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Application of bacterial-derived long cellulose nanofiber to suspension culture of mammalian cells as a shear protectant¹

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¹**Abbreviations:** AC, acetyl cellulose; BC, bacterial cellulose; CFD, computational fluid dynamics; CHO, Chinese hamster ovary; CMC, carboxymethyl cellulose; HPC, hydroxypropyl cellulose; IVCC, integral viable cell concentration; LDH, lactate dehydrogenase; NFBC, nanofibrillated bacterial cellulose; NFC, nanofibrillated cellulose

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ABSTRACT

Nanofibrillated bacterial cellulose (NFBC) is a bio-compatible long-fiber nanocellulose produced by cellulose-synthesizing bacteria. It forms an entangled network structure in the suspension state, thereby imparting greater viscosity than conventional media additives. In this study, we examined its application as a shear protectant in the suspension culture of mammalian cells to mitigate hydrodynamic stress imposed on the cells. The media supplemented with hydroxypropyl cellulose-adsorbed NFBC (HP-NFBC) exhibited an increase in shear viscosity according to rheometric analysis, similar to FP003, a commercially available medium additive. Suspension culture of Chinese hamster ovary cells in HP-NFBC-containing media under a high stirring rate (120 rpm) demonstrated higher cell growth and lower cell death compared to those in the medium without additives and in FP003. A 0.10 (w/v)% concentration of HP-NFBC showed the highest viable cell number among the tested concentrations. Computational fluid dynamics simulation revealed a decrease in shear rate and flow velocity within the spinner flask owing to the addition of HP-NFBC or FP003. It is suggested that the decline of these parameters in high-viscosity media suppresses the hydrodynamic stress on cells. This study highlights the potential of HP-NFBC as a shear protectant in mammalian cell suspension culture.

42 **KEYWORDS**

43 Nanofibrillated bacterial cellulose; Mammalian cell culture; shear protectant

44

1 INTRODUCTION

Cellulose, a natural linear polymer of glucose linked by β -(1,4) glycosidic linkages [1], is primarily synthesized by plants. It finds extensive use in clothing, paper production, and various applications, serving as an indispensable material in our daily lives. Various cellulose derivatives such as carboxymethyl cellulose (CMC), hydroxypropyl cellulose (HPC), and acetyl cellulose (AC) are produced by chemical modification at the hydroxyl group in the glucose unit [2,3].

Certain bacteria, such as acetic acid bacteria, are capable of producing a form of cellulose known as bacterial cellulose (BC). Generally, BC is synthesized as a gel-like membrane (pellicle) at the air-liquid interface through the incubation of acetic acid bacteria in a liquid medium [4]. This pellicle exhibits a three-dimensional network structure comprising 50–100 nm ribbons [5] and has excellent mechanical properties, such as high mechanical strength and elastic modulus. Owing to its biocompatibility, which depends on its high purity and lack of hemicellulose or lignin, BC has also been investigated for its application as a scaffold material in mammalian cell cultures [6,7].

Nanofibrillated cellulose (NFC) is a high-aspect-ratio fiber with nanosized width, typically derived from wood pulp through either mechanical degradation or chemical treatment known as a top-down process [8,9]. It possesses remarkable attributes,

including lightweight, high strength, large specific surface area, minimal thermal deformation, and durability [10,11]. Even at low concentrations, NFC suspensions exhibit excellent viscoelastic properties due to their high aspect ratios [12,13], making them advantageous as scaffold materials in mammalian cell cultures [14,15].

Kose et al. successfully synthesized nanofibrillated BC (NFBC) with dispersed microfibrils from low-molecular-weight biomass (e.g., glucose, beet molasses, and waste glycerol) by cultivating cellulose-synthesizing bacteria under aeration and agitation in a dispersant medium to inhibit microfibrils aggregation [16,17]. NFBC prepared by the bottom-up process is characterized by its extremely long ($>15\ \mu\text{m}$) fiber length and high uniformity [18], in contrast to NFC prepared through top-down methods, where mechanical and/or chemical treatments can cause severe fiber damage. NFBC exhibits excellent mechanical strength owing to its high crystallinity. It is also safe, as it is prepared through a bioprocess using food-derived raw materials and bacteria isolated from fruit. Furthermore, the properties of NFBC can be tailored by the choice of dispersant, enabling a range of applications, such as resin reinforcement [19], the preparation of antimicrobial materials [20], food additives (for modifying physical properties and enhancing productivity), binders [18], and drug carriers [21,22]. Its long-fiber length allows NFBC to be effective even when added in small amounts.

