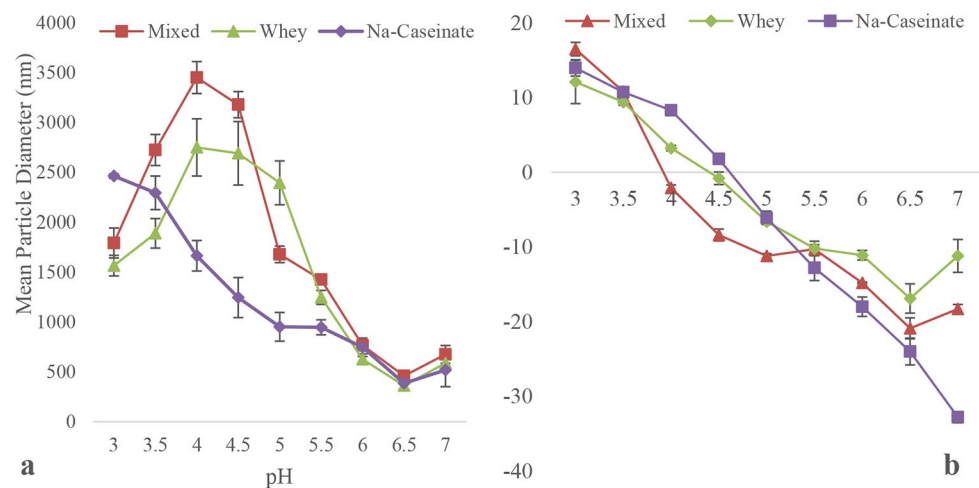
The data was collected in triplicates and statistical analysis was conducted using SPSS software (SPSS Version 16, Chicago, IL, USA). The one-way analysis of variance was performed and the significant differences among the means were identified using the post hoc Tukey's test with a confidence interval of 95%.

Results and discussion

Electrical properties of protein constituents

The solutions of colostrum whey and Na-caseinate were monitored under a pH change from 4 to 7 to conduct the electrophoresis measurements (Fig. 1a, and 1b). The isoelectric points (pI: points of zero charge) for both protein components (pH 4.6 for Na-caseinate and 4.8 for whey) were similar to those obtained in previous studies [22]. At these conditions of pH, both amino and carboxyl groups of proteins balance the negative and positive charge to zero. At acidic pH (<4.5) the particles show higher positive charge on their surface, as the pH was below the pI of proteins (Fig. 1b). At this pH, the carboxyl groups (-COOH) of proteins were neutral, while the amino groups (-NH₃⁺) were positively charged. On increasing the pH, positive charge decreased partly due to carboxyl groups (-COOH⁻) gained negative charge and partly due to the amino groups (-NH₃) which become neutral. At elevated pH (>4.5) the particles show net negative charge on their surface as the negatively charged groups were higher than the positively charged groups [23]. The value of ζ-potential for whey at pH 6.5 was -16.9 mV which decreased to -11.2 mV at pH 7. The magnitude of negative charge on the Na-caseinate was -24 at pH 6.5, which decreased significantly with the decrease in pH. Since the aggregation stability depends on the suitable pH conditions as it highly impacts the charge densities of the protein particles and hence their binding ability [24]. The experimentation of mixing whey and Na-caseinate in distilled water at a 1:1 ratio (0.2% w/v) indicated that pH 6.5 was most suitable to develop the NPs based on the structural charge densities of the individual proteins in the mixture (-20.3). Furthermore, the information obtained from particle

Fig. 1 Effect of pH (4–7) on (a) mean particle diameter (nm), and (b) ζ -potential (mV) of proteins constituents (0.2% w/v of each component) and protein mixture (0.2% w/v of Na-caseinate and 0.2% w/v of Whey)



diameter showed that the average particle diameters were less than 500 nm for whey, Na-caseinate and mixture at pH 6.5 which increased altering the pH value above or below from this point (Fig. 1a). The electrostatic interactions were the highest within the proteins at the pH value 6.5, which caused the formation of stable complexes [10]. The interactions between casein and whey proteins were maximum at pH values of 6.75–6.8 [25] since at pH values just below 7 all the whey protein structures tend to bind with casein [26].

