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Enrichment of bovine milk-derived extracellular vesicles using surface-functionalized cellulose nanofibers

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ABSTRACT

The isolation of extracellular vesicles (EVs) from milk, a complex mixture of colloidal structures having a comparable size to EVs, is challenging. Although ultracentrifugation (UC) has been widely used for EV isolation, this has significant limitations, including a long processing time at high g-force conditions and large sample volume requirements. We introduced a new approach based on nature nanoentities cellulose nanofibers (CNFs) and short time and low g-force centrifugation to isolate EVs from various milk fractions. The flexible and entangled network of CNFs forms nanoporous, which entraps the EVs. Further, positively charged CNFs interact with anionic EVs through an electrostatic attraction, promoting their isolation with efficiency comparable with UC. The functionality and toxicity of isolated milk EVs were tested in Caco2 cells. Overall, the newly developed approach provides straightforward isolation and biocompatibility and preserves the natural properties of the isolated EVs, enabling further applications.

1. Introduction

Extracellular vesicles (EVs) are a heterogeneous group of membrane-bound particles discharged from cells that range in size from 30 to 1000 nm, with a nomenclature based on their origin and dimensions (van Niel et al., 2018). Exosomes are EVs with a size of 30–200 nm, produced by the interior budding of a late endosome lumen in the form of a multivesicular body (MVB) and secreted using fusion with a plasma membrane (van der Grein & Nolte-'t Hoen, 2014). Larger EVs produced by the budding of the plasma membrane having a diameter of 200–1000 nm are termed microvesicles. Apoptotic bodies, produced during apoptosis, have size in the range of 1–4 μm (Théry et al., 2009). Exosomes are involved in intercellular communication via transporting proteins, nucleic acids, and lipids from original cell to others (Maas et al., 2017). Thus, they contain RNA and are enriched with some specific proteins such as CD9, CD63, CD81, Hsc70, and TSG101, which differentiate them from other vesicles (Théry et al., 2009; Valadi et al., 2007).

Bovine milk provides abundant source of EVs potentially harnessed in various future therapeutic and diagnostic applications (Adriano et al., 2021; Kandimalla et al., 2021; Zhang et al., 2020). Bovine milk derived EVs withstand adverse environmental condition (Pieters et al., 2015; Rahman et al., 2019) and long-term storage (Adriano et al., 2021; Wijenayake et al., 2021). However, milk consists of a complex mixture of colloidal structures similar in size to EV, including milk fat globules (0.1–15 μm) and casein micelles (100–200 nm), making EV isolation challenging (Jukkola & Rojas, 2017; Somiya et al., 2018). These lipoprotein particles, non-EV proteins (serum albumin, lactoglobulin, immunoglobulins, etc.), and sugar can interfere with particle counts and protein evaluation (Adriano et al., 2021). Thus, the separation of EVs from soluble proteins and non-EV lipid particles is crucial for biomarker

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discovery and validation.

Although ultracentrifugation (UC) techniques have become widely used for EV isolation from various sources, the main drawbacks associated with this approach include long processing time at high g-force conditions and large volume requirements (Stam et al., 2021). These drawbacks have inspired the development of alternative approaches to improve the EV isolation process, purity, quality (intact morphology and molecular activity), and recovery efficiency (Liangsupree et al., 2021). For instance, approaches based on polymers (e.g., ExoQuick) (Wijenayake et al., 2021), isoelectric precipitation (Yamauchi et al., 2019), and size exclusion chromatography (Blans et al., 2017; Vaswani et al., 2017) have been investigated as potential UC substitutes. Although these approaches display some promising features, they often have insufficient EV purity or require demanding treatment protocols. Therefore, more straightforward processes that enable high-capacity isolation of EVs with higher quality are still required.

We introduced a novel methodology based on functionalized cellulose nanofibers (CNFs) and short time and low g-force centrifugation for enriching milk-derived EVs. CNFs are flexible, nanosized strands of biopolymeric cellulose chains composed of hundreds to thousands of repeating glucopyranose units containing three hydroxyl groups that can be easily chemically modified to perform various functions CNFs (Kontturi et al., 2018; Zhang et al., 2019). Cellulose nanomaterials have been used in various biomedical and biological systems, including tissue engineering and drug delivery (Hujaya et al., 2018; Hujaya et al., 2019). However, nanocellulose (NC) interactions with EVs or their use for the isolation of EVs have not been examined earlier. CNF naturally form nanoporous networks; therefore, it can potentially entrap nanosize EVs close to the CNF pore's size. Moreover, different chemistry interactions such as via hydrogen bonding, electrostatic forces, or other specific interactions might enhance the isolation efficiency. To verify this hypothesis, four different functionalized CNFs were synthesized from wood cellulose fibers, and their potential for enriching EVs from raw milk, skim milk, and whey fraction was proven. The isolated EVs are carefully characterized for morphology, total protein content, and proteomic profiles to evaluate the quality and specificity of the NC-based EV isolation approach. Furthermore, the biological functionality of isolated EVs were evaluated using the intestinal epithelial cell model (Caco2 cell).

2. Experimental section/methods

2.1. Materials and reagents

Choline chloride (>98.0 %) was obtained from TCI (Germany), and EDA, ethylene glycol, HCl, hydroxylamine hydrochloride, methanol, NaOH, PBS, sodium borohydride ($NaBH_4$), sodium chlorite ($NaClO_2$), sodium periodate ($NaIO_4$), and urea (99.0 %–100.5 %) were purchased from Sigma (Sigma-Aldrich, US & Germany) and used without further purification. Acetic acid was obtained from Merck (Germany). Commercial bleached birch kraft pulp and dissolving pulp (Domsjö Fabriker AB, Sweden) were used as cellulose raw materials. The properties of cellulose are presented elsewhere (Hujaya et al., 2018).

