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Author Contribution

Data collection, interpretation, drafting the article was done by PS. AN, PL and VS co-wrote and helped for the data analysis of nanofibril characterization. SS, SA and CP co-wrote and helped for the data analysis of nutrients part. RS and CP designed the study, co-wrote, supervised and approved for the submission.

Sincerely

Dr. Ramesh

Utilizing protein nanofibrils as a scaffold for enhancing nutritional value in toned milk

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Abstract

Toned milk is a lower-fat, healthier alternative to whole milk that still contains all essential nutrients. A number of methods have been developed to improve the functionality of toned milk and make it more appealing to the consumers. However, these methods often involve extensive processing techniques and can be expensive. Therefore, alternative methods are needed. Proteins are well known for their ability to form well-defined nanofibril materials that can be used as a scaffold for various applications. In this article, a straightforward self-assembly process was used to load inulin into protein nanofibrils, creating unique composite nanofibrils. Characterization using AFM and SEM revealed well-defined composite nanofibrils with an average diameter of 4-6 nm and lengths ranging from 0.25 μm up to 10 μm. FT-IR and *in-vitro* release assays show that inulin was successfully attached to prepared protein nanofibrils. The composite nanofibrils were tested on toned milk to enhance the physico/chemical properties and nutritional values. The findings can be applied to the food industry to create a number of novel functional food products cost-effectively.

Keywords: Nanofibrils, Proteins, Polysaccharides, Self-assembly and Toned milk.

1. Introduction

Strong food security and agriculture are closely interconnected in achieving the Sustainable Development Goals proposed by the United Nations. However, land degradation, desertification, labor shortages, climate change, and drought create enormous problems in the global food supply chain. Hence, reducing food losses and waste is the prime objective to ensure that everyone suffering from hunger and malnutrition has access to nourishing food, especially with the world's population continuing to rise (Umesha 2018). There are numerous bioactive ingredients available naturally; however, most of them are not directly consumed in daily life. The development of functional foods incorporating such bioactive ingredients using a cost-effective methodology offers significant advantages for sustainable food production and contributes to the growth of agricultural production (Vagsholm 2020).

Self-assembly is a low cost, high throughput method for producing nanosized materials. Self-assembly is a spontaneous process; well-defined structure forms instantly from basic building blocks of atoms/molecules *via* hydrophobic, hydrophilic, π-π interactions and hydrogen bonding (Skink 2012). The self-assembly method of preparing nanofibrils allows for morphology control with straight and strong fibrils with a hierarchical design. These nano-sized materials have improved physical and chemical properties because of their high surface-to-volume ratio (Yadav 2020, Li 2021). Peptides and protein-based biopolymers can form well-defined nanostructures by tuning the parameters like pH, temperature, concentration, ionic conditions and agitation. There are a number of protein-based biopolymers, such as β-lactoglobulin, k-casein, and ovalbumin, that can be self-assemble and form well-defined nanofibrils. These nanofibrils is the continuous presence of β-sheets along the fibril axis, stabilized by a number of non-covalent interactions. Cross-β structure formation is the common core of these nanofibrils, leading to high mechanical rigidity, which makes these fibrils stable and an interesting candidate for functional nanomaterials. Hendricks et al., reported that the peptides /protein based amphiphilic molecules can self-assemble into cylindrical nanofibrils with high aspect ratios through electrostatic interactions between the charged head groups and hydrophobic collapse of the alkyl region. Lara *et al*, has investigated two morphologically distinct nanofibrils by tuning the ovalbumin concentration (Lara 2012). Ovalbumin could form flexible and thin fibrils at low concentrations, but rigid and thicker fibrils at high concentrations. According to Feng et al., ovalbumin loaded with curcumin has higher pH stability, resolubility and storage stability and forms a stable structure (Feng 2016). Martins *et*

al., reported that edible nano-coating of ovalbumin on food can retain colour, enzymes, and antioxidants, prevent browning, and prevent gas and moisture exchange (Martins 2018).

Therefore, nanofibrils can act as a scaffold to attach other functional molecules to increase food's nutritional and medicinal values. Inulin is found naturally in the roots of many foods and is used as a dietary fiber (Arcia 2011). It is a functional food in the food industries because of the health benefits like maintenance of gut health, blood sugar control, weight and appetite control and calcium absorption (Ahmad 2020). It is a water-soluble fiber that dissolves in the stomach and produces a gelatinous substance which aids in the reduction of cholesterol absorption in the digestive system. Inulin is well-known for stimulating the growth of beneficial bacteria, including Bifidobacteria and Lactobacilli, in the gut (Luis 2011). These bacteria consume undesirable pathogens (bad bacteria), preventing infection and stimulating our immune system (Ahmad 2020). For instance, inulin forms a creamy white liquid with water that can replace fat as demonstrated by Shoaib *et al* (Shoaib 2016). Food products containing inulin are more popular among consumers as functional foods (Luis 2011). Compared to other available carbohydrates, inulin is widely used as a dietary fibre, and it degrades into fructose that can be completely metabolised in the liver (Usman 2021).

