Carboxymethylated Cellulose Nanofibrils with Controlled Substitution: Physicochemical Properties and *In Vitro* Biosafety Assessment

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Cellulose nanofibrils (CNF) are increasingly explored for biomedical applications; however, the relationship between surface functionalization and biological responses remains incompletely understood. In this study, CNF were carboxymethylated to controlled degrees of substitution (DS) using kraft pulp as a starting material, and their physicochemical properties and *in vitro* biosafety were evaluated. Increasing DS altered crystallinity and surface charge, resulting in measurable changes in zeta potential and structural characteristics. Cell viability was assessed in HepG2, HEK293, and RAW 264.7 cells across a concentration range of 100 to 1000 µg/mL using complementary assays. All tested materials exhibited concentration-dependent trends in cell viability. However, under the tested conditions, the observed effects remained within the non-cytotoxic range. Among the tested samples, CM CNF with DS 0.2 showed stable cell viability and limited apoptosis-related responses comparable to those of hyaluronic acid (HA), while samples with lower or higher substitution levels showed modest reductions in viability at higher concentrations. Apoptosis-related gene and protein expression analyses further indicated limited transcriptional and translational changes relative to the vehicle-treated control. Overall, the findings suggest that the degree of carboxymethylation influences cell–material interactions in a concentration-dependent manner, while maintaining biosafety within the tested DS range.

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INTRODUCTION

Soft tissue fillers are widely used for wrinkle correction and facial contouring, among which hyaluronic acid (HA)–based products have become the most common option in current clinical practice (Kruglikov and Wollina 2015; Hong *et al.* 2024; Kyriazidis *et al.* 2024). However, diverse reports have indicated that HA fillers can induce various adverse reactions, including inflammation, edema, injection-site pain, and, in rare cases, hypersensitivity responses or even vascular complications (Taylor *et al.* 2009; Shi *et al.* 2016; Fino *et al.* 2019). Consequently, there is a growing demand for safer alternatives

with superior biocompatibility.

Cellulose is a carbohydrate composed of anhydroglucose units linked by β-1,4 glycosidic bond that form parallel crystalline structures through inter- and intramolecular hydrogen bond (Altaner *et al.* 2014; Etale *et al.* 2023). Cellulose derivatives such as carboxymethyl cellulose (CMC) offer advantages in terms of viscoelastic modulation and improved injectability, but their potential cytotoxicity and ability to provoke inflammatory responses have not yet been systematically validated (Varma *et al.* 2014; Leonardis and Panange 2015). In addition to such chemically modified cellulose derivatives, cellulose nanofibrils (CNF) have recently attracted considerable attention as an emerging class of advanced cellulose-based materials. CNF are obtained by disintegrating native cellulose into the nanoscale through mechanical, enzymatic, or chemical processing (Im *et al.* 2018a; Choi *et al.* 2025). Owing to their high mechanical strength, low density, excellent film-forming capability, and tunable surface chemistry, CNF have emerged as promising next-generation bio-based materials across diverse industries, including packaging, paper and printing, composites, electronics, construction, cosmetics, and pharmaceuticals. In particular, numerous studies have demonstrated the potential of CNF-based hydrogels and scaffolds in skin tissue engineering and wound healing, suggesting further opportunities for its application in soft-tissue fillers and skin-contact medical device materials (Zhang *et al.* 2022; Datta *et al.* 2025; Mohan *et al.* 2025).

Although CNF is also generally regarded as a non-toxic material, its widespread commercialization has gained momentum only within the past decade, and the toxicological and immunological data accumulated thus far have shown inconsistent results. Several *in-vitro* studies have reported that CNF do not induce significant cytotoxicity or inflammatory responses across various cell lines (Nordli *et al.* 2016; Lopes *et al.* 2017); however, other studies have demonstrated that, under certain conditions, CNF can elicit oxidative stress, increased secretion of pro-inflammatory cytokines, genotoxic effects, and inflammatory reactions in lung tissue (Menas *et al.* 2017; Ede *et al.* 2019; Kujita and Moriyama 2025).