2.2. Preparation of various NCs

Four different CNF samples with various surface functionalities were fabricated using a mechano-chemical approach. Neutral, chemically nonfunctionalized CNFs (NCNFs) were prepared using a combined treatment of a DES system and high-pressure microfluidization (Sirviö et al., 2015). The NCNF was oxidized to DACNFs using sodium periodate, which were then converted to DCCNFs using sodium chlorite (Liimatainen et al., 2012). EDACNFs were in turn synthesized by reductive amination of DACNF. Fig. 1a shows the synthetic routes of the NCs. The details step of all different CNF synthesis is explained in Supporting Information section.

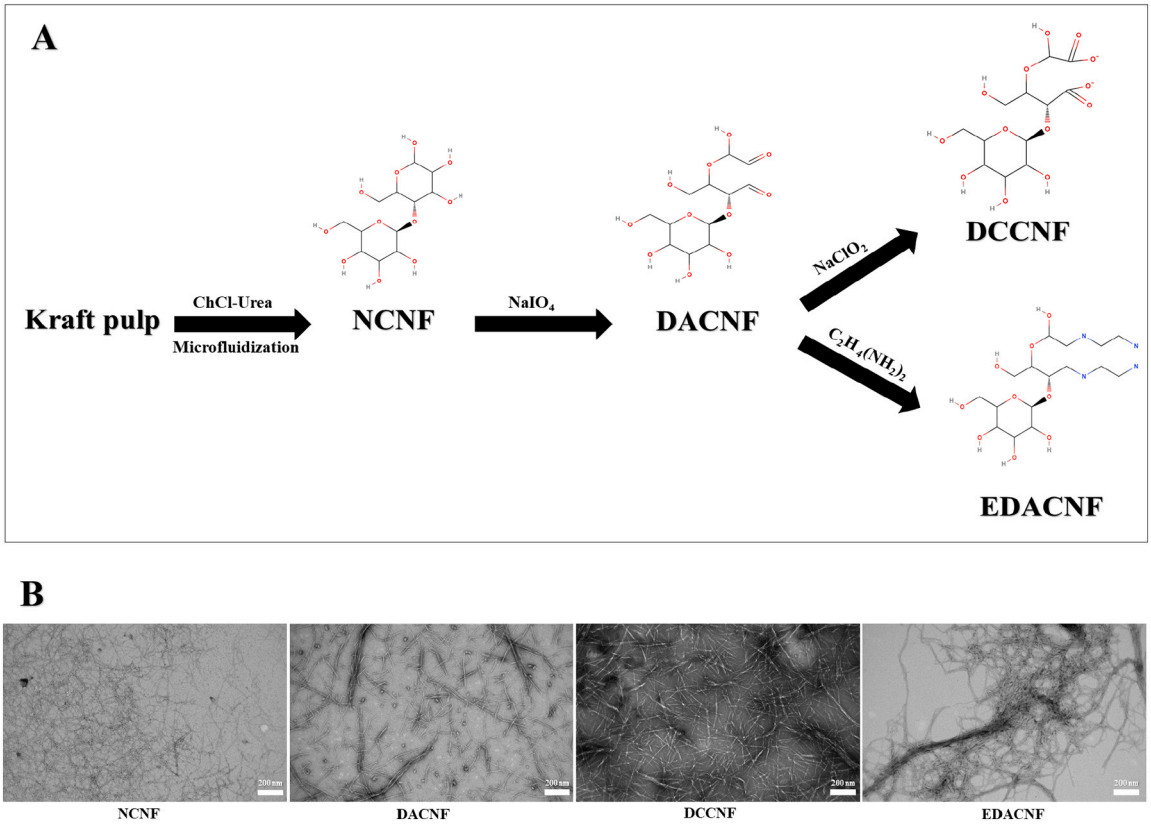


Fig. 1. (a) Synthesis steps for NCNF, DACNF, DCCNF, and EDACNF and (b) TEM images of CNF.

2.3. EV isolation and characterization methods

Different milk processing and acid treatment prior EV isolation were explained in in Supporting Information.

2.3.1. EV isolation using NCs (Scheme 1 left)

First, NC (0.2 %-wt aqueous suspension) was added to a milk specimen, and the sample was incubated for 1 h. The supernatant was discarded after spinning the solution at 10,000 g for 10 min. The pellet was washed several times with PBS before being repalletized at 10,000 g for 10 min. The EV-rich pellet was resuspended in PBS and stored at −20 °C for further investigation. To investigate the overall capabilities of various types of NCs to interact and enrich EVs, preisolated (acetic acid-treated) EV fractions of milk using UC were used.

2.3.2. EV isolation using UC (Scheme 1 right)

Both acetic acid-treated and untreated milk samples (obtained after precentrifuged at 10,000 g for 10 min at 4 °C and filtration with a 0.22 μm filter) were ultracentrifuged at 150,000 g for 90 min using a Sorvall™ WX+ Ultracentrifuge with rotor type TH-641 (Thermo Scientific, USA). After washing with PBS, the EV pellet was ultracentrifuged at 150,000 g for 90 min. The EV-rich pellet was resuspended in 1 mL PBS and stored at −20 °C for further analysis.