Various modern biotechnology methods have been employed to enhance the variety of agricultural food products. Among these methods, self-assembly is a particularly useful technique for improving food quality, protein modeling, and creating beneficial variants that enhance bioavailability (Oluwole 2021). The loading of bioactive compounds can be effective for the formulation that is nutrient-rich, especially given the growing interest on a global scale in biotechnological tactics in contemporary and sustainable agriculture (Zabot, G L, 2022). In this context, the utilization of nano-scale loading for bioactive ingredients has gained significant attraction in the food industry, primarily due to its potential to bioactive food products with high added value through nanoscale encapsulation (Senthilkumar 2022a; Scott 2014). The primary objective of this study is to employ protein nanofibrils as a scaffold for encapsulating inulin, thereby enhancing the functionality of toned milk. Toned milk, chosen for its low-fat content and ease of digestion, is particularly favoured among obesity and diabetic patients. Incorporating inulin with protein nanofibrils into toned milk not only boosts its protein content with minimal caloric impact but also reduces cholesterol absorption, functions as a health-conscious sweetener, and stimulates the immune system (Ahmad 2020).

2. Materials and methods

2.1 Materials

Ovalbumin (A5503) was purchased from Sigma Aldrich, Phosphate Buffer Saline (PBS), Hydrochloric Acid (HCl) and Sodium Hydroxide (NaOH) were purchased from Isochem laboratories. Inulin was purchased from Sigma Aldrich (I2255), USA. Toned milk was purchased from local suppliers (Grocery markets).

2.2 Preparation of nanofibrils and inulin loaded nanofibrils

20 mg of proteins (β-lactoglobulin, k-casein and ovalbumin) *i.e.* 2% (w/w), was added to 1 ml of double distilled water and denatured using HCl (0.1 N) and adjusted the pH to 2 (Yadav 2020). The prepared solution was centrifuged at 4000 RPM for 30 minutes, and removed the residues by filtration. The solution was constantly agitated at 1000 RPM in eppendorf thermo mixer C – F 1.5 for 24 hours at 80°C. For the preparation of inulin loaded nanofibrils, 20 mg of inulin was dissolved in 1 ml of double distilled water (2% w/w) and 0.5 ml of this solution was added to the 0.5 ml of prepared ovalbumin nanofibrils. The resulting solution was agitated at 1000 RPM in vortex for a few seconds to load inulin in nanofibrils. The resulting solution was kept in 4˚C for further characterization.

2.3. Morphological and spectral characterization of nanofibrils The nanofibrils solution was diluted 100 times, deposited uniformly on a fresh mica sheet surface and air dried for surface topography observation. The surface morphology of nanofibrils was studied using Atomic Force Microscopy (AFM) (NTM-DT, NTEGRA, Russia) and Scanning Electron Microscopy (SEM) (ZeissEvo18, USA) at 15000 X magnification (Li 2021). FT-IR was used to study the presence of the functional groups in the inulin-loaded nanofibrils (Mahdi 2017). We filtered the inulin-loaded solution using a 0.5 µm Millipore membrane filter and washed the filter three times to eliminate any free inulin, ensuring that only the loaded inulin remained attached and not the unbound inulin. The re-suspended precipitate was then used for subsequent characterization. The prepared nanofibrils were diluted for 1000 times at pH 2 and scanned using IR Affinity -1S Fourier Transform Infrared spectroscopy (Shimadzu MIRacle 10) (32 scans / minute in 4000-400 cm^{-1} wavenumber range). Before characterization, the background spectrum of the empty chamber (water vapours and carbon dioxide) was removed in order to gain information about the prepared samples.

2.4. Nutrients enhancement of toned milk using inulin loaded nanofibrils

10 ml of inulin loaded ovalbumin nanofibrils (1 ml of inulin loaded nanofibrils solution was diluted in 9ml of distilled water) was added in 240 ml of toned milk and pasteurized for further analysis (heated at 72°C for 20 seconds and cooled down to room temperature) (Silva 2020). Nutritional analysis, pH, Total titrable acidity, anti-oxidant activity, loading efficiency, *in-vitro* release analysis in PBS buffer, and sensory evaluation were studied. The analysis was conducted over a 7-hour period with 1-hour intervals. To test the shelf-life of toned milk before and after adding nanofibrils, we monitored the Total Plate Count (TPC) for seven days using Disk diffusion methods.

2.5 Analysis of inulin loaded toned milk

2.5.1. Nutritional analysis

Nutritional parameters such as fat, moisture, protein, ash, lactose, carbohydrates, and energy were measured in inulin loaded toned milk and toned milk. The measurements were made in accordance with ISO standards (the ISO numbers for fat, moisture, protein, ash, and lactose measurements were 1211, 5537, 8968, 9877, and 22662, respectively). For carbohydrates and energy, IS 1656 and ISSN 0259:2916 method was followed.

2.5.2. pH and titrable acidity

Using a pH-metre, the pH value of 30 mL of fortified milk and 30 mL of toned milk was determined (HI-98107). The total acidity of inulin loaded toned milk and toned milk was determined by titrating 20 ml of inulin loaded toned milk and toned milk separately against 0.1 N NaOH. The final stage was the appearance of a pale pink colour. The formula 1 was used to calculate the total titrable acidity (Seethu 2020).

$$\text{Total Acidity (\%)} = \frac{\text{NaOH used (ml)} \times \text{milli equivalent factor}}{\text{Volume of sample}} \times 100 \qquad (1)$$

2.5.3. Anti-oxidant activity

The anti-oxidant activity for the inulin loaded toned milk and toned milk was measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging radical method (Senthilkumar 2022b). Using 1 ml of DPPH solution, different concentrations of the nanofibrils (50, 100, 150, 200 and 250 µl) were dissolved. For a period of 30 minutes, the dissolved solution was incubated in the dark at room temperature. Absorbance was measured at 517nm, and the DPPH scavenging activity was calculated using the formula 2 (Jayan 2019).

$$\text{DPPH Scavenging \%} = (A_c - A_s) \times 100 \qquad (2)$$

Where, A_C is the absorbance of the control, and A_S is the absorbance of the sample.