These discrepancies are believed to result from variations in physicochemical properties, including raw material sources, manufacturing processes, fiber length and diameter, crystallinity, surface charge, and degree of functionalization. As a result, there is a growing consensus that the biological safety of CNF must be evaluated individually for each material and application scenario (Ede *et al.* 2019; Yamashita *et al.* 2024). In particular, the ionic properties of CNF play a crucial role not only in determining their colloidal stability but also in governing their interactions with cells, the formation of protein coronas, cellular internalization, and the activation patterns of immune cells (Fleischer and Payne 2012; Nordli *et al.* 2016; Arezki *et al.* 2022). Surface modification by introducing negatively charged functional groups, such as through carboxymethylation, can lead to changes in the size and morphology of CNF particles, consequently affecting their electrostatic interactions with cell membranes and the propensity for phagocytosis (Im *et al.* 2018b; Aimonen *et al.* 2022).

These observations imply that the degree of carboxymethylation is important in determining the biological safety of CNF. To date, most studies reported have evaluated CNF with a single or limited range of DS values, typically using only one or a few cell lines. Systematic comparisons of CNF with different DS across multiple human- and animal-derived cell types that assess cytotoxicity, inflammatory responses, and apoptosis in parallel remain insufficient. Few studies have compared quantitatively the relative safety of CNF with clinically or industrially relevant reference materials, such as HA and CMC.

To advance CNF as candidate materials for soft-tissue fillers and skin-contact biomedical applications, it is essential to determine how different levels of surface carboxymethylation influence their safety profiles across diverse human organ-derived cells, and to establish how their biosafety benchmarks against existing commercial materials.

In this study, CNF with different DS were prepared from kraft pulp, and their biological responses were systematically evaluated in multiple human-and animal-derived cell lines. By comprehensively analyzing cell viability, pro-inflammatory cytokine expression, and apoptosis-related indicators, this study examined the optimal DS range for CNF and establish their potential biosafety as alternative soft-tissue filler materials.

EXPERIMENTAL

Materials

To investigate the effect of degree of substitution (DS) in carboxymethylated CNF on human-derived cell lines, never-dried bleached eucalyptus kraft pulp was used as a raw material, which was supplied by Moorim P&P, Korea. For the carboxymethylation reaction, monochloroacetic acid (MCA, 99.0%, Sigma-Aldrich), sodium hydroxide (98.0%, Samchun Chemical), and isopropanol (99.5%, Duksan Reagents) were used without additional purification. Hyaluronic acid (HA, Average M_w 125 kDa) and carboxymethyl cellulose (CMC, Average M_w 250 kDa, DS 0.7), which were used as reference materials for carboxymethylated CNFs, were purchased from Merck (Korea). To evaluate the cytotoxic effects of carboxymethylated CNF with different DS, as well as CMC and HA, human liver-derived cell line (HepG2), human embryonic kidney cell line (HEK293), and a mouse macrophage cell line (RAW 264.7) were obtained from the American Type Culture Collection (ATCC, USA) and used in this study. For cytotoxicity assays, fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco/BRL (USA). Trypsin–EDTA (0.25%) was purchased from Thermo Fisher Scientific (USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Sigma-Aldrich Co. (USA), and plastic cell culture ware, including 96-well plates, was purchased from Nunc Inc. (USA).

Preparation of Carboxymethylated CNF

Carboxymethylation was performed following the procedure described by Im *et al.* (2018a). Briefly, 30 g of pulp fiber was alkalized by dispersing in 1,500 mL of isopropanol (IPA) containing NaOH for 30 min at 35 °C for alkalization, Subsequently, MCA was introduced into the reaction system for esterification, which was conducted for 60 min at 65 °C. During the carboxymethylation, the amounts of NaOH and MCA were adjusted to control DS of carboxyl groups. After finishing the reaction, pre-treated pulp was washed with distilled water until the pH was neutral.

The DS of carboxymethylated (CM) pulps was determined by 1H NMR spectroscopy (AVANCE III, Bruker, Germany). CM pulps prepared under different reaction conditions were hydrolyzed with sulfuric acid, followed by secondary hydrolysis in D_2O. After filtration, the hydrolysates were analyzed by 1H NMR, and the spectra were processed using TopSpin software. The DS was calculated by integrating the anomeric and carboxyl-related proton signals according to a previously reported method (Cao *et al.* 2021). The resulting DS values and corresponding reaction conditions are summarized in Table 1.

Table 1. Reaction Conditions for Carboxymethylation as a Pre-treatment

Classification	Pulp fiber (g)	NaOH (g)	MCA (g)	IPA (mL)	Degree of substitution (DS)
CM CNF_0.1	30	2.22	2.17	1,500	0.1
CM CNF_0.2	30	13.33	13.00	1,500	0.2

A high-pressure homogenizer (GEA, Niro Soavi Panda Plus, Italy) was used to prepare CM CNF with different DS. The pre-treated pulp suspensions with different DS were passed through the homogenizer seven times at a pressure of 800 bar. After nano-fibrillation, CM CNF were diluted using distilled water for cytotoxicity assay to 100, 400, 800, and 1000 µg/mL.