In recent years, mammalian cell culture has attracted attention for its applications in regenerative medicine, cultured meat, and biopharmaceutical production [23]. Suspension culture methods have been investigated to produce large quantities of mammalian cells [24–26]. Stirred-tank bioreactors are widely used due to their ease of operation and scalability. However, while agitation is essential for increasing nutrient and oxygen supply to floating cells, can induce hydrodynamic stress, leading to cell necrosis and apoptosis [27]. To mitigate this, shear protectants like polysaccharides [28,29] and poloxamers [30,31] have been used. Gellan gum-based polysaccharides, such as FP001 and FP003, are notable for imparting viscoelasticity to media by forming intramolecular network structures with divalent cations [32,33]. They are commonly used for 3D culture to inhibit cell sedimentation and excessive aggregation [34–41]. Furthermore, their addition has been shown to suppress hydrodynamic stress owing to increased medium viscoelasticity [42,43]. Owing to its elongated structure composed of hundreds of cellulose molecules, NFBC may exhibit distinct effects compared to conventional shear protectants molecularly dispersed (dissolved state), particularly in enhancing the high mechanical strength of cell-containing sols.

We aimed to explore the application of NFBC as a shear protectant in mammalian cell suspension cultures and assess its impact on inhibiting hydrodynamic

stress. Chinese hamster ovary (CHO) cells, which are commonly used in industry and available in suspension culture, were used as the model mammalian cells. The non-cytotoxic nature of NFBC on CHO cells was confirmed via adhesion culture. Subsequently, the shear viscosity of NFBC-supplemented media was measured. To establish a baseline in the rheometric analysis, the same measurements were performed using FP003, a widely used polysaccharide, as a control. Viscosity curves obtained were fitted to the Cross model, a viscosity model for pseudoplastic fluids, to determine the flow characteristic parameters of the medium. The same NFBC-containing media were used to study the effect of the medium during suspension culture by comparing live/dead cell concentrations under high-speed agitation, similar to the comparison with FP003. Additionally, computational fluid dynamics (CFD) simulations were performed to clarify the relationship between culture results and hydrodynamic parameters of the media with and without NFBC in suspension culture.

2 MATERIALS AND METHODS

2.1 Materials

HP-NFBC, prepared with HPC as the dispersing agent, was purchased from Kusano Sakko Inc. (Ebetsu, Japan). The fiber length, width, and HPC content are shown

in Table S1. FCeM[®] Advance Preparation kit (FP003) was purchased from Nissan Chemical Corp. (Tokyo, Japan) and used as a control for suspension culture experiments. The concentration of FP003 was determined using a moisture meter MOC63u (Shimadzu Corp., Kyoto, Japan).

2.2 Cell line and media

A CHO cell line, CHO DP-12 clone#1934 (ATCC CRL-12445), which produces an anti-IL-8 monoclonal antibody, was used for mammalian cell culture. The culture medium comprised Ham's F-12 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 1.06 g/L sodium bicarbonate (Fujifilm Wako Pure Chemicals Corporation, Japan), 0.90 mL/L penicillin-streptomycin (Merck KGaA, Darmstadt, Germany), and 10 vol% newborn bovine serum (Thermo Fisher Scientific, MA, USA). NFBC and FP003 were dispersed by adding medium, the volume of which was adjusted to achieve the desired final concentration.

2.3 Preparation of rheometric analysis

To investigate the viscous properties of the medium containing NFBC, we performed shear viscosity measurements using a rheometer (MCR 702; Anton-Paar, Graz, Austria). After pre-shearing at 10 s⁻¹ for 1 min, the shear rate ($d\gamma/dt$) was varied from 0.1 to 1000 s⁻¹, and the viscosity (μ) was measured at each shear rate. The obtained viscosity

curves were fitted using Equation (1) (Cross model [44]). Each parameter was determined using the least-squares method and used for the CFD analysis.

$$\mu = \mu_{inf} + \frac{(\mu_0 - \mu_{inf})}{1 + (m \frac{d\gamma}{dt})^n} \dots (1)$$

where μ_0 is the viscosity at $d\gamma/dt=0$, μ_{inf} is the viscosity at $d\gamma/dt=\infty$, m is the relaxation time, and n is the power-law index.

2.4 Cell culture

CHO cells were seeded into 100 mm tissue culture dishes (Corning, NY, USA) and cultured in an MCO-18M incubator (Sanyo Biomedical, Gunma, Japan) at 37 °C and 5 % CO₂. After two passages (on days 4 and 9 from seeding), the inoculum was transferred to 100-mL spinner flasks (Shibata Hario, Tokyo, Japan) containing 20 mL of culture medium to achieve a seeding concentration of 2.0×10^5 cells/mL. A schematic of the suspension culture apparatus and spinner flasks is shown in Fig. 1.