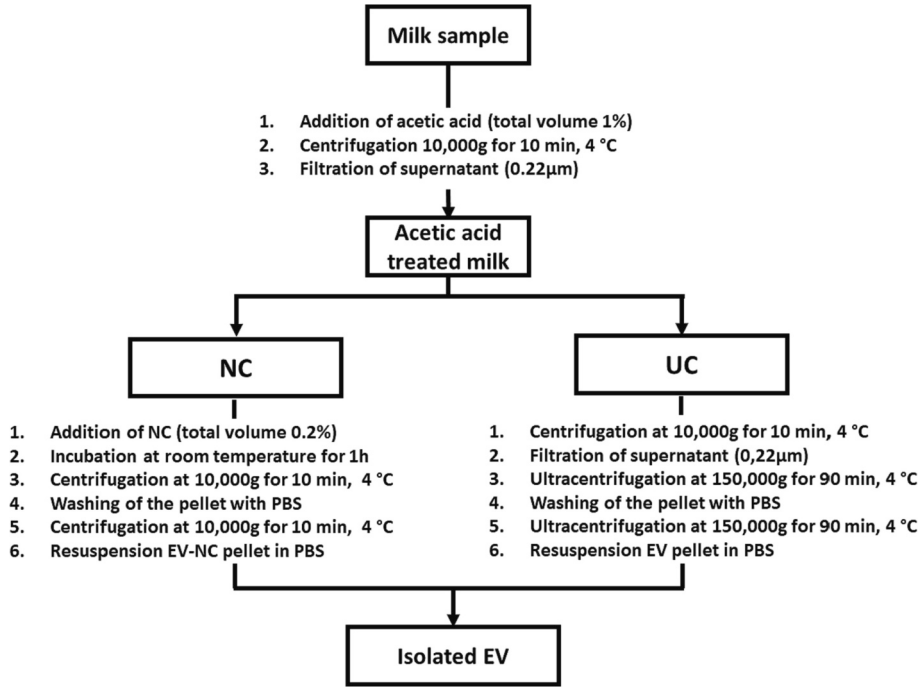
2.4. Proteome analysis

Stained gels containing equal numbers of proteins were sliced, washed with MilliQ water, and in-gel digested. MS analysis was conducted at Turku Proteomics Facility, Finland. Digested peptide samples were dissolved in 45 μL of 0.1 % formic acid, with 5 μL of each sample submitted to LC-ESI-MS/MS analysis. One wash run was submitted between each sample to minimize the potential carry-over of peptides. The LC-ESI-MS/MS analyses were conducted on a nanoflow HPLC system (Easy-nLC1000, Thermo Fisher Scientific, Germany) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a nanoelectrospray ionization source. Peptides were loaded in a trapping column before being separated in line on a 15 cm C18 column (75 μm × 15 cm, ReproSil-Pur 5 μm 200 Å C18-AQ, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The mobile phase was either water with 0.1 % formic acid (solvent A) or acetonitrile/water (80:20 (v/v)) with 0.1 % formic acid (solvent B). A linear 60 min gradient from 6 % to 39 % B, followed by a wash with 100 % B, was used to elute peptides. MS data were acquired automatically using Thermo Xcalibur 3.1 software (Thermo Fisher Scientific, Germany). An information-dependent acquisition approach included an Orbitrap MS Survey scan in the mass range of 300–2000 *m/z*, followed by HCD fragmentation for the 10 most intense peptide ions. In further, the detailed procedure for protein identification and software information is explained in detail in supporting information.

3. Results and discussion

3.1. Synthesis different functionalization CNF

In the present study, we designed a quick and simple approach for EV isolation from various milk fractions, i.e., raw milk, skim milk, and whey fraction using CNFs synthesized from wood pulp. As a reference approach, UC was harnessed for the EV isolation. Four different types of CNFs with varied surface functionalities were fabricated, as shown schematically in Fig. 1a, and the details of their synthesis are described in the Experimental Section and Supporting Information. The neutral, nonfunctionalized CNFs (NCNFs, with intact cellulose structure) were produced using deep eutectic solvent (DES) according to a previously published approach (Sirviö et al., 2015). NCNF is later oxidized with aqueous sodium periodate to produce highly reactive dialdehyde CNFs (DACNFs). The DACNF was subsequently oxidized to anionic dicarboxylic acid CNFs (DCCNFs). Furthermore, a positively charged NC (ethylenediamine CNF [EDACNF]) was obtained from DACNF by conjugating with ethylenediamine (EDA). These four CNFs possessing different surface chemistries (but similar elongated nanofibrous morphology) were used to understand the impact of cellulose surface chemistry on CNF–EV interactions and CNF efficiency in EV isolation. All the CNFs had typical nanoscale dimensions (diameter ranging from a few nanometers to tens of nanometers) and flexible and fibrillar structures (Fig. 1b) (Kontturi et al., 2018). A successful synthesis of individual nanocellulose species was approved by TEM imaging.



Scheme 1. Workflow for EV isolation based on the ultracentrifugation and nanocellulose.

Nanofilaments with almost uniform structure and cylindrical shape can be observed. An aggregation was indicated in EDACNF more than in other CNFs because of strong hydrogen bonds between the amine and hydroxyl group.

3.2. Separation of preisolated milk EVs using various CNF

A standard EV sample (preisolated milk EVs diluted with phosphate-buffered saline [PBS]) was used to address the ability of the different NCs to bind EVs and facilitate EV isolation during short-term and low g-force centrifugation. The aqueous CNF suspension was added to an EV containing fluid (CNF dry matter content 0.20 % of total volume), properly mixed, and incubated for 1 h before centrifugation at 10,000 rpm for 10 min. Preisolated raw milk EVs in PBS with no additional NC but treated with the same protocol were used as a control sample (PBS only). After the separation process, all used CNFs formed pellets under spinning, whereas the control samples remained suspended. Presumably, the nanopores structures formed of a network of CNFs (Fig. S1) entrapped the EVs partly using size exclusion and, due to the high density of CNFs, turned them into pellets during centrifugation. In contrast, other smaller contaminants stay in the supernatant.