The morphological properties of the CM CNF depending on DS were investigated using a field emission scanning electron microscope (FE-SEM, Carl Zeiss, Sigma, UK). An X-ray diffractometer with a Cu K α X-ray source (XRD, Bruker, D8 Advance, Germany) was used to evaluate the effect of the crystallinity of CM CNF with different DS and CMC on cytotoxicity. The crystallinity index was estimated using the Segal method, calculated from the height ratio between the crystalline peak intensity (I_{200} - I_{am}) and the total intensity (I_{200}), as described in Eq. 1.

$$Crystallinity\ (\%) = ((I_{200} - I_{AM})/ I_{200})\ x\ 100 \quad (1)$$

The zeta potentials of CM treated samples, including CNF and CMC, were also evaluated using Zetasizer (Nano Zs, Malvern Instruments, Ltd) at 25 °C.

Cell Culture

The cytotoxicity of CM CNF with different DS were evaluated using human- and mouse-derived cell lines. All cell lines were cultured in Dulbecco's modified eagle's medium (DMEM), a widely used cell culture medium containing essential nutrients such as glucose, amino acids, vitamins, and inorganic salts required for cell growth and proliferation (Schultheiss *et al.* 2013). To support cell attachment, growth, and survival, the culture medium was supplemented with 10% fetal bovine serum (FBS, Gibco BRL, USA). FBS is derived from fetal calf blood and is rich in growth factors, hormones, and proteins that promote cellular proliferation and viability. In addition, 1% antibiotic–antimycotic solution was added to the culture medium to prevent bacterial and fungal contamination during cell culture.

Cell Viability Assay

To evaluate the effects of the DS of CM CNF and CMC on cell viability, proliferation, and metabolic activity, cell viability assays were performed using both the Cell Counting Kit-8 (CCK-8) assay and the MTT assay. The use of two complementary assays was intended to enhance the reliability and reproducibility of the cytotoxicity evaluation, as previously reported (Cai *et al.* 2019). Cells were seeded into 96-well plates at a density of 5×10^4 cells per well in 100 µL of complete culture medium and incubated for 24 h. Stock dispersions of CM CNF with different DS, CMC, or HA were prepared in distilled water and added to the wells (10 µL per well) to obtain final concentrations of 100, 400, 800, and 1000 µg/mL in a total volume of 110 µL per well. The cells were incubated for an additional 24 h under the same conditions.

For the CCK-8 assay, 10 µL of CCK-8 reagent was added to each well, followed

by incubation for 4 h at 37 °C in 5% CO_2. The absorbance of the generated formazan, which is proportional to the number of viable cells, was measured at 470 nm using a microplate reader (Synergy-HT, BioTek, Winooski, VT, USA).

For the MTT assay, 100 µL of serum-free medium containing 0.5 mg/mL MTT reagent was added to each well. The plates were incubated for 2 h at 37 °C in 5% CO_2. Subsequently, the medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 540 nm using a microplate reader.

Analysis of Apoptosis-related Responses by qRT-PCR and Western Blot

Apoptosis-related cellular responses induced by CM CNF with different DS, CMC, and HA were evaluated at transcriptional and protein levels using quantitative Real-Time PCR (qRT-PCR) and Western blot analysis. For qRT-PCR, transcriptional changes in apoptosis-related genes (BAX, p53, and BCL2) were evaluated in HEK293 cells after exposure to the above materials at the concentration of 1000 µg/mL. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the SuperScript® IV First-Strand Synthesis System (Invitrogen, Waltham, MA, USA). Quantitative PCR was carried out using Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) with gene-specific primers. Each reaction mixture contained 2 µL of cDNA, 12.5 µL of 2× SYBR Green master mix, 1 µL each of forward and reverse primers, and nuclease-free water to a final volume of 25 µL. PCR cycling conditions were optimized for each target gene. Relative gene expression levels were normalized to 18S ribosomal RNA and calculated using the vehicle-treated group as the reference. Data analysis was performed using One-Step System software v2.1 (Applied Biosystems).