2.5.4. Loading Efficiency

The efficiency of loaded nanofibrils (inulin-loaded ovalbumin nanofibrils) was measured by adding (20 µl) nanofibrils to 80 % ethanol (100 ml), and the resulting solution was shaken for 10 minutes (Thermo mixer C- F.15, eppendorf). The prepared solution was filtered using Whatmann filter paper, and UV spectrophotometer to measure the absorbance at 306 nm (Inanc, 2019). The efficiency was calculated using the formula 3.

$$\text{Loading efficiency}(\%) = \frac{\text{Amount of Nanofibrils in pellet } (R_T)}{\text{Total amount of Nanofibrils unentrapped } (R_F)} \times 100 \qquad (3)$$

Where, R_T is the total amount of inulin added for loading, and R_F is the amount of inulin un-entrapped.

2.5.5. *In-vitro* release analysis in PBS (Phosphate Buffer Saline)

To study the release of inulin loaded nanofibrils in toned milk, toned milk was used as control. 5 ml of inulin toned milk and 1.5 ml of PBS buffer saline were placed in the dialysis bag. 50 ml of PBS solution (release medium) was taken in the beaker, and the dialysis bag was dipped into it and the temperature was maintained at 37 ℃ with constant agitation. At 1hour intervals, 1 ml of PBS (release medium) was taken and replaced with fresh PBS. PBS absorbance was measured at 305 nm at 1-hour intervals using visible spectroscopy (Yang 2021).

2.5.6. Sensory Evaluation

Sensory evaluation of prepared inulin loaded toned milk and toned milk was evaluated by consumers of all age groups. Both the samples were served to the consumers at random. To avoid the taste of carryover milk, mineral water was served to cleanse the palate of the members A 10-point scale was used, with 10 being extremely likeable and 1 being extremely dislikeable. A few minutes were allotted for evaluation, and customers were asked to select their favourite milk (Senthilkumar 2022b). Taste, colour, appearance, texture, taste, flavour, and aftertaste were all measured and plotted on a spider graph.

2.6. Statistical Analysis

All the characterizations and analysis were made in triplicate. Statistical analysis was carried out with excel software by using a one-way analysis of Anova (Analysis of variation). Tukey's multiple comparison test was analyzed to find the significant differences for the comparison of

198 data ($p \leq 0.05$). All the graphs were included with mean values and standard deviations using
199 whiskers.
200
201 **2.7. Ethical Clearance**
202 Ethical clearance was obtained from the IEC (Internal Ethical Clearance) in compliance with
203 the institutional research ethics policies and ICMR guidelines (ISBN: 978-81-910091-94) for
204 sensory evaluation
205
206 **3. Results and discussion**

207 Numerous approaches, including phase separation, electrospinning, template synthesis, and
208 self-assembly, can be utilized to create functional nanofibrils. However, self-assembly has the
209 most advantages due to the cost-effective and simple preparation steps. The schematic
210 representation of the self-assembly of the functional nanofibrils loaded with inulin and its
211 application on the toned milk is shown in Figure 1. We have also conducted studies on β-
212 lactoglobulin and k-casein inulin-loaded nanofibers, which yielded similar results (data not
213 shown). Therefore, this article presents a detailed characterization study of inulin loaded
214 ovalbumin nanofibrils.