To visualize the entrapped EVs, the EV milk samples were labeled with lipophilic carbocyanine DiOC18(3) dye (DiO) and imaged using confocal microscopy (Fig. 2a). The green fluorescence was detected in abundance in EV samples treated with EDACNFs. Since the DiO are lipid-based dyes, they can only effectively bind and generate high fluorescence to lipid-like structures; when CNF is mixed with only DiO without EV, no fluorescence is observed (last image in Fig. 2a). We used Qubit protein assay to examine total proteins and sulfovanilin assay to quantify the lipid of resuspended pellets obtained from different CNF, PBS, and UC technique to evaluate the quality of isolated EV (Jang et al., 2019) (Fig. 2b). The quantity of isolated EV (both lipid and protein number) using UC is higher than EDACNF but the purity between these two methods is not significantly different (according to Tukey post hoc test with $p < 0.05$ in Fig. S2a). This is probably because the ultrahigh centrifugal force and very long processing time of UC, which is more favorable for isolating EVs in a broader size range while NC-based isolation only entraps EV having a size that similar the nanofibrous nanopores (Ziaei et al., 2018), which is also supported by more uniform size of EV (Fig. S3).

Among all CNFs (Fig. 2b), the EDACNF has the highest ratio of lipid to protein, implying that the surface functionality of CNFs plays a critical role in EV separation. The DACNF and NFCNF ratio values are similar, that is, 0.144 and 0.129, respectively, roughly fourth fold higher

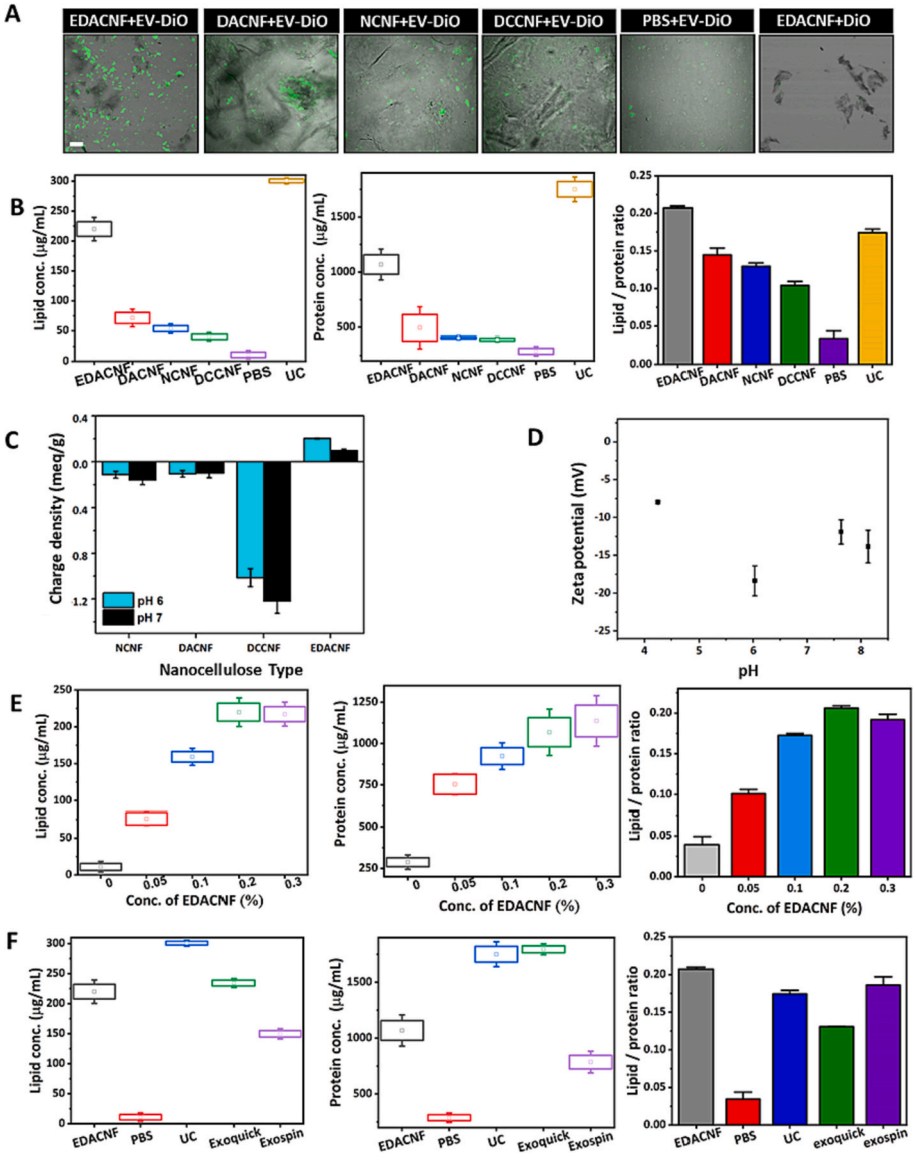


Fig. 2. (a) confocal microscopy images (scale bar: 50 μm) and (b) lipid, protein, and ratio between lipid and protein concentration of isolated milk EVs using various nanocelluloses at a constant dose of 0.2 %, (c) the charge density of various nanocelluloses at pH 6 and 7, (d) the zeta potential of EVs for various pH, (e) the effect of the EDACNF concentration on the lipid, protein, and ratio between lipid and protein concentration of isolated milk EV, and (f) comparison of isolated milk EV using NC to other methods such as UC, exoquick, and exospin. ($N = 4$, mean ± standard deviation).

than the PBS (0.030). The lipid/protein ratio value of DCCNF (0.103) shows the lowest among all CNF yet is still threefold higher than PBS may indicate the morphology of CNF, in general, is favorable for EV isolation.

To understand the isolation mechanism of EVs promoted by CNFs, we first measured the charge density of NCs at pH 6 and 7 and the zeta potentials of standardized EVs (Fig. 2c and d, respectively). The NCNF, DACNF, and DCCNF displayed a net negative surface charge (Liangsupree et al., 2021; Midekessa et al., 2020), while the EDACNF was positively charged. The EV in turn had a negative zeta potential throughout the pH range under study. Therefore, the electrostatic attraction between EVs and EDACNF enhanced EV precipitation during the incubation and centrifugation, explaining the best recovery efficiencies among other CNFs (Deregibus et al., 2016). DCCNF's negative surface charge in turn induced electrostatic repulsion with EVs, resulting in the poorest recovery efficiency.