Effect of whey and Na-Caseinate concentration on the complexation of NPs

Protein-protein interactions were used to fabricate various structures of complexes ranging from nm to μm using more than one forces of interactions, such as, π - π stacking, host-guest, H-bonding, van der Waals forces, and hydrophobic forces [27]. Protein concentration affects the aggregation step, rather than unfolding of whey proteins and with increasing concentrations, there is increase in the formation of higher Mw aggregates [15]. The effect of protein concentrations to fabricate the NPs depending on the minimum particle size were determined to increase the stability and avoid aggregation of complex over time. The nature of NPs formed through complexation was ascertained by estimating the particle size and ζ -potential as a function of Na-caseinate and whey protein concentrations. The aqueous protein solutions of different concentrations were prepared and mixed. The addition of CaCl_2 make solution cloudy, indicating the presence of complex formation large enough to scatter light. The solutions prepared at the concentration of 0.4% (w/v) of Na-caseinate showed the highest complexation, and no sedimentation was observed for these NPs within the experimentation time (24 h). At this concentration of Na-caseinate, the minimum particle diameter was obtained (116.1 ± 30) with a PDI (poly dispersibility index) value of 0.32 (Fig. 2). The

whey concentration affected the complexation significantly as the concentration increased above 0.5% (w/v), the rate of sedimentation also increased and clear phase separation was observed after 12 h, indicating aggregation and instability of NPs. The higher concentrations also affect the particle diameter and result in higher values of PDI (Fig. 2) and lower values of ζ -potential (Fig. 3). The excess amount of protein constituents (whey > 0.5%) in the same volume of solution could be the reason for the segregation and instability of the NPs. The Na-caseinate concentration of 0.4% (w/v) was suitable for the formation of complexes, as in these volumes of solution the minimum particle size (116.1 ± 30) was obtained that showed maximum ζ -potential (-21.2 ± 0.6) and good stability during the experimentation time (24 h). A further increase in the concentration of Na-caseinate did not affect the complex formation significantly.

The whey protein concentration showed significant effect on the size and solubility of the formed NPs, changing the overall ratio of whey/ casein effect the size of complex [28]. Whey protein possesses both hydrophilic and hydrophobic regions which can be readily adsorbed on the caseinate–water interface through electrostatic interactions [29]. The NPs were formed from caseinate when the CaCl_2 was added in the solution which generate the insoluble form of caseinate which adopted the form of NPs due to our medium conditions since the Ca^{+2} changes the molecular charge density, self-assembling and aggregation behavior of caseinate through the binding with phosphoserine residues. The formation of whey protein complex with caseinate was due to hydrophobic force of interactions between both proteins. The complexation of whey protein with caseinate due to charge neutralization action and/or bridging effect could be the origin of NPs formation since, it was observed in previous study that k-casein on the surface of casein was involved in the complex formation with whey proteins [26]. The k-casein is found in the distributed form on the

Fig. 2 Effect of whey protein (0.25–1%, w/v) and Na-caseinate concentrations (0.1–0.5% (w/v) on the particle diameter (nm) (bar label is indicating the PDI value of each treatment)

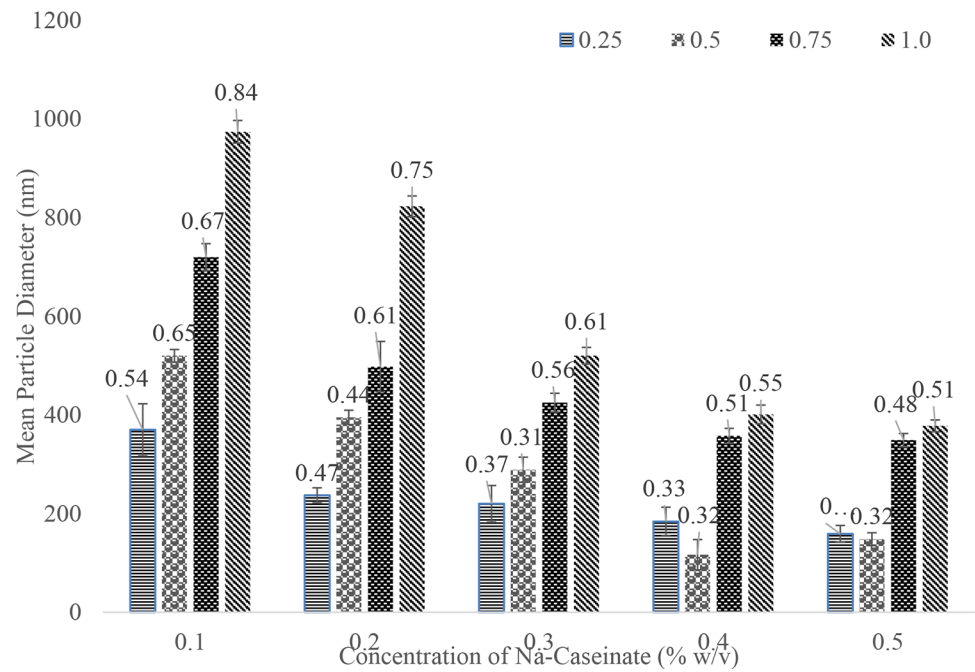
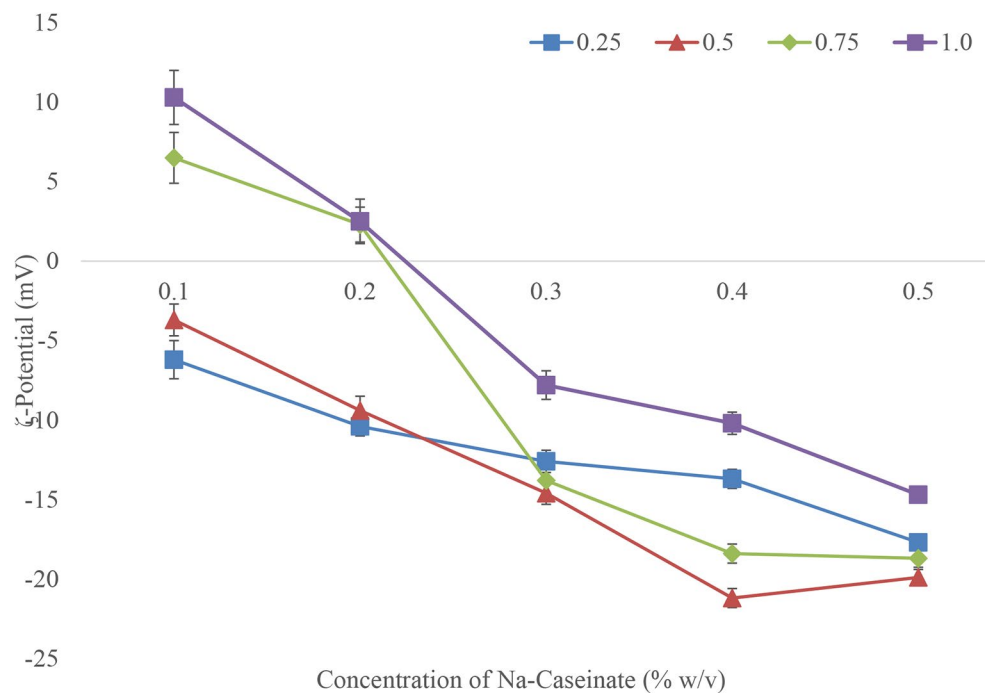


Fig. 3 Effect of whey protein (0.25–1%, w/v) and Na-caseinate concentrations (0.1–0.5% (w/v) on the ζ -potential of particles



surface of casein creating a sterically stabilizing hairy layer in which gaps are found to attach the whey protein molecules [30]. The b-Lg and BSA can also show interactions with casein via thiol group with k-casein and α_2 , with the minor assistances of hydrophobic and electrostatic interactions [31]. However, α -La contains many binding sites for Ca^{2+} at the junction of α and β -domains, and at neutral pH it is found in many partially unfolded forms with secondary structures but not well-defined tertiary structure due to

absence of Ca^{2+} . The binding of α -La with Ca^{2+} strongly influence the stability of molecule and it is necessary for the accurate refolding of proteins [32].

Further knowledge about the stability of protein complexes (NPs) was obtained through the measurement of ζ -potential of particles as a function of different concentrations of protein constituents (Fig. 3). The minimum values of ζ -potential were observed at the lower concentration of Na-caseinate (< 0.2%, w/v). The lower concentration of

casein has not the ability to hold all the available whey protein molecules in the solutions. With the increasing concentrations of caseinate, the diameters of the whey protein-caseinate nanoparticles were found decreasing, which indicate the strong interactions between these two molecules [27], [33]. The conditions of solution, such as pH, salt, and protein concentrations, significantly affect these forces to form the complexes. In a previous study it was observed that the interaction between whey proteins and casein increases at pH values around 6.75–6.8 [25] as observed in our study the maximum interaction was around pH 6.5, at pH value just below 7 all the whey proteins structures tend to bind with casein [26]. The concentrations of both proteins show major impacts on particle aggregation and stability. The higher concentrations of whey (0.75–1%, w/v) result in the binding of more than one whey molecule on the surface of Na-caseinate, which possibly increases the size of the complex and the rate of sedimentation, as observed for these concentrations. Consequently, these colloidal systems were unsuitable to be studied further. The difference in charge densities of both protein components (at pH 6.5) also affected the overall charge density of NPs. The pI of Na-caseinate was around 4.7, which was shifted ($\text{pH} \leq 4.8$) due to the higher concentration of Igs in whey. Since the pI of Igs was higher (pH 6.1–8.5), that could cause a significant effect on the electrical charge of fabricated NPs [22]. The more binding of Igs on the NPs surface can increase the charge density on their surface. The low concentration of whey (0.5%) as compared to Na-caseinate (0.4%) in the solution provides the most suitable conditions of concentration to form stable NPs. Furthermore, the pH dependence of ζ -potential indicated (Fig. 1) that the sodium caseinate at $\text{pH} \leq 6.5$ (at 0.2%w/v) was capable of binding more whey protein molecules as compared to low values of solution pH (≤ 6.0) to form the complexes. This trend was further supported by the fact that complex formation was more likely at this pH value due to higher particle charge densities. Since specific amount of CaCl_2 (200 mM) was also added to each sample which influenced the charge densities of particles. There are binding sites available for Ca^{+2} on the surface of whey proteins and it is possible that there could be more binding sites on the surface of IgG proteins than other whey components [34]. The Ca^{+2} induced aggregation of proteins can be due to electrostatic shielding of negative charges