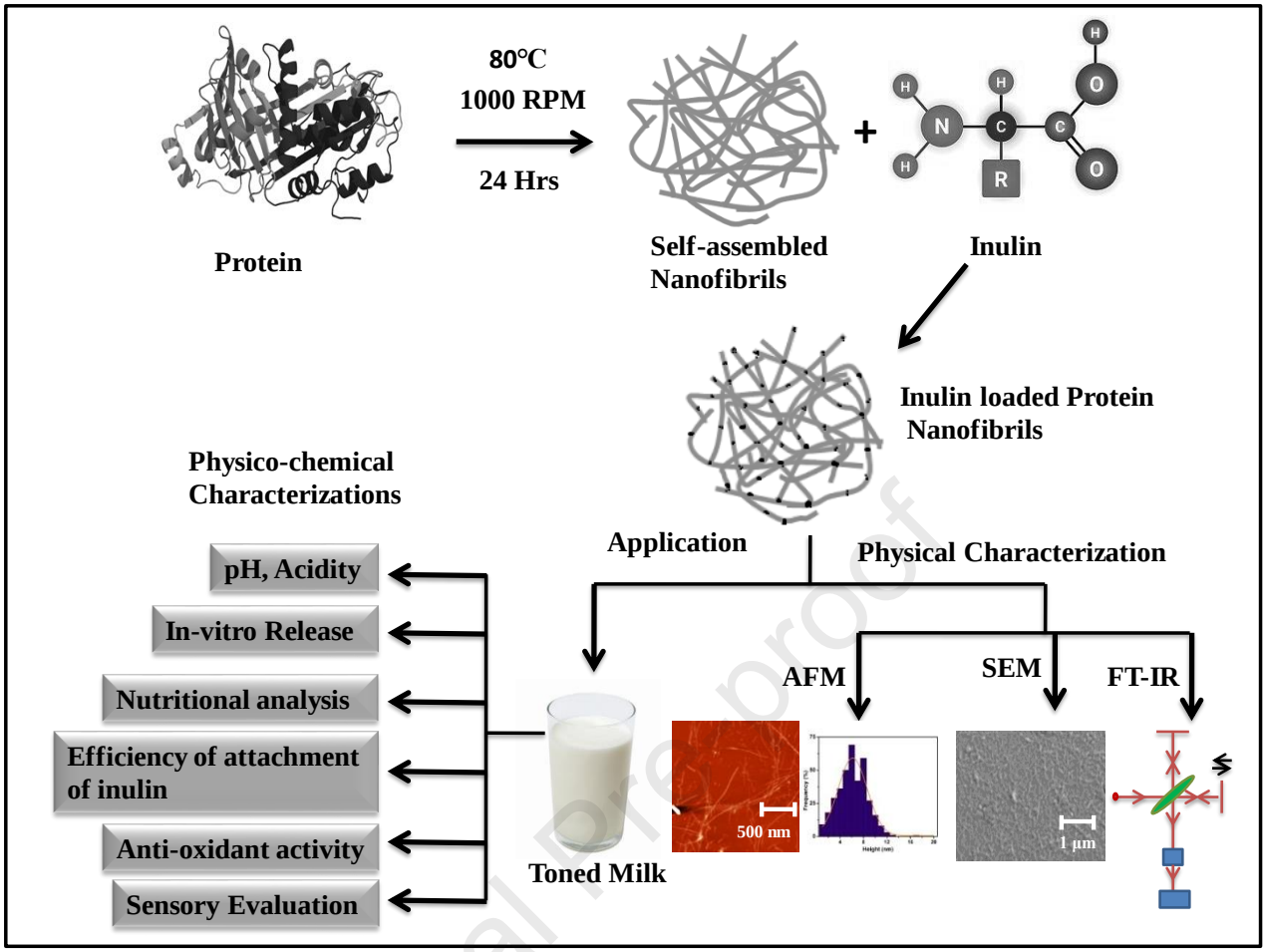


Figure 1. The preparation of inulin-loaded protein nanofibrils and their application in toned milk is depicted schematically.

3.1. Characterization of ovalbumin nanofibrils

Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) were used to examine the nanofibril's morphology and structural details. The representative AFM images of prepared ovalbumin nanofibrils are shown in Figure 2a. The diameter of nanofibrils varies between ~2 nm to 6 nm. Figure 2b shows the height histogram of ovalbumin, with the majority of the fibrils ranging in size from 4 to 8 nm. 12-18 nm nanofibrils were credited to the bundling effect of the fibril formation. The length of the ovalbumin nanofibrils varies from a few nanometers to several micrometres (Figure 2c). SEM analysis reveals the fibrils of diverse diameters and length, which complements the AFM images (Figure 2d). The results showed that nanofibril formations were comparable to those previously reported in the literature. At pH 2, Arnaudov et al reported the formation of two fibrils, thin fibrils with 2.5 nm diameters and thick fibrils with 4.0 nm diameters, which was comparable to our findings (Arnaudov

2005). Kamada and colleagues reported self-assembled nanofibrils in 2017, in which long and straight fibrils formed at low concentrations (3% protein) and short and worm-like fibrils formed at high concentrations (7.5% protein) (Kamada 2017). Mahdi *et al* prepared ovalbumin nanofibrils by self-assembly method for loading hydrophobic bioactive molecules in physiological conditions due to their high stability at a range of pH 2 (Mahdi 2017). Similarly, Sambani *et al.* prepared and characterized self-assembled elastin nanofibrils using AFM. The results obtained indicated that a 1% w/v concentration of elastin in ultra-pure water facilitated the most efficient self-assembly process, leading to the creation of elastin nanofibrils at the nanoscale level (Sambani 2023). Li *et al.* attempted the preparation of peptide nanofibrils from resins, and AFM imaging revealed the formation of thin nanofibrils. These nanofibrils exhibited lengths typically exceeding several micrometres, which is consistent with our own findings. These observations imply that the majority of nanofibrils are formed due to interhelical ion pairing and other polar interactions (Li 2020). Thus, through the creation of effective functional groups, the self-assembly approach provided a pathway for creating well defined nanofibrils. A primary advantage was that the essential bioactive component (inulin) could be integrated into the nanofibril structures. The general mechanism of self-assembly was elucidated by Ozsvar and colleagues, wherein interactions and cross-linking of peptide molecules play a pivotal role in forming self-assembled structures. At low pH and with prolonged denaturing temperatures, disulfide bonds reform, causing protein unfolding and exposing the hydrophobic core of the protein. This promotes fibrillation and hierarchical assembly through interactions such as hydrogen bonding, van der Waals forces, hydrophobic interactions, π-π interactions, and electrostatic forces. When such fibrils form, the surfaces of the nanofibrils expose both hydrophobic and hydrophilic groups, allowing inulin (or any other active ingredients) to attach to the surface of the nanofibrils. (Ozsvar 2021)