The pore size/structure is another important factor defining the isolation efficiency. Even though nanofibers do not have internal pores, they form a continuous network with open pores, in which the dimensions of pores depending on nanofiber diameter and nanofiber concentration. This structure contains both pores with a typical diameter of 10–20 nm, but also larger holes having dimensions in sub micrometers-scale (Fig. S1). It is supposed that these nanopores can entrap efficiently EVs which have their dimensions <200 nm (Hujaya et al., 2019; Ukkola et al., 2021). We have presented in Figs. 2e and S2b the influence of EDACNF concentration on EV isolation efficiency. This data indicates that the protein and lipid recovery significantly increase as the pore structure decreases when the CNF network is denser (concentration from 0.05 % up to 0.20 %).

In addition, EV isolation using NC (EDACNF) was compared with other methods such as UC, Exoquick, and Exospin in Figs. 2f and S2c. It shows that Exoquick has a higher protein yield than the NC technique (EDACNF). However, the purity (lipid/protein ratio) of the Exoquick method is significantly lower than obtained with our technique. Exospin has a comparable purity level with EDACNF methods. Moreover, the EV size distribution of both Exoquick and Exospin is more heterogenous, as shown in NTA data (Fig. S3). In the following section, isolation EV from various milk using EDACNF based NC will be referred as "NC technique".

3.3. Characterization of EVs isolated from different milk sources using NC in comparison to UC

We used bovine milk as a sample to evaluate the applicability of NC for EV isolation from complex biofluid. Three different kinds of bovine milk specimens were used. Raw milk was unprocessed, fresh from the cow, and had a significant level of fat. Skim milk was defatted raw milk, with only 0.1 % of fat, and whey was the liquid remaining from further processed milk, mainly containing milk proteins. Prior UC and NC isolation approaches, we removed soluble non-EV proteins, fats, and other contaminants from milk samples using the previously reported acetic acid precipitation (Yamauchi et al., 2019). Since the pH of the isoelectric point of caseins is low, they can be precipitated under acidic conditions and using low speed centrifugation (Rahman et al., 2019). The supernatant was further used for EV isolation using either the UC or NC approach.

Transmission electron microscopy (TEM) imaging was used to visualize the EVs isolated from milk fraction (Fig. 3). The isolated EVs using NC displayed typical morphology of donut/cup-shaped particles with an intact membrane having diameters ranging from 30 to 120 nm, therefore, indicating exosomes (Théry et al., 2002). A similar EV size range was evidenced with the UC approach, indicating the feasibility and effectiveness of exosome isolation using NC. Furthermore, EVs obtained using the NC approach were more homogeneous in size than the UC approaches (Fig. S3).

The protein yield of isolated milk fractions from both enrichment approaches was also determined. A decrease in total protein yields with acetic acid precipitation and UC/NC isolation implies that some non-EV proteins precipitated during acid pretreatment were removed by washing (Table 1) (Somiya et al., 2018). The quantity of protein and lipid EV isolated from the same milk source is not significantly different using UC and NC methods. The ratio between lipid and protein of EV isolation for the NC approach with whey, raw milk, and skim milk was 0.227, 0.205, and 0.175, respectively. Thus, the EV purity using both techniques is similar. Because of the lower content of interfering non-EV components, the whey fraction had the highest quantity and purity isolated EV among other milk sources.

Cross-comparison of EV protein distributions of three different milk fractions obtained from UC and NC-based isolation was made with 10 %

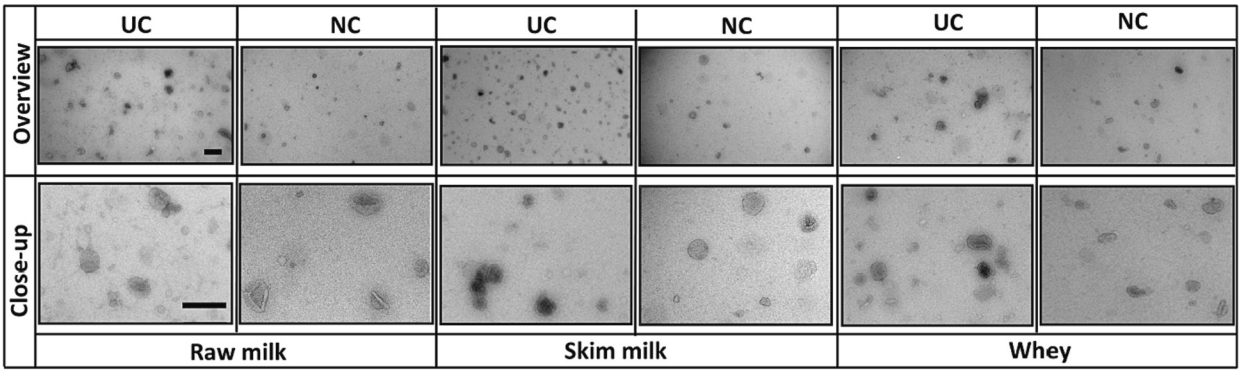


Fig. 3. TEM images of milk EVs isolated using UC and NC (scale bar: 200 nm).

Table 1
Protein concentration and yield of various milk samples after UC and NC treatment.