Western blot analysis was performed to assess apoptosis-related protein expression following treatment with CM CNF and control materials (Li *et al.* 2021). After overnight incubation with the test materials, culture media were removed, and the cells were washed with phosphate-buffered saline (PBS). The cell lysates were then clarified by centrifugation, mixed with sample buffer, and denatured at 100 °C for 5 min. Protein samples (40 µL) were separated by SDS–PAGE and transferred onto PVDF membranes. The membranes were blocked with 10% skim milk, incubated with primary antibodies followed by HRP-conjugated secondary antibodies, and visualized using enhanced chemiluminescence (ECL). β-Actin was used as a housekeeping protein and loading control to confirm equal protein loading and transfer efficiency across samples.

RESULTS AND DISCUSSION

Characteristics of CM CNF

Two CM CNF samples with different DS were prepared by adjusting the amounts of the reactants NaOH and MCA, and their morphological characteristics are presented in Fig. 1. Two CM CNF samples exhibited similar morphology and width distribution after seven homogenization passes, despite differences in the DS level. These results indicated that sufficient electrostatic repulsion for complete nano-fibrillation was present even at a DS level of 0.1. Based on optical microscopy observations of the morphological changes shown in Fig. 2, the DS for CM CNF preparation was restricted to under DS of 0.2 because

significant pulp swelling at higher DS values prevented the formation of CNF structures. This behavior can be attributed to the reaction mechanism of carboxymethylation. The carboxymethylation reaction begins with an equilibrium alkalization between sodium hydroxide and pulp (2), followed by an etherification reaction between alkali cellulose and MCA (3) during which hydroxy group onto pulp fiber are substituted with carboxymethyl groups. When the alkalization step in the initial stage of carboxymethylation proceeds excessively, significant pulp swelling occurs, as shown in Fig. 2 (You *et al.* 2021; Lee *et al.* 2022). To examine biological responses at higher degrees of substitution, commercially available CMC with a DS of 0.7 was used as an additional reference material in this study.

$$Alkalization: ROH + NaOH \leftrightarrow RONa + H_2O \quad (2)$$

$$Etherification: RONa + ClCH_2COONa \leftrightarrow ROCH_2COONa + NaCl \quad (3)$$