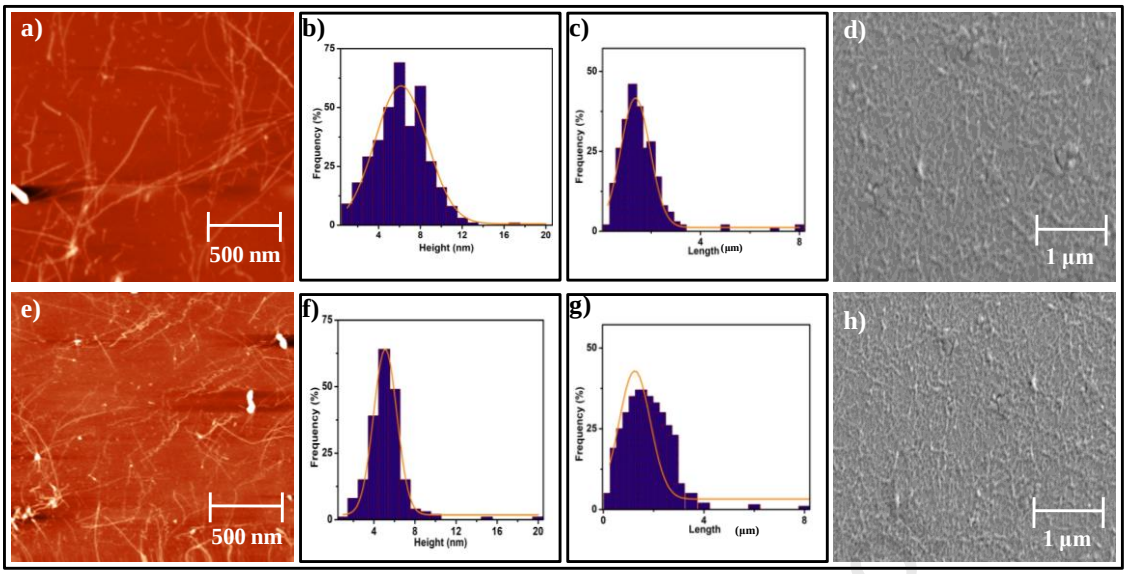


Figure 2. a) AFM, b) Height, c) length histogram and d) SEM images of ovalbumin nanofibrils, e) AFM, f) Height g) length histogram and h) SEM images of inulin loaded ovalbumin nanofibrils

Figures 2e and 2h show that inulin-loaded ovalbumin nanofibrils were successfully formed. Similar to ovalbumin nanofibrils, the obtained SEM results supplemented the AFM results. Inulin-loaded nanofibrils formed more aggregates and bundling than ovalbumin nanofibrils (figure 2a) due to inulin attachment to ovalbumin nanofibrils. However, the measured heights (4 to 8 nm, n=115) and lengths (0.1 to 5 µm, n=115) were identical to the ovalbumin nanofibrils, as shown in figures 2f and 2g. Due to the lack of high-resolution imaging and periodicity, it was not possible to demonstrate the attachment of inulin to ovalbumin nanofibrils directly using AFM and SEM, primarily because of the size of the inulin molecules (with molecular weights ranging between ~3.5-5.5 kDa), therefore, complementary analysis was conducted.

3.2. Fourier Transform Infrared Spectroscopy

FT-IR spectra were used to confirm the loading/attachment of inulin in the ovalbumin nanofibrils by the functional groups in the nanofibrils solution. The appearance of the alcoholic group was responsible for the broad peak observed for O-H stretching vibration at 3531 and 3107 cm^{-1} (figure 3a). Figure 3b shows the source of 1004 cm^{-1} for the stretching vibration peak of C-O bond in inulin. A peak at 1109 and 1042 cm^{-1} shifted to 1004 cm^{-1}

shown in figure 3c represents the phosphate group present in ovalbumin and disappeared after the loading of inulin matrix by hydrogen bonding. α-helical and amide II peaks were observed in ovalbumin nanofibrils at 1620 and 1529 $cm^{-1,}$ respectively, which shifted to 1701 and 1610 cm^{-1} for inulin loaded ovalbumin nanofibrils due to the loading of inulin (C=O stretching of -NH group). In addition, a peak at 821cm^{-1} was observed for 2-keto furanose for the confirmation of loading of inulin. The obtained spectrum confirms the presence of inulin in the ovalbumin nanofibrils. Similarly, Taherinia *et al.* investigated peptide nanofibrils and identified four characteristic peaks at 1722, 1646, 1177, and 1067 cm-1, corresponding to (C=O), (N–H), and (C–O) vibrations, respectively (Taherinia 2019). In line with this, Aydogdu et al. described the FT-IR spectra of protein nanofibrils containing bioactive compounds, observing peaks at 1058, 1095, and 1145 cm-1, which were associated with stretching vibrations of the C-O complex. Other distinctive peaks were located around 1680 and 1790-1, indicative of N-H and C=O bonds, respectively. These observations confirmed the attachment of bioactive compounds to the nanofibrils (Aydogdu 2018). Consistent with the literature, our observations support those characteristic peaks signifies interaction between the functional groups of ovalbumin and inulin.