Milk sample	Protein concentration (μg/mL)		Protein yield (μg/mL)	
	Untreated sample	Acetic acid treated sample	UC method	NC method
Raw milk	4707.5 ± 68.1	4701.7 ± 91.8	346.0 ± 66.2	299.8 ± 107.8
Skim milk	4920.3 ± 40.0	4171.7 ± 11.3	239.9 ± 43.6	188.2 ± 38.1
Whey	4890.4 ± 107.8	4563.6 ± 59.8	858.7 ± 25.7	840.2 ± 27.8

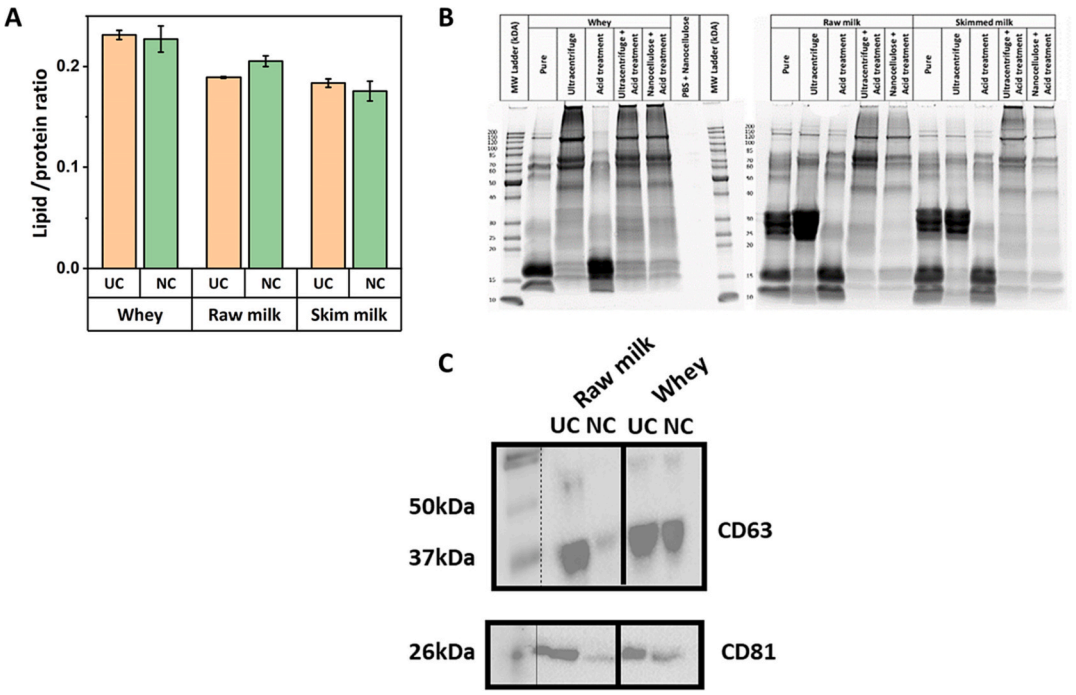


Fig. 4. (a) Ratio between lipid and protein concentration of isolated EV from the various milk fractions using UC and NC ($N = 4$, mean ± standard deviation). (b) SDS-PAGE stained with SYPRO for raw, skim, and whey fractions of milk samples and isolated EV. (c) Western blot of EV isolated from raw milk and whey using UC and NC

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (Fig. 4b). Furthermore, SDS-PAGE revealed that EDACNF did not exhibit any detectable background proteins. In cow's milk, the most abundant proteins are caseins (α-S1-, α-S2-, β-, and κ-forms), representing approximately 78 % of the total protein concentration, followed by whey proteins that make up 17 % (β-lactoglobulin, α-lactalbumin, lactoferrin, and lactoperoxidase) (Vincent et al., 2016). Despite the low resolution, it was evident that protein concentrations in several bands of isolated EVs were significantly reduced, both with UC and NC approaches such as in the 15–17 kDa (immunoglobulin and α-lactalbumin) for all milk and 20–30 kDa (casein) and 50–70 kDa (butyrophilin, lactadherin, and lactotransferrin) for raw and skim milk (Samuel et al., 2017). Western blotting was conducted with antibodies against CD81 and CD63 (Fig. 4c) to determine the presence of EV protein marker in milk fractions' EV obtained from UC and NC. These results reveal that EVs isolated using both UC and NC contain the exosome membrane markers CD63 and CD81 (Reinhardt et al., 2012).

3.4. Proteomics analysis of EVs isolated from different milk sources

The details of EV protein composition in NC and UC isolated EV fractions of various milk samples were further investigated using mass spectrometry (MS). Since EDACNF did not exhibit any detectable background proteins, the NC containing EV fractions can be used directly for proteomic analysis of EVs without having to elute the EV from NC. Fig. 5 shows that the majority of proteins in EVs recovered using NC from the three milk samples were identical to those isolated with UC ($N_{raw\ milk}(UC \cap NC) = 152; N_{skim\ milk}(UC \cap NC) = 190; N_{whey}(UC \cap NC) = 224$).

EVs isolated from raw and skim milk using both approaches were free of the apoptotic body markers as indicated by the absence of calreticulin, GRP78, histone, and other organelle biomarkers (Reinhardt et al., 2012; Wickman et al., 2013); only whey specimen contained calreticulin (Table 2). Since there is an overlap in size of exosomes and other microvesicles, it was expected that neither approach could fully separate them (Zempleni et al., 2016). Surprisingly, there was very low contamination of other multivesicular bodies caused by plasma membrane blebbing as confirmed by the absence of surface markers of integrin-β1, p-selectin, CD40 (Munagala et al., 2016), and endoplasmic reticulum (ER) marker calnexin (Baietti et al., 2012) in all milk sources with both approaches.