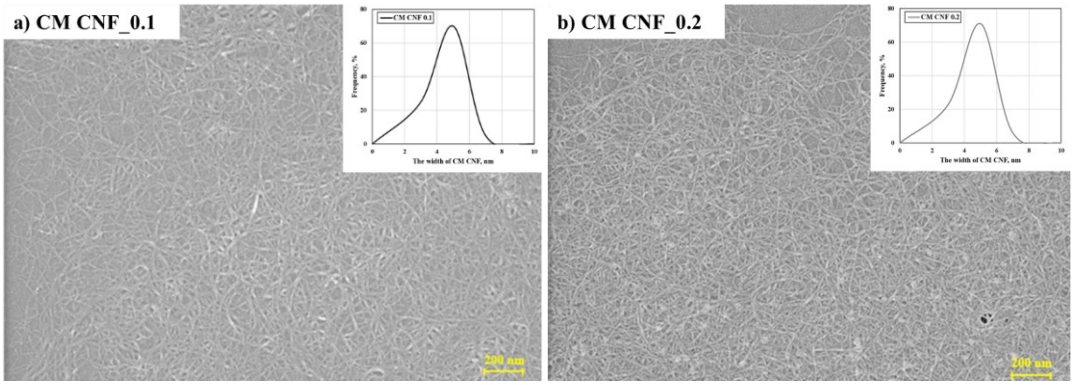

Fig. 1. The morphology and width distribution of CM CNF with different DS

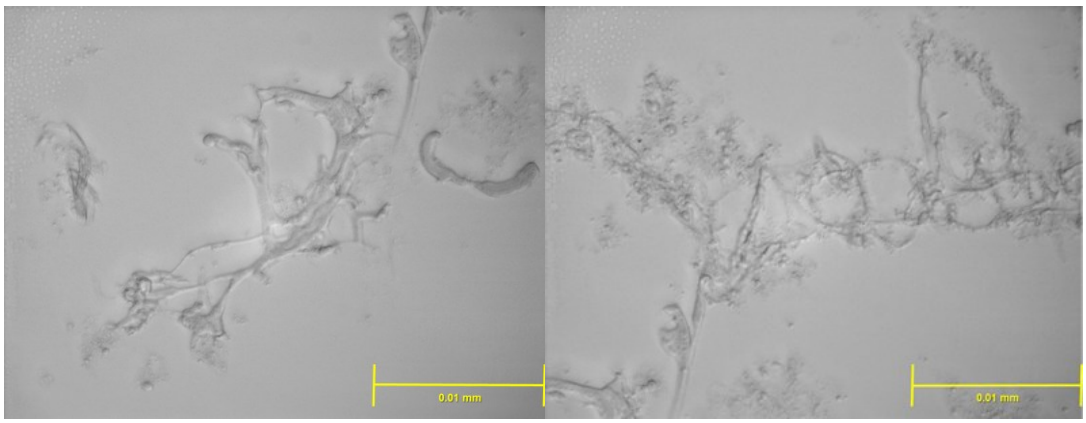

Fig. 2. Swelling behavior of pulp fiber during carboxymethylation

Both the effects on crystallinity and the charge properties associated with carboxymethylation treated samples can enhance biological responses (Liu *et al.* 2011; Souza *et al.* 2018; Zhang *et al.* 2021). To investigate their effects, the crystallinity and zeta potential of CM CNF and CMC with different DS was analyzed by XRD analysis and Zetasizer, respectively. Figure 3 presents the XRD patterns, crystallinity, and zeta potential of CM CNF with different DS and CMC. In the case of crystallinity of CM samples, the crystallinity of the carboxymethylated samples decreased with increasing DS. As shown in Fig. 2, this tendency can be attributed to excessive alkalization, which induces cellulose

swelling and disruption of crystalline structure of cellulose. As the DS value increased, the zeta potential of the CM-treated samples increased due to the increased carboxyl group content on the cellulose fibrils.

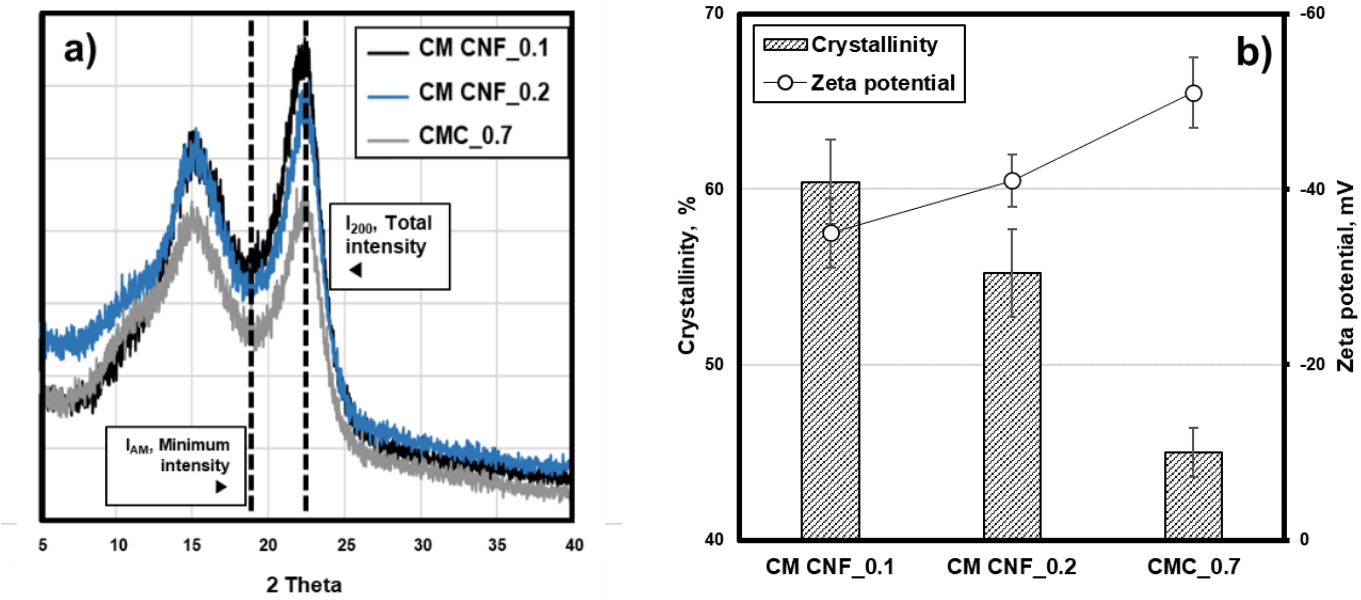

Fig. 3. The XRD pattern (a) and crystallinity and zeta potential of carboxymethylated samples (b)

Cytotoxic Response Depending on DS of Carboxymethylation

The cytotoxic and immunological effects of CM CNF with different DS, commercial CMC, and HA were evaluated using HepG2, HEK293, and RAW 264.7 cells to represent liver, kidney, and immune system responses, respectively. Cytotoxicity was assessed using both MTT and CCK-8 assays to enhance reliability. In HepG2 cells (Fig. 4a and 4b), all materials exhibited negligible cytotoxicity at low concentrations (≤ 200 µg/mL), while a gradual decrease in cell viability was observed at higher concentrations (400 to 1000 µg/mL). According to ISO 10993-5 and previous studies, CM CNF_0.2 and HA, which maintained cell viability ≥ 90%, were classified as non-cytotoxic, whereas CM CNF_0.1 and CMC_0.7 showed relatively greater reductions in viability but remained within the non-cytotoxic to slightly cytotoxic range (Giti *et al.* 2021; Rosa *et al.* 2023). The relatively higher reduction in viability for CM CNF_0.1 and CMC_0.7 can be affected by their DS, which leads to imbalances in surface chemistry and physicochemical properties.

At CM CNF_0.1, the lower degree of carboxymethyl substitution resulted in relatively higher crystallinity and lower surface charge compared to CM CNF_0.2. These physicochemical characteristics may be associated with the small differences observed in cell viability; however, all samples remained within the non-cytotoxic range under the tested conditions. The findings suggest that subtle variations in surface chemistry and structural organization may influence cell–material interactions to a limited extent. Previous studies have reported that supramolecular structure can affect cellular responses to cellulose-based nanomaterials (Daicho *et al.* 2018). For CMC_0.7, the higher degree of substitution leads to increased charge density and molecular flexibility in the dissolved state. Highly substituted polyelectrolytes may interact with cellular components differently from fibrillar CNF. Nevertheless, the reduction in cell viability observed at higher concentrations remained within the non-cytotoxic regime.

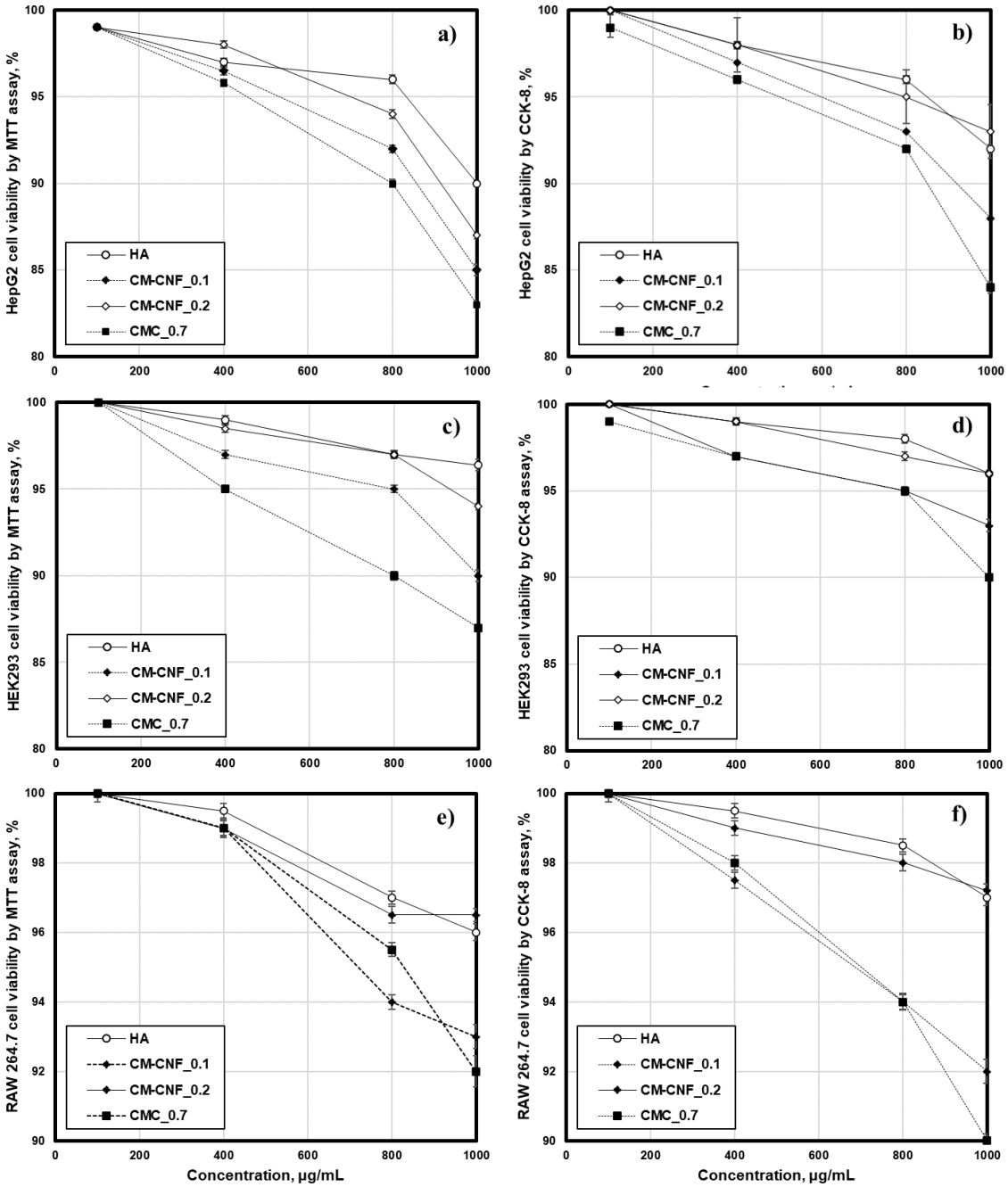

Fig. 4. Concentration-dependent cytotoxicity of two CM CNF, CMC, and HA in HepG2, HEK293, and RAW 264.7 cells

Previous reports on anionic nanomaterials indicate that surface charge can modulate intracellular interactions under certain conditions (Fröhlich 2012). In this study, the responses observed in the present study likely reflect the combined influence of surface charge, structural characteristics, aggregation state, and concentration. Similar concentration-dependent trends were observed in HEK293 (Fig. 4c and 4d) and RAW 264.7 cells (Fig. 4e and 4f); however, the extent of cell viability reduction was generally more pronounced in HepG2 cells. In HEK293 cells, cell viability remained above 90% for

all tested samples even at higher concentrations. This indicated minimal nephrotoxic effects, with only a slight decrease observed for CMC_0.7 in the MTT assay. In RAW 264.7 macrophages, all materials maintained high cell viability across the entire concentration range, suggesting negligible immunotoxicity. Previous studies on carboxymethylated polysaccharides have also reported low cytotoxicity, suggesting that carboxymethylation does not result in notable cellular toxicity (Liu *et al.* 2011; Zhang *et al.* 2021).

Effect on Inflammatory Cytokines

Although both CM CNF with different DS, as well as CMC, rarely induce direct cytotoxicity, their physicochemical properties, including crystallinity and surface chemistry, have been reported to trigger inflammatory responses (Menas *et al.* 2017). Evaluation of inflammatory signaling is therefore essential to use as an alternative material, as immune activation without significant cytotoxicity may still affect tissue compatibility, chronic inflammation, and the overall safety profile of biomaterials.

For the evaluation of the inflammatory response, HEK293 cells were used because they are immortalized kidney cells derived from a human embryo and are among the most widely used cell lines in basic, pharmaceutical, and medical research (Tan *et al.* 2021). Figure 5a and 5b present the apoptosis-related responses induced by the tested materials at both the transcriptional and protein levels, as assessed by qRT-PCR and Western blot analysis. At the mRNA level (Fig. 5a), relative gene expression was calculated using the vehicle-treated group as the reference (set to 1). The expression of the pro-apoptotic genes BAX and p53 showed minor variations among the treatment groups. CMC_0.7 and CM CNF_0.1 exhibited slightly higher mean values compared to HA, whereas CM CNF_0.2 showed expression levels generally comparable to those of HA. In contrast, the anti-apoptotic gene BCL2 showed a modest tendency toward reduced expression in CMC_0.7 and CM CNF_0.1, while relatively stable expression levels were observed in CM CNF_0.2 and HA. However, the magnitude of these changes was limited, and all values remained close to the vehicle-treated control, indicating no pronounced induction of apoptosis at the transcriptional level. Li *et al.* (2021) reported that the length and crystallinity of nanocellulose, including cellulose nanocrystal (CNC) and CNF, are critical roles in the inflammatory response. In particular, CNC with shorter lengths and higher crystalline domains exhibited increased cellular uptake, which was considered a major mechanism contributing to their cytotoxicity and inflammatory responses. However, the relatively low crystallinity of the materials used in this study may limit their inflammatory potential. These transcriptional trends were generally reflected at the protein level (Fig. 5b).

Western blot analysis showed a mild increase in the expression of pro-apoptotic proteins *p53* and *Bax* in CMC_0.7 and CM CNF_0.1, whereas CM CNF_0.2 exhibited the lowest protein expression levels, similar to those of HA. Conversely, the expression of the anti-apoptotic protein *Bcl2* showed a slight reduction in CMC_0.7 and CM CNF_0.1, while higher levels were preserved in CM CNF_0.2 and HA. In Western blot analysis, β-actin was used as an internal loading control to normalize protein expression and verify equal protein loading and transfer efficiency across samples, as confirmed by the consistent β-actin signals. From these results, appropriate carboxymethylation establishes a balance between surface charge and crystallinity, which supports favorable biological responses of CNF and provides a rational basis for its safe design for soft-tissue filler and skin-contact biomedical applications.

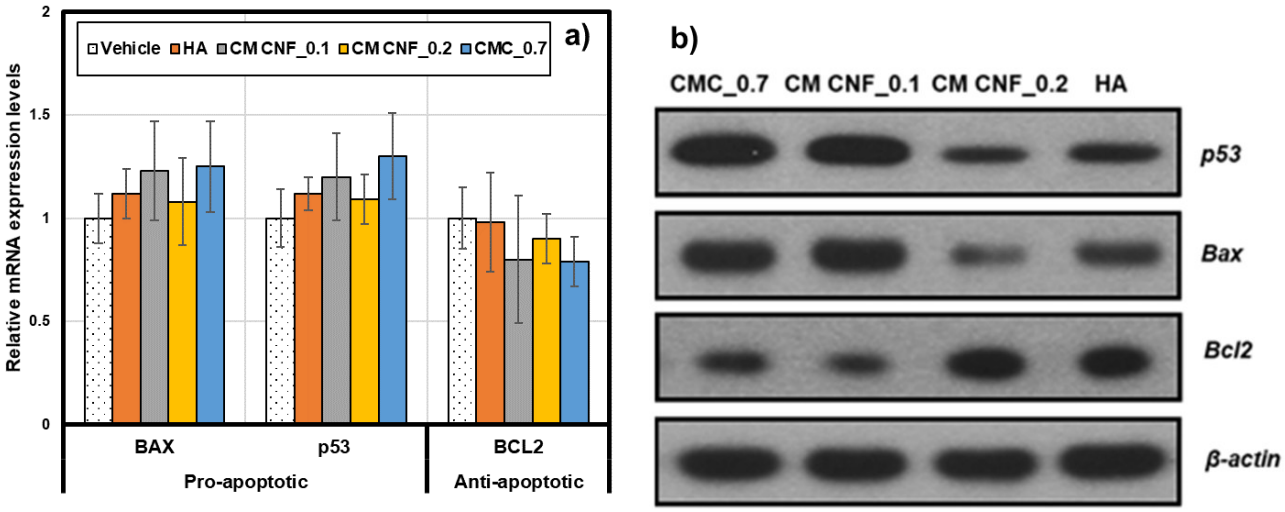

Fig. 5. Apoptosis-related mRNA and protein expression in HEK293 cells

CONCLUSIONS

1. Carboxymethylated cellulose nanofibrils (CM-CNF) with different degrees of substitution (DS) were prepared from kraft pulp, and their physicochemical properties and biological responses were comprehensively evaluated using multiple human- and mouse-derived cell lines. Increasing DS reduced crystallinity and increased surface charge, resulting in measurable differences in physicochemical characteristics. All tested materials exhibited concentration-dependent cellular responses; however, cell viability remained within the non-cytotoxic range across the examined concentrations in HepG2, HEK293, and RAW 264.7 cells.
2. CM CNF with DS 0.2 demonstrated stable cell viability and limited apoptosis-related responses comparable to those of hyaluronic acid (HA), whereas materials with lower (DS 0.1) or higher substitution (CMC_0.7) showed modest but concentration-dependent reductions in viability at higher exposure levels.
3. Within the experimentally accessible DS range, the results indicate that surface carboxymethylation modulates cell–material interactions without inducing pronounced cytotoxic or apoptotic effects. These findings support the importance of controlled functionalization in the safe design of CNF for soft-tissue filler and skin-contact biomedical applications.

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