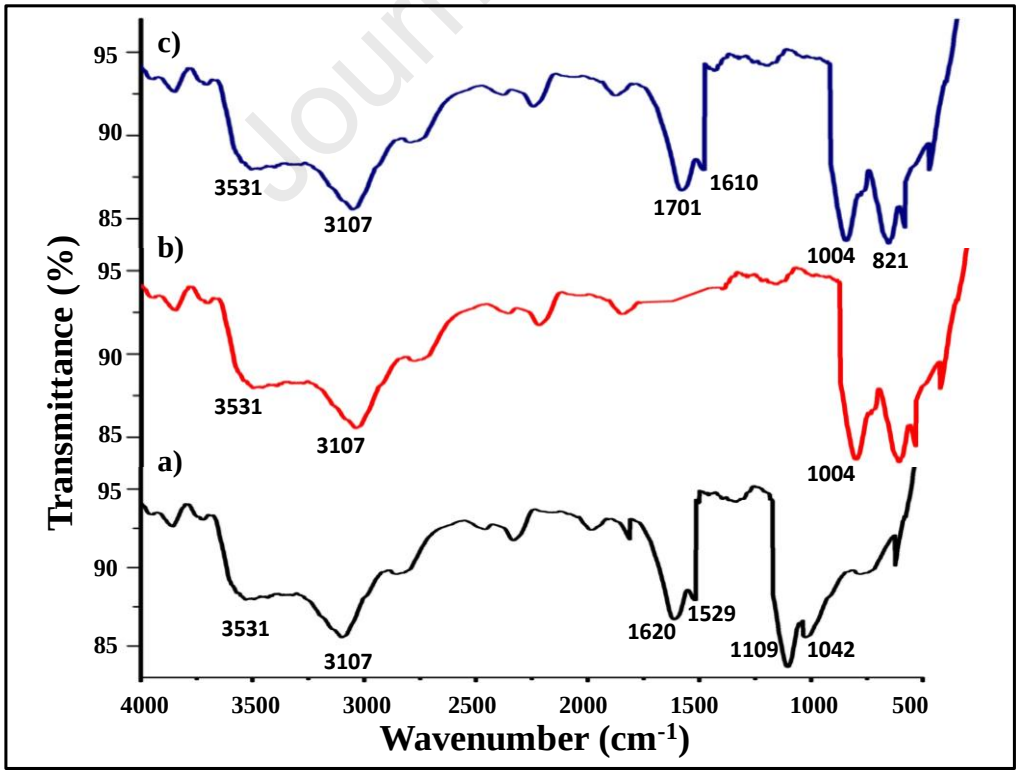


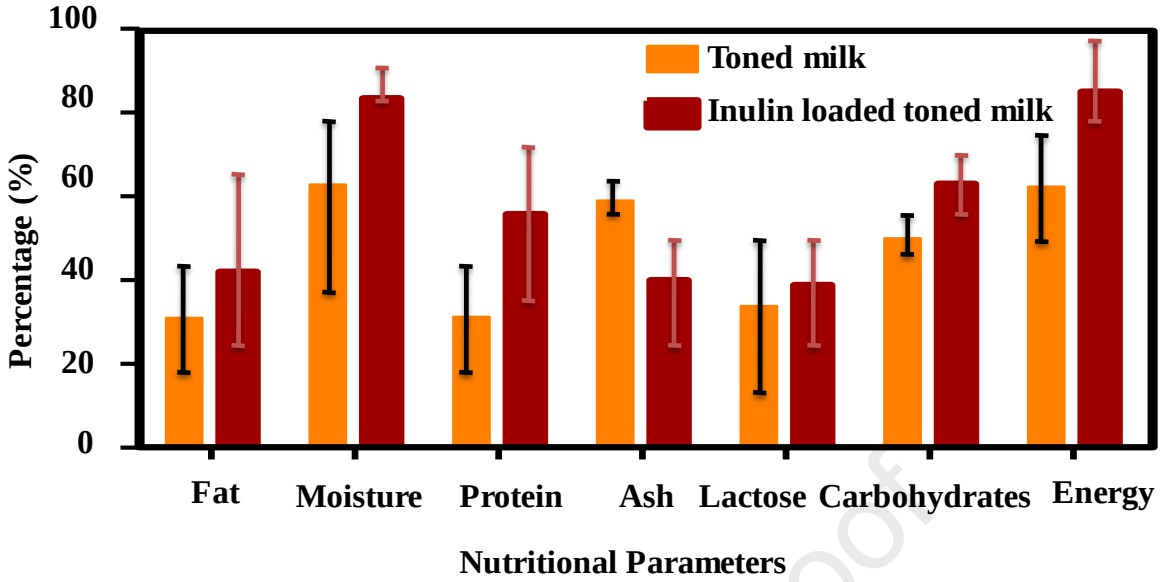
Figure 3. FT-IR spectra of a) ovalbumin, b) inulin and c) inulin loaded ovalbumin nanofibrils

3.3. Analysis of nanofibrils added toned milk

The measured physical and chemical characterizations confirmed the successful formation of nanofibrils and the attachment of inulin to the nanofibrils. To improve bioavailability and nutritional value, these novel nanofibrils were added into toned milk.

3.3.1. Nutritional analysis

Humans' and plants' growth and development rely on essential nutrients, which encompass vital mineral elements. Among the commonly consumed dairy products worldwide, milk holds a prominent position. However, its production often occurs in nutrient-deficient conditions, particularly in resource-limited regions (Banerjee S, 2023), emphasising nutrition-based beverages' potential impact. The recommended daily intake of milk for the average adult ranges from 400 ml to 600 ml of dairy products per day to meet their calcium and nutrient needs. In this context, the development of nutritional toned milk presents an opportunity for significant advancements. A comparative analysis was conducted between the developed nutritional toned milk and toned milk enriched with nanofibrils. The obtained nutritional values, such as fat, moisture, and ash, were similar and did not differ significantly. Protein, lactose, and carbohydrate values increased to 78%, 15%, and 26%, respectively, and energy increased to 24% compared to toned milk. The increase in the percentage of nutritional value after adding nanofibrils is helpful for meeting the recommended daily intake. Figure 4 shows the comparative nutritional values of both milks. Mohite *et al.* investigated the micro-addition of bioactive compounds to fortified milk. Their study demonstrated the feasibility of preparing fortified milk products through the micro-addition of appropriate bioactive compounds to enhance nutritional values, a finding similar to our research (Mohite 2020). The total plate count of toned milk before adding nanofibrils was ~0.8 CFU/ml, and after adding nanofibrils, it was ~1 CFU/ml (the plate count of toned milk, as per IS 5887, is ≥ 1), confirming the shelf life of the toned milk (over the period of seven days at 4°C), which indicates the stability of the nanofibrils.