Milk EV isolates were enriched in CD9, CD63, and CD81 (Table 2)

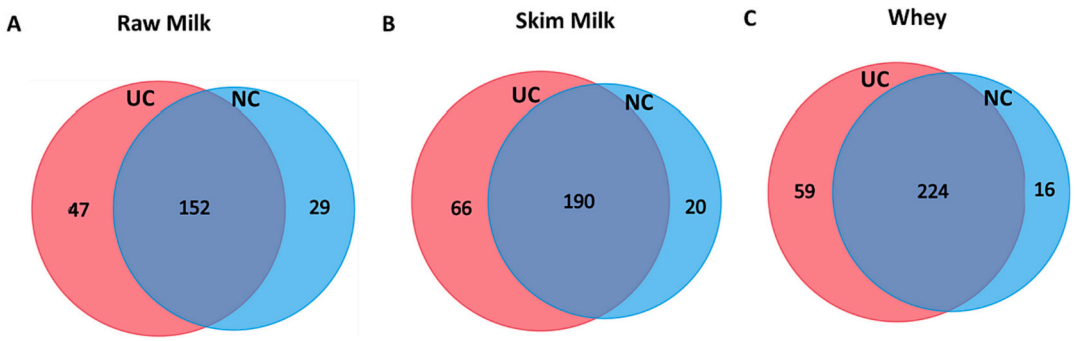


Fig. 5. Venn diagrams of EV proteins based on MS in (a) raw, (b) skim, and (c) whey milk fractions subjected to UC and NC treatments.

Table 2
Proteomics analysis of milk EVs isolated using ultracentrifugation (UC) and nanocellulose (NC) treatments. The occurrence of marker in sample is marked by (+) and absence by (−).

Exosome markers	Raw milk		Skim milk		Whey	
	UC	NC	UC	NC	UC	NC
CD9	+	+	+	+	+	+
CD63	−	+	+	+	+	+
CD81	+	−	+	+	+	+
Hsp70	+	+	+	+	+	+
HSP90β	+	+	+	+	+	+
HSC70	+	+	+	+	+	+
14-3-3	+	+	+	+	+	+
CD-47	−	+	−	−	−	+

MV and ER markers	Raw milk		Skim milk		Whey	
	UC	NC	UC	NC	UC	NC
Integrin-β1	–	–	–	–	–	–
p-Selectin	–	–	–	–	–	–
CD40	–	–	–	–	–	–
Calnexin	–	–	–	–	–	–

Apoptotic body markers	Raw milk		Skim milk		Whey	
	UC	NC	UC	NC	UC	NC
Calreticulin	–	–	–	–	+	+
GRP78	–	–	–	–	–	–
Histone	–	–	–	–	–	–

compared with minimal levels of the EV marker proteins in other suitable milk fractions like milk fat globules and other vesicles (Melnik et al., 2016; Sanwlani et al., 2020). Thus, tetraspanins on the surface of EV can distinguish them from other vesicles like apoptotic bodies, other microvesicles from the cell membrane budding, and ER (Raposo & Stoorvogel, 2013). For skim and whey milk, all these proteins were found in both approaches. However, CD63 and CD81 were missing from isolated whey milk using UC and raw milk using NC, respectively. Exosomes in most milk sources contain cytoplasmic proteins like molecular chaperon/heat shock protein Hsp70 and endosomal sorting complex required for transport-associated proteins HSC70 and HSP90β (Pieters et al., 2015).The EV proteome can reflect other functions of the secretory cells and mechanisms that affect immune cells in milk. CD-47, which is related to the "don't eat me" signal, was discovered in EV isolated from whey and raw milk using NC and UC, respectively.

3.5. Functionality and biocompatibility of isolated milk EVs

Functionality and biocompatibility of raw milk EVs isolated with UC and NC were examined using cellular uptake and cytotoxicity in Caco2 cell model. The Caco2 cell culture system represents a well-characterized in vitro system that closely mimics drug and nutrient absorption and transport processes across the human intestinal epithelium. The NCs containing milk's EVs were sonicated prior to incubation with the cell using a sonication bath at 37 kHz and 50 % power at 4 °C for 1 h to stimulate EVs release from CNF without destroying the structure of EV (Fig. 6a). Fluorescence microscopy images and fluorescence intensity quantification revealed that the uptake of both EVs in Caco2 cells increased linearly for a specific time interval of 30 min–8 h and reached saturation afterward (Fig. 6a). Furthermore, the uptake of NC isolated EVs was slightly slower than that of UC isolated EVs,