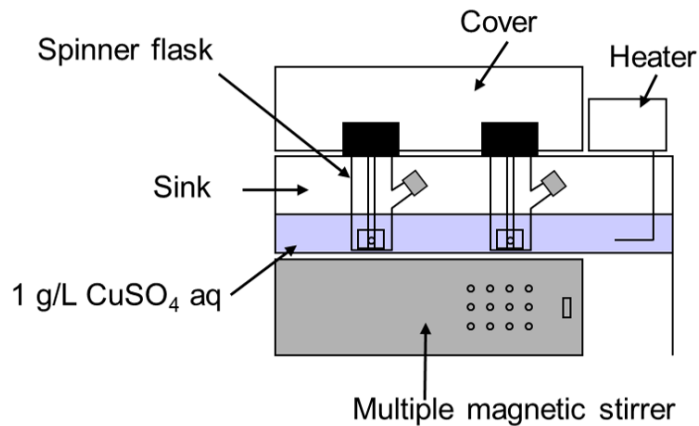


Fig. 1. Schematic of suspension culture apparatus.

The tank was placed on a magnetic stirrer and filled with 1 g/L CuSO₄ aq. The temperature was maintained at 37 °C using a heater (8053900, Taitech, Saitama, Japan). Air containing 5 % CO₂ was supplied to the apparatus, and a series of suspension incubations were performed at 120 rpm for 5 days.

2.5 Measurement of culture medium contents

Daily, 0.5 mL of culture medium was collected from each spinner flask to analyze cell viability, cell concentration, nutrients, and metabolites in the supernatant. Viable cell concentrations were determined using the trypan blue exclusion method with a hemocytometer. Dead cell amounts were estimated using lactate dehydrogenase (LDH) activity levels measured by the LDH Cytotoxicity Assay Kit (Dojindo Laboratories,

Kumamoto, Japan). Colorimetric assays for the LDH levels were performed using a Model 550 fluorescence microplate reader (Bio-Rad, Hercules, CA, USA).

2.6 CFD simulations

CFD simulation was conducted by using Comsol multiphysics 6.1 (Comsol AB, Stockholm, Sweden) based on the finite element method with steady state. The spinner flask geometry is illustrated in Fig. S1. A realizable k - ϵ model [45] was used for the simulations. A total of 24422 meshes were used. The medium viscosity was calculated using the Cross-model constants. The medium density was determined by weighing 10 mL of each medium. (Table S2).

2.7 Statistical analyses

Statistical significance was tested for $n = 3$ experiments using Tukey's test with R version 4.1.0 [46].

3 RESULTS AND DISCUSSION

The effect of NFBC on culture media viscosity, cell proliferation or death, and hydrodynamic parameters from CFD analysis were investigated. These were compared with outcomes from FP003, a gellan gum-based functional polymer known to behave as a viscoelastic fluid, suppressing cell sedimentation and excess aggregation. Furthermore,

FP003 supplemented medium has been reported to suppress hydrodynamic stress under rotating culture conditions in multi-well plates [42,43], and similar effects were anticipated within spinner flasks.

NFBCs were anticipated to mitigate hydrodynamic stress by enhancing the viscoelasticity of the medium. Unlike FP003, NFBCs disperse while retaining their long nanofibrous structure, enabling the formation of a robust three-dimensional network structure [Under review]. This property is expected to inhibit hydrodynamic stress on CHO cells, even at high stirring rates. NFBC variants include anionic hydrophilic CM-NFBCs prepared with CMC as a dispersant and non-ionic amphiphilic HP-NFBCs prepared with HPC as a dispersant, with HP-NFBC used in this study. Initially, we examined the effect of HP-NFBC on the shear viscosity, cell proliferation or death, fluid dynamics and compared with FP003. Then, the effect of concentration of HP-NFBC from 0 (No addition) to 0.20 (w/v)% was investigated.

3.1 Effect of the addition of HP-NFBC on the rheological properties of the medium

Prior to the study, we assessed the rheological properties of HP-NFBC-supplemented medium and confirmed the non-cytotoxicity of HP-NFBC by testing its effect on cell proliferation or cell death in adherent cultures and comparing it with FP003-added media. The concentration of FP003 was determined using a moisture meter

and prepared at 0.016 (w/v)% (according to the manufacturer's instructions) and 0.10 (w/v)% (the same concentration as HP-NFBC). The results confirmed that NFBC had no cytotoxic effects on cell proliferation or viability at the experimental concentration range used in this study (Fig. S2).

Fig. 2 displays shear viscosity measurements. Media with HP-NFBC and FP003 exhibited higher viscosity compared to the medium without HP-NFBC. Specifically, the medium with HP-NFBC demonstrated approximately 10 times higher than that with FP003. Both HP-NFBC (0.10 (w/v)%) or FP003 (0.016 or 0.10 (w/v)%) exhibited shear-thinning behavior, with the viscosity decreasing with increasing shear rate, suggesting the formation of physical or chemical cross-links. The viscosity curves were well-fitted by the cross model [44], with constants detailed in Table S2 for subsequent CFD simulations within spinner flasks.

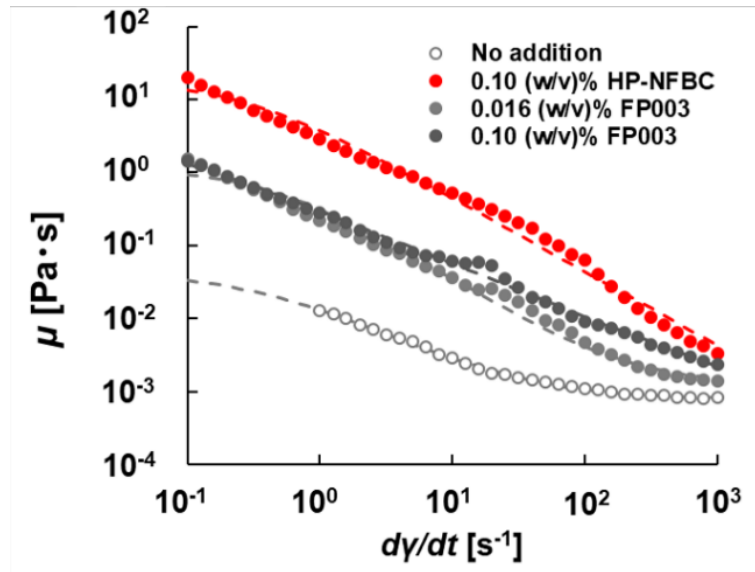


Fig. 2. Shear viscosity of the media with and without HP-NFBC and FP003. Dotted lines indicate calculated values from the Cross model (Eq. (1)). Constants for each medium are shown in Table S2.

3.2 Effect of the addition of HP-NFBC on suspension culture

The impact of HP-NFBC on CHO cell proliferation in suspension culture was examined and compared with that of FP003. To clearly confirm the effect of reducing the hydrodynamic stress, we varied the stirring rate from 70 to 120 rpm, which was used in the normal suspension culture. Preliminary experiments examining the effects of increased stirring rate and NFBC addition on viable or dead cell numbers showed that increasing the stirring rate (120 rpm) without additives increased dead cell concentration and subsequently decreased viable cell concentration. However, conditions supplemented

with 0.10 (w/v)% HP-NFBCs resulted in high cell numbers and low dead cell concentration even at high stirring rates (120 rpm) (Fig. S3).

Fig. 3(a) shows the proliferation curve of CHO cells in suspension culture at 120 rpm with and without the media supplemented with HP-NFBC (0.10 (w/v)% or FP003 (0.016 or 0.10 (w/v)%). The maximum cell concentration increased notably with the greater viscosity of the medium (Fig. 2), owing to the additives (HP-NFBC or FP003). In the absence of additives, the maximum cell concentration was the lowest among those examined, reaching only approximately twice the seeding concentration. However, in media containing HP-NFBC or FP003, the maximum cell number count surpassed three times the seeding concentration, with the stationary phase extended to day 4. Particularly in the medium with HP-NFBC, the cell number reached about 9.06×10^5 cells/mL, exceeding both concentrations of FP003 (6.68×10^5 cells/mL on 0.016 (w/v)% and 7.56×10^5 cells/mL on 0.10 (w/v)%) (Fig. 3(a)).

Next, we investigated LDH activity, an enzyme released from membrane damaged or lysed cells during medium stirring, serving as a damage marker to assess dead cell concentration. As shown in Fig. 3(b), dead cell concentration decreased with additive supplementation, reaching its lowest in the medium with HP-NFBC after day 4. The cumulative dead cell concentration (shown on day 5) in the absence of additives

exceeded 4×10^5 cells/mL, whereas it was almost halved in the media with HP-NFBC or FP003. This reduction in cell death contrasted with the increase in viable cell concentration, suggesting that the inhibition of cell death led to the rise in cell number. Notably, suppression of cell death corresponded with increased medium viscosity, indicating the mitigation of hydrodynamic stress with high viscosity.