325

326 **Figure 4.** Nutritional values of toned milk and inulin loaded toned milk ($p \leq 0.05$)

327

328 **3.3.2. pH and titrable acidity**

329 There are no significant differences in pH between inulin-loaded toned milk and toned milk
330 (figure 5a). The total titrable acidity was measured to determine whether the milk was less
331 acidic due to the presence of nanofibrils (Wen 2017). The acidity was measured for 5 hours,
332 with 1hour intervals (figure 5b). The titrable acidity values of inulin loaded toned milk and
333 toned milk were found to be comparable.

334

335 **3.3.3. Anti-oxidant activity**

336 The antioxidant activity of inulin-loaded toned milk and toned milk was measured using the
337 DPPH scavenging radical method. Toned milk with inulin has higher anti-oxidant activity due
338 to protein nanofibrils containing amino acids (figure 5c) compared to the control. The activity
339 increases gradually from 30 to 50% when the concentration of nanofibrils is increased from 50
340 to 250 mg/ml because of the ovalbumin conjugation with reducing sugars. The inulin's
341 molecular properties could be the cause. The reductive hydroxyl group terminals of
342 polysaccharides, which could receive and remove free radicals, were associated with the
343 antioxidant properties of inulin which was a polysaccharide (Shang 2018). Agustin *et al.*
344 investigated the DPPH scavenging activity of protein hydrolysates at various concentrations,

revealing a sharp increase in activity over time and demonstrated the formation of short peptides and free amino acids during hydrolysis. It was evident from their findings that these factors significantly influenced the DPPH activity (Agustin 2021). Additionally, since most *in vivo* antioxidant reactions involve two-electron processes, the single-electron radical scavenging abilities of food components are not relevant to antioxidant defense in the human body (Sadowska-Bartosz 2022). Our results are in line with the literature, complementing the understanding that DPPH activity plays a greater role in the hydrolysis of amino acids in dairy products and contributes to the defense against radicals in the human body.

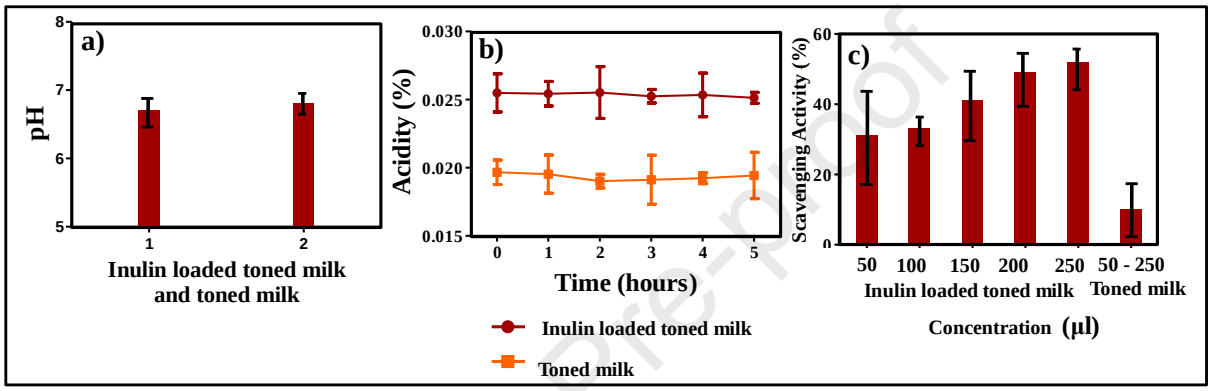


Figure 5.a) pH, b) acidity, and c) anti-oxidant activity of inulin loaded toned milk and toned milk ($p \leq 0.05$)

3.3.4. Loading efficiency

Nanofibril concentration was 2 % (w/w) constantly maintained, whereas inulin concentrations varied such as 0, 2 and 3 % (w/w) for the attachment in nanofibrils. Figure 6b shows that as the concentration of inulin increases, loading efficiency decreases from 84% to 46% when, as shown in figure 6b. This result shows that higher loading is obtained in the lower concentration of inulin, due to the uniform loading of inulin in the fibrils without the formation of bundles, whereas high concentration of inulin leads to the formation of aggregates (Yang 2021). Li and Du studied that pH 2 nanofibrils have 100% loading efficiency, similar to our results. The inulin loading improvement is due to the hydrogen bond's stability with water, deprotonation, and separation from the polymer upon water addition. Inulin carries a negative charge when introduced into water. As a result, there was no change in size due to microparticle oxidation. This suggests that loading efficiency would improve at lower material concentrations and a pH of 2 (Afinjuomo 2019). Preserving active ingredients, extending shelf life, and regulating the release of bioactive components constitute the key functions considered for microloading. With

the growing interest in nanoloading, more emphasis has been placed on functions related to size reduction, such as increased surface area and enhanced intracellular uptake at low nanoloading concentrations (Shaaban 2022).

3.3.5. *In-vitro* release from PBS (Phosphate Buffer Saline)

An *in-vitro* release test was performed using the dialysis bag method to confirm the release and attachment of inulin from loaded ovalbumin nanofibrils (Altay 2015). The release of inulin-loaded ovalbumin nanofibrils (loaded toned milk) was 15% in the first hour and gradually increased to 60% in the seventh hour, whereas toned milk remained constant until the seventh hour (figure 6a). The release of resveratrol attached with zein nanofibrils interface with proteinaceous loading was responsible for the initial time (Maria 2020). The release of nanofibrils proves the bioavailability of toned milk. Paramera *et al.* reported a sustained release of inulin from protein nanofibrils in toned milk, demonstrating the similarities shown in figure 6a (Paramera 2011). Numerous scientists have investigated various kinetics models to elucidate the release of loaded molecules from bonded nanofibrils. According to these kinetics models, the prolonged release of inulin was attributed to factors such as the relaxation of protein matrices, diffusion, and the surrounding medium (Antoniraj 2020). Tang *et al.* employed proteins to create nanostructures for the oral delivery of dairy products, intended for use as food additives in beverages, meals, and dietary supplements. Many of the beneficial health effects (antioxidative, anti-inflammatory, anticarcinogenic, or antihypertensive) associated with dairy products can be attributed to these bioactive molecules (Pateiro 2021).