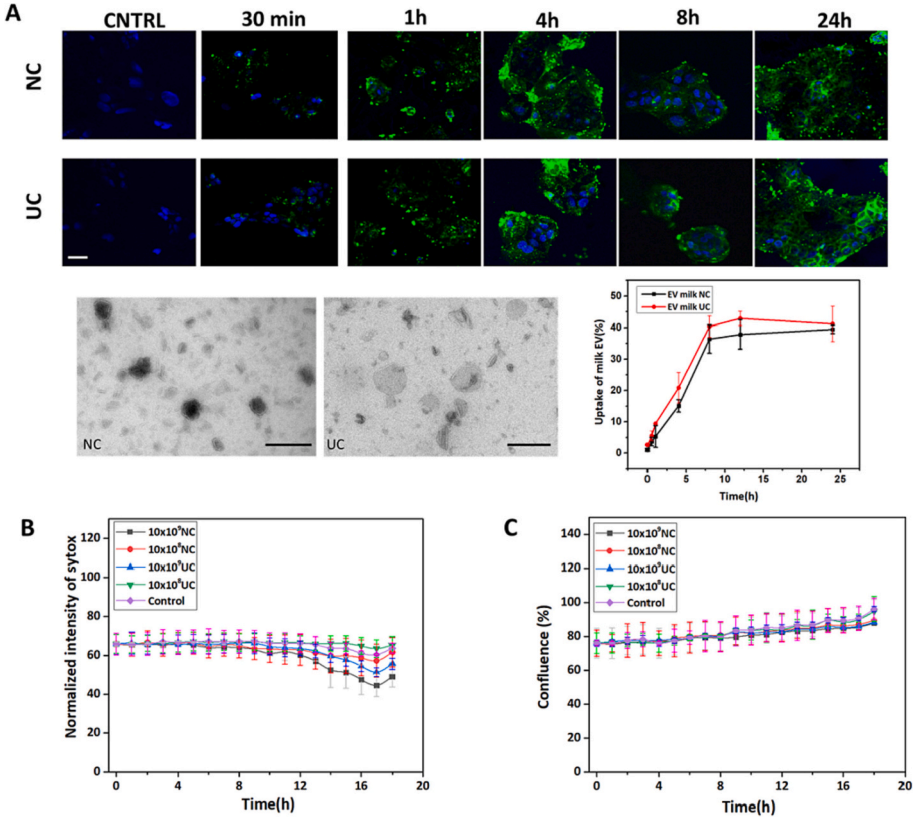


Fig. 6. (a) Visualization and quantification of time-dependent uptake of milk EVs in Caco2 cells (blue: DAPI staining nucleus, green: milk EV). Scale bar: 40 μm. Cytotoxicity of EVs in Caco2 cells for 24 h in vitro analyzed based on (b) green intensity value of SYTOX® Green and (c) cell confluence ($N = 3$, mean ± standard deviation).

Table 3
Comparison of advantages and disadvantages of ultracentrifugation and nanocellulose-based EV separation approaches.

	Advantages	Disadvantages
Ultracentrifugation	• No additional reagent is required • Commonly used approaches	• Lengthy operation time (3–18 h) • Require high-volume of sample • High instrument cost • High g-forces lead to co-purification of contaminants and EV damage • Prone to water-soluble proteins contamination
Nanocellulose	• Short treatment time (<2 h) • Use regular centrifuges with low g-forces • Possible to use with starting low sample volume • Cheap and inert materials • Possible to directly be used for further application without EV elution	• Could not separate subpopulations of EV • Only entrap EV in the size range of nanopores

perhaps because of the slow release of EVs from the NC.

The cytotoxicity of UC and NC isolated raw milk EVs in Caco2 cells was investigated by incorporating SYTOX™ Green dead cell stain and monitoring the fluorescence intensity over time. SYTOX® Green is a high-affinity nucleic acid stain that quickly penetrates cells with damaged plasma membranes but does not permeate the membranes of living cells. Thus, the green fluorescence intensity equals the number of dead cells. Even at the highest EV concentration, the percentage viability of cells treated with EV either the fluorescence intensity or confluence of cell percentage value remained close to that of the control cell (Fig. 6b and c) up to 18 h. We conclude that EVs do not show cytotoxicity and do not affect cell growth in vitro despite the presence of NC.

3.6. Comparison of the EV isolation methods

UC is one of the most used isolation approaches for EVs. The UC approach is based on the differences in physical properties between exosomes and other constituents, such as density, size, and shape, and it typically consists of a series of long centrifugation cycles at high rotational speeds and washing steps to remove cells, cell debris, and organelles and then pelleting exosomes from supernatants (Veerman et al., 2021). Thus, the process is time consuming and requiring high-performance instruments. UC showed a greater number of different proteins associated with exosomes in the current study, but it also has the risk of introducing other water-soluble proteins and other types of contamination (Niu et al., 2017; Ziaei et al., 2018). Repeated centrifugation can minimize the number of contaminants coisolated with the EVs but may also decrease particle yield because of lost and damaged EVs (Vaswani et al., 2017). Furthermore, the ultrahigh centrifugal force (100,000–200,000 g) can cause EV's aggregation and fusion, increase the size of captured EV, damage the captured EV, and lead to inaccuracy in downstream analyses (Chen et al., 2020).

Elongated and surface-functionalized CNFs were harnessed as a straightforward and quick approach to enhance EV isolation and resulted in EV yield comparable with that of UC. The NC approach significantly shortened the required processing time (total treatment time < 2 h), and the good isolation efficiency was attained with low centrifugal force (10,000*g*) using a regular centrifugation instrument. The isolated EVs could directly be used for proteomic analysis without the EV elution step from the NC. Furthermore, this approach is biocompatible and preserves EV's molecular function (see Table 3).

4. Conclusions

We have developed simple approach based on functionalized CNFs and short-term and low g-force centrifugation to isolate milk-derived EVs. In this study, four different functionalized CNFs were synthesized from wood cellulose fibers, and their potential for the enrichment of EVs from raw milk, skim milk, and whey fractions were demonstrated. The efficiency of isolated EVs from complex biological matrices using a combination of positively charged NC, low speed, and short-time centrifuge is comparable with that of long-time ultracentrifuge approaches. Furthermore, the biological functionality of isolated milk EVs using this technique remains preserved; thus, these isolated EVs can be potentially used for a diagnostic and drug delivery system.

CRediT authorship contribution statement

Jonne Ukkola: Conceptualization, Methodology, Writing **Feby Wijaya Pratiwi**: Conceptualization, Methodology, Writing- Reviewing, **Santeri Kankaanpää**: Methodology, Writing, **Seyedamirhosein Abdorahimzadeh**: Methodology, Writing, **Mohammad KarzarJeddi**: Methodology, Writing- Reviewing, **Prateek Singh**: Methodology, Writing, **Artem Zhyvolozhnyi**: Methodology, Writing, **Olha Makieieva**: Methodology, **Sirja Viitala** Supervision, Methodology, Writing- Reviewing, **Anatoliy Samoylenko**: Methodology, **Hely Haggman**: Supervision, Reviewing, **Seppo J. Vainio**: Conceptualization, Supervision, Methodology, Reviewing, **Caglar Elbuken**: Supervision, Reviewing, **Henrikki Liimatainen**: Conceptualization, Supervision, Methodology, Writing-Reviewing.

Author contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

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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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.120069.

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