causing hydrophobic aggregation of proteins. Other mechanism are ion-specific hydrophobic interactions generated by ion induced conformational change and crosslinking of anionic molecules via proteins- Ca^{+2} -protein bridge [31]. The protein- Ca^{+2} interactions can disrupt the hydrogen bond of proteins with water leading to increase in diameter of proteins due to calcium bridging [35]. The CaCl_2 generates the divalent Ca^{+2} ions above the pI of proteins which are more effective at shielding the electrostatic interactions and binding the oppositely charged protein components [36]. Furthermore, in the absence of CaCl_2 or at the concentrations below 200mM, the size of particles increased significantly around the pI of whey proteins indicating the large aggregation [23].

Interestingly, the NPs prepared in the absence of a basic amino acid (L-arginine) showed the minimum stability and higher particle diameter while the NPs prepared in the presence of L-arginine showed a smaller mean diameter and improved stability (Table 1). The PDI obtained was also lower than the PDI of NPs prepared without L-arginine. The presence of L-arginine in the solution prevents the clumping of casein NPs due to the development of electrostatic forces between carboxyl moieties ($-\text{COOH}$ residues of glutamic and aspartic acids) and amino groups of casein. These interactions between carboxyl moieties and L-arginine make the surface of NPs more hydrophilic and minimize the chances of aggregations. Furthermore, the stability of NPs at room temperature was also improved due to the presence of L-arginine. A similar effect of L-arginine on the stability and size of NPs was also reported in a previous study where the Na-caseinate NPs were loaded with folic acid [37].

Effect of CaCl_2 concentration on particle diameter and ζ -potential

Using the coacervation technique, the casein NPs were prepared by the addition of CaCl_2 to aqueous medium. Since the Ca^{2+} specifically attaches to the phosphoserine residues of casein, it can modify the surface charge density. The CaCl_2 , therefore, modifies the charge distribution of molecules, self-assembling behavior, as well as flocculation and aggregation behavior [38]. The different concentrations of salt showed significant effects on the charge densities and mean hydrodynamic diameters of the particles (Table 2). The increase in salt concentration increased the cloudiness of the solution, indicating the complex formation, but the change from 200 to 300 mM of salt concentration showed no significant increment in particle size and charge. A significant increase in the ζ -potential was observed upon increasing the salt concentration up to 200 mM, while further increases in salt concentration increased the particle size and decreased the ζ -potential (less stability). Further addition of salt

Table 1 Effect of L-arginine as a stabilizing agent on the particle diameter and ζ -potential (Na-caseinate 0.4% and whey 0.5% w/v). NPs-1 prepared without stabilizing agent while NPs-2 prepared with stabilizing agent

Sample	Stabilizing agent	Mean diameter (nm)	PDI	ζ -potential (mV)
NPs-1	None	176 ± 12	0.44 ± 0.04	-14.6 ± 0.5
NPs-2	L-Arginine	116.1 ± 30	0.32 ± 0.03	-21.2 ± 0.6

Table 2 Effect of CaCl₂ concentration (50–300 mM) on the surface charge density (ζ -potential) and mean particle diameter of NPs prepared at pH 6.5

1 M CaCl ₂ (mM)	Hydrodynamic diameter (nm)	PDI	ζ -Potential (mV)
50	344.3 ± 21.7 _a	0.41 ± 0.1	-12.9 ± 1.1 _a
100	239.2 ± 16.3 _b	0.31 ± 0.03	-12.4 ± 0.6 _a
150	188.1 ± 15.3 _c	0.38 ± 0.04	-10.6 ± 0.8 _b
200	123.1 ± 12 _d	0.31 ± 0.01	-21.2 ± 0.7 _c
250	170.7 ± 14.6 _c	0.37 ± 0.04	-19.4 ± 4.5 _d
300	279.7 ± 18.3 _d	0.34 ± 0.02	-13.3 ± 2.4 _a

resulted in large scale aggregation of proteins and particle sedimentation was observed after 12 h. The increase in salt concentration increased the particle size, due to destabilizing the complex because of electrostatic shielding, and the electro kinetic potential value decreased due to higher ionic strength [39]. Absence of Ca²⁺ in the medium enhances the instability and sensitivity of α -La to pH and ionic concentrations due negative charge interactions at cation binding site [32].

Structural analysis of proteins and NPs

An FTIR analysis was conducted to evaluate the structural changes of proteins and their interactions with each other (Fig. 4). The peak at 3600–3000 cm⁻¹ showed hydroxyl (O-H) bond and N-H bond stretch from proteins with decreased intensity in Na-caseinate and NPs as more Na-caseinate (double than whey) was involved as compared to whey in complex formation. The intense and wide band near 3281 cm⁻¹ was an overlap of bands linked to the stretching vibration of O–H in the casein related to proline residues [40]. The peaks of absorption from 2950 to 2810 cm⁻¹ were due to CH₂ symmetric and antisymmetric stretching from fatty acids, and absorption peak at 1747 cm⁻¹ showed C=O stretching from milk fats that was slightly absorbed in NPs, since no fat was involved in complex formation (samples

were filtered to remove the floating fat content from mixtures). The peak at 2853 cm⁻¹ in whey almost diminished in NPs, indicating deformation of CH₂ bonds in samples. The two main vibrational peaks of the proteins are the amide I (1700–1600 cm⁻¹), due to stretching of C-O bonds and amide II (1600–1500 cm⁻¹), due to stretching of C-N bonds and deformation of N-H bonds [36]. Spectral regions at 1629 cm⁻¹ and 1250–910 cm⁻¹ correspond to amide I, mainly C-O stretching of proteins. The secondary structures (β -sheets ~1629 cm⁻¹) showed no changes indicating that secondary structures were intact. In whey and casein, the peak at 1629 cm⁻¹ shifts to 1637 cm⁻¹ in NPs due to overlap of casein and whey amide band. The absorption at 1527 cm⁻¹ was due to the N–H and C–H bending vibration of amide II from proteins [39], [41]. The spectral peak at 1436 cm⁻¹ shifts to 1427 cm⁻¹ due to attachment of whey peptides, while the absorption band at 1390 cm⁻¹ due to symmetric stretches of the carboxylic groups vanishes in NPs [42]. This effect can be related to the formation of COO–Ca bond between extended carboxyl groups of casein and Ca²⁺. The absorption peak at 1390 cm⁻¹ in the casein indicates the carboxylate group (COO⁻) because Glu and Asp residues were deprotonated. The signal for glycoside residue was at 1066 cm⁻¹ in the fingerprint region [36]. The absorption peaks at 1066 cm⁻¹ in Na-caseinate shifted to 1074 cm⁻¹ in NPs were linked to the covalently bonded phosphate group in casein protein. The overlapping of bands and shifting of peaks indicates the attachment of whey with casein.

Thermal stability analysis of NPs

Thermal treatment of proteins leads to denaturation, aggregation, and gelation. The thermograms of NPs and other components were studied using DSC (Fig. 5). The melting of samples starts at a temperature of (onset temperature) 87, 106, 98.4, and 126.5 °C for whey, casein, arginine, and NPs respectively. The melting temperatures (peak temperatures)

Fig. 4 Illustration of structural changes in NPs and constituent proteins

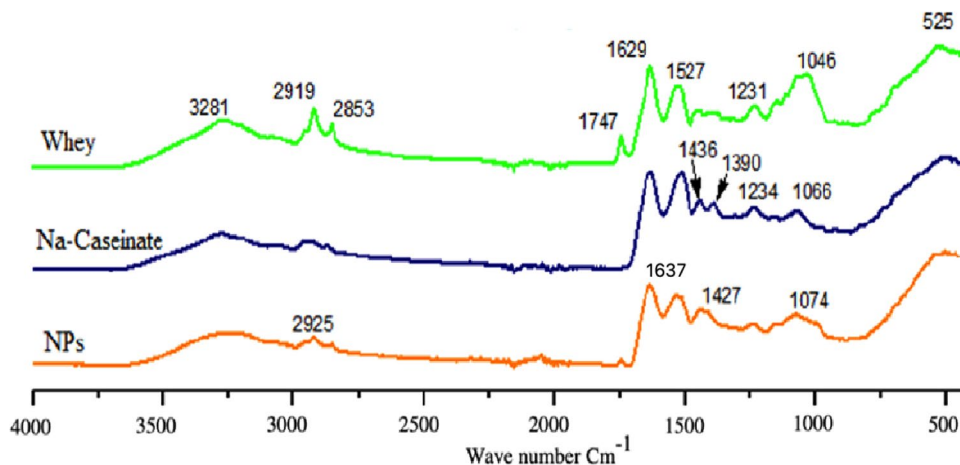
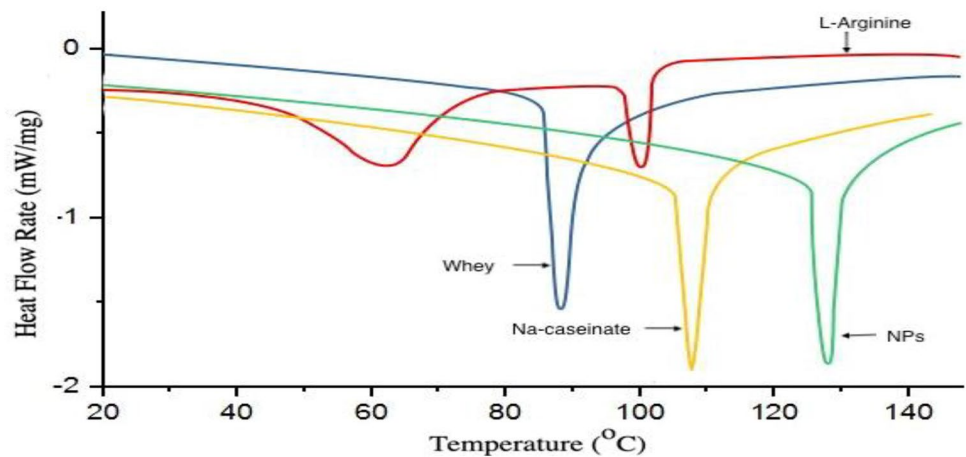


Fig. 5 Differential scanning thermograms of NPs and protein components



obtained were 89.4 °C (ΔH_f 8.97 J/g) for whey, 108 °C (ΔH_f 10.71 J/g) for casein, 99.2 °C (ΔH_f 6.66 J/g) for arginine and 128.3 °C (ΔH_f 11.37 J/g) for the NPs. The melting temperature obtained in this study were in complete agreement with the melting temperature observed in previous studies for whey [3] and casein [43]. The main source of improvement in heat stability of NPs was due to changes in structures of native proteins due to overlapping of amide bands (Fig. 5). The interactions between native proteins and Ca^{+2} provided the additional stability to NPs structure, which become more compact and stronger. The structures of native proteins were random while it was uniformly distributed structure of spherical shapes for NPs (Fig. 6).

Morphological observation of NPs

Milk proteins contain a high ability to form a variety of structures suddenly under specific physicochemical conditions, so the samples were analyzed through SEM for morphological observation (Fig. 7a-d). The casein (Fig. 7a) and whey (Fig. 7b) contained larger particles of irregular shapes which were modified to form NPs. The obtained structure of NPs consisted of a network of spherical amasses of proteins detached by many voids of varying shapes and sizes (Fig. 7c and d). The casein structure can easily carry the small molecules as well as it can interact with large molecules such as lactoferrin, β -Lg, and α -La. It was clear from SEM images that the smaller particles were making large clusters of proteins and overall size of the resulting semi spheres was less than 300 nm. The spherical nanoparticle of caseinate were also obtained in a previous study due to effect of Ca^{+2} [44]. The NPs of BSA in sphere shapes were also reported in a previous study [45]. The β -Lg also possesses a good tendency to bind with amphiphilic compounds such as Na-caseinate. From obtained images it was observed that the peptides were interacting with each other to make a big porous structure which was also responsible

for higher solubility index of samples (data not shown). All the structures were tightly clumped with each other which results in good stability of the NPs.

Bio accessibility of Igs content

The concentration of certain substances in the blood can reflect an animal's physiological condition. Blood biochemical factors such as total protein, immunoglobulins (Igs), and triglycerides play a crucial role in assessing metabolic activity, energy levels, and growth, thereby serving as valuable indicators for determining an animal's overall health [46]. To cure systemic disorders through oral administration of Igs, it's required that the Igs reach the distal part of the gut without their denaturation. The obtained results show that the recovered Igs content decreased significantly within the five hours of intestinal digestion of NPs (IgG 68 to 21.7%, IgA 14.5 to 6.5%, and IgM 19.5 to 10.5%) and control (IgG 42.7 to 10.3%, IgA 9.7 to 1.2, and IgM 14.4 to 2.3%) (Fig. 6)). In the gastric conditions, NPs showed more stability in terms of Igs because the pepsin preferably attacks the peptide bonds of casein. Due to the addition of CaCl_2 solution, the peptide residues were trapped inside the network of proteins due to their hydrophobic nature. In SSIF, the Igs concentration was rapidly decreasing at the start, but it was slower and continuous in releasing the Igs due to a repulsion effect between the whey and casein at pH 7.0 [47]. The control (whey) sample showed a significantly lower amount of recovered Igs as compared to NPs. This behavior of whey illustrates the resistant nature of casein since the presence of casein in NPs provides protection to Igs and renders them more available in the intestinal phase. Furthermore, it was also observed that the rate of digestion in the small intestine was slower for NPs when compared to control. It can be shown that the NPs release the Igs at a slower rate, so Igs retain their structure and become available for a longer period in the small intestine. It was also

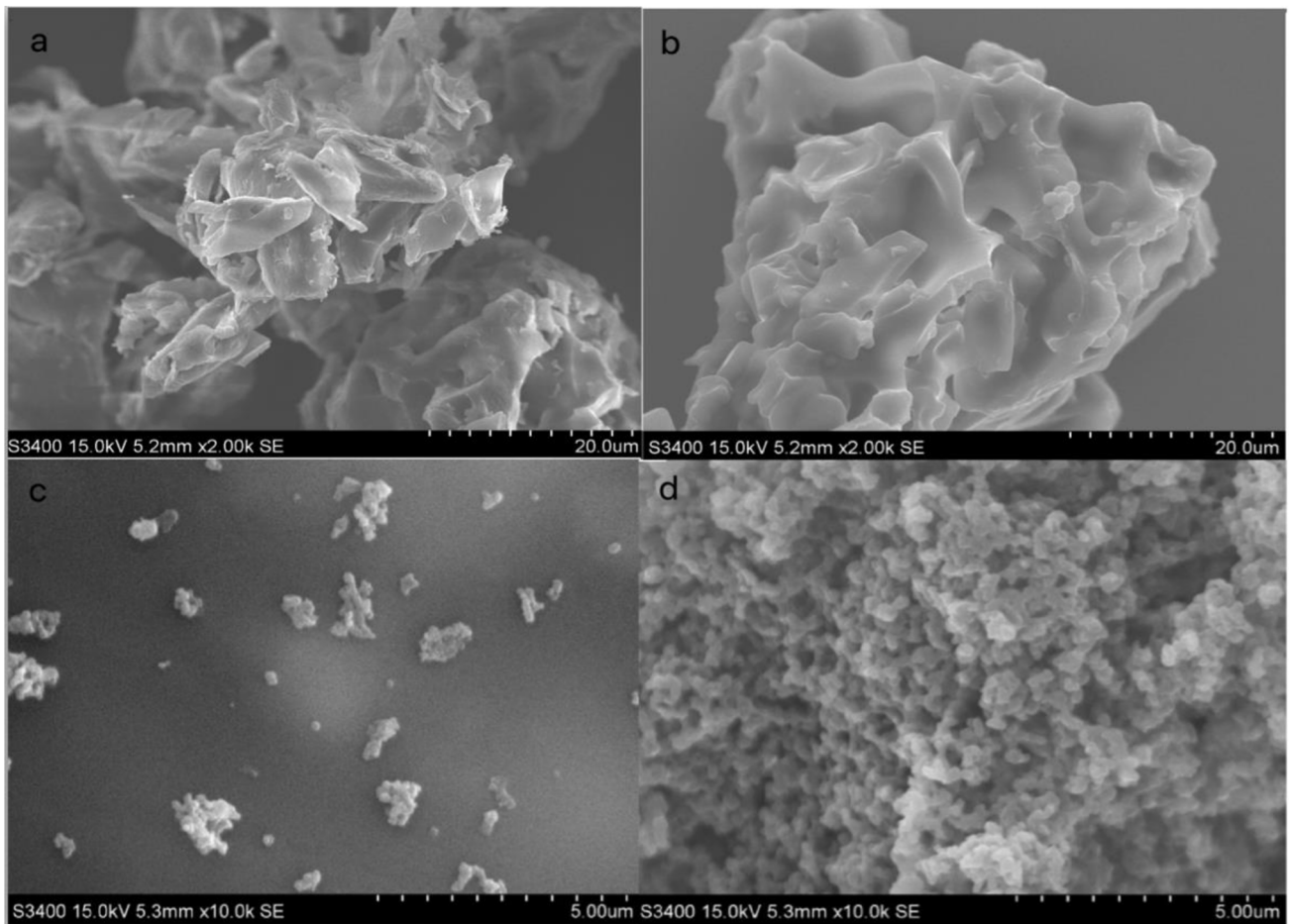
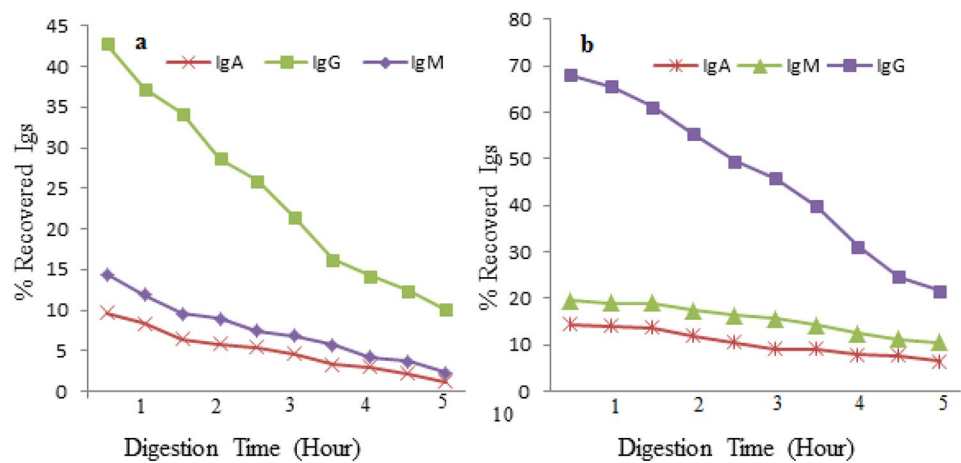


Fig. 6 The Igs Digestion profile in SSIF after gastric digestion, where (a) The control sample (whey) and (b) the developed NPs.

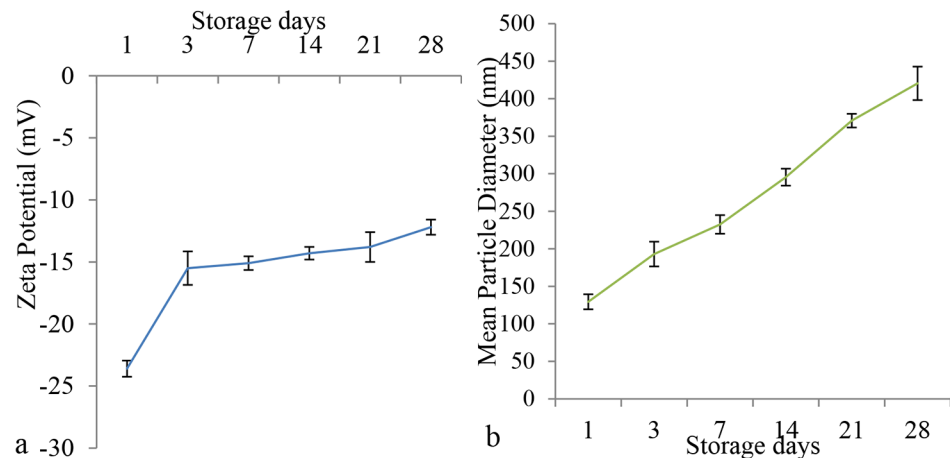
Fig. 7 Outer surface SEM images of freeze-dried samples at indicated magnifications, **a)** Na-Caseinate at 2kX, **b)** Whey at 2kX, **c)** NPs at 10kX, and **d)** NPs at 10kX



observed that the IgGs are more resistant to digestion in the gastric phase as compared to IgA and IgM, and therefore can provide specific nutritional requirements for intestinal disorders and diseases which cannot be provided with other dietary proteins. The rate of digestion in SSIF was higher for IgG as compared to IgM and IgA as the percentage of

IgM and IgA reduced at a slower rate. The main digestive enzymes for proteins in the human digestive system are pepsin, chymotrypsin, and trypsin, which break the IgG into Fab dimers and monomer fragments, but these IgG fragments also retain binding and antigen-neutralizing activity even after proteolytic digestion until they are denatured, as

Fig. 8 Changes in charge density and particle size of NPs in aqueous phase stored at room temperature (NPs were prepared at pH 6.5, Na-caseinate 0.4% and whey 0.5% w/v)



demonstrated in previous *in vitro* studies [48]. The higher amount of recovered active IgG could be due to the nature of its components to maintain the antigen-neutralizing efficacy as estimation of Igs was done using an antigen-neutralizing technique (ELISA). Some previous *in vitro* studies indicated similar results for the proteolytic digestion of Igs with a decreased antigen-neutralizing activity. The bovine hyper immunized powder was studied against pathogens in many studies, which showed the amount of recovered IgG varies from 10 to 49% [49].

Storage stability of NPs

Because of their high surface energy at the nanoscale, most colloidal systems are thermodynamically unstable at unfavorable conditions of temperature, pH, salt, and constituent concentrations. The changes in particle characteristics such as ξ -potential and mean diameter were monitored for a storage period of 28 days (Fig. 8). The NPs showed slight changes during this storage duration. The particle charge decreased from -23.6 – -12.2 mV while the size also increased from 129.4 to 420.5 nm. The NPs showed good stability as no phase separation or sedimentation was observed during this period.

Conclusion

Colostrum is gaining more interest due to its high concentration of bioactive components and its ability to cure several diseases and disorders. The bioactive components of colostrum are highly sensitive to thermal and processing unit operation, which was the major problem hindering its applications. This study shows that the ionic concentration and pH have significant impact on the casein-whey interactions which was utilized to generate NPs. The particles were

highly unstable near the pI of casein resulting in coagulation or precipitation. The surface charge modification of casein resulted in preparation of stable NPs with colostrum whey proteins at pH 6.5. At this pH the particles show highest stability with minimum size and higher charge density. The NPs show higher storage and heat stability than the native protein components which is good for the incorporation in other nutraceuticals. However, the *in vitro* digestibility indicated good stability of Igs in gut phase and improved availability in the small intestine. Colostrum whey is rich source of bioactive proteins and fabrication of NPs increased the bio accessibility of these components (Igs) which can be economical approach for the oral administration of these bioactive proteins. These NPs can be used as a good source of Igs and other colostrum proteins and can be further incorporated into functional beverages and nutraceuticals to have specific health benefits or to cure the diseases, gastrointestinal disorders and to boost the immunity.

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Data availability The data that supports the findings of this study are available in this article.

Declarations

Conflict of interest The authors declare that they have no known com-

peting financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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