3.3.6. Sensory Evaluation

Since product quality influences consumer acceptance and demand, the ability to measure sensory perception is required to develop and produce products that meet consumer expectations (Sharif 2017). Panel members assessed the acceptability of prepared inulin loaded toned milk versus toned milk. Around 30 people took part and were regular consumers of toned milk and semisolid dairy products at least once a day. Inulin loaded toned milk did not show any irritation for the panel members. The toned milk has good mouthfeel and taste compared to inulin loaded toned milk. Figure 6c shows the panel members' overall acceptance of inulin-loaded toned milk. Both milks appeared to have the same colour, appearance, OAA, and aftertaste. Taste and flavour were better in inulin loaded toned milk because of the sweeteners,

whereas texture was better for toned milk. Similarly, in line with studies conducted by Dimitrelloua et al., the addition of probiotic cells during the production of fermented milk did not impact the sensory qualities of the final product. The milk product showed excellent potential as a wall material for probiotic incorporation (Dimitrelloua 2019). Additionally, extended shelf-life milk was prepared with high processing of bioactive compounds. In this study, 89.5% of the panel members chose the processed milk, similar to our findings (Lim 2022). Su *et al.* also prepared milk with different fat content and reported an overall acceptance score of 7.4. Moreover, overall preference significantly improved with the addition of attributes like sweetness, post-milk scent, protein-like characteristics, mellowness, and thickness (Su 2022).

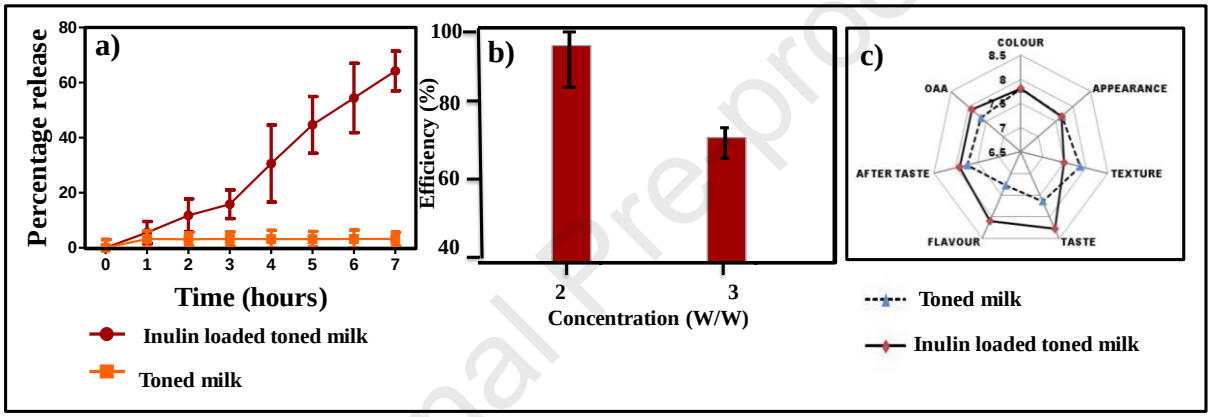


Figure 6. a) *In-vitro* release analysis for inulin loaded toned milk and toned milk, b) loading efficiency of inulin loaded toned milk, and c) sensory evaluation of inulin loaded toned milk and toned milk ($p \leq 0.05$).

4. Conclusion

In this study, we used a simple self-assembly technique to create inulin-loaded protein nanofibrils with diameters of 4-6 nm and lengths of several micrometres. The physicochemical properties of prepared nanofibrils were investigated using AFM, SEM, and FT-IR. The nanofibrils were loaded with inulin and added to toned milk to improve their functionality and palatability. pH, acidity, antioxidant activities, sensory evaluation, and *in-vitro* release analysis revealed that fortified toned milk had higher nutrient values and consumer acceptance than regular milk. In addition, the obtained result shows that higher loading is obtained in the lower concentration of inulin, due to the uniform loading of inulin in the fibrils without the formation of bundles, whereas high concentration of inulin leads to the formation of aggregates. The

shelf-life of toned milk, both before and after adding the nanofibrils, does not spoil for seven days at 4°C, which indicates the stability of the nanofibrils. Overall, we suggest that protein nano-fibrils can be used successfully as scaffold materials to create functional foods, thereby increasing the health benefits of various food products.

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7. Conflict of Interest

The authors report no conflicts of interest.

8. Author Contribution

Data collection, interpretation, drafting the article was done by PS. AN, PL and VS co-wrote and helped for the data analysis of nanofibril characterization. SS, SA and CP co-wrote and helped for the data analysis of nutrients part. RS and CP designed the study, co-wrote, supervised and approved for the submission.

9. Data Availability

The data that support the findings of this research are available from corresponding author upon request.

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Highlights

- Conventional methods for improving toned milk's quality often involve complex processes.
- This study introduces innovative methods to enhance its functionality in a simpler, more cost-effective manner
- Nanofibrils were prepared and used as scaffold to attach the active ingredients.
- Inulin was successfully loaded onto ovalbumin nanofibrils to improve functionality and nutrient values of the tonned milk.
- Similar bio-active ingredients can be combined to prepare novel functional foods that will find widespread use in the food industry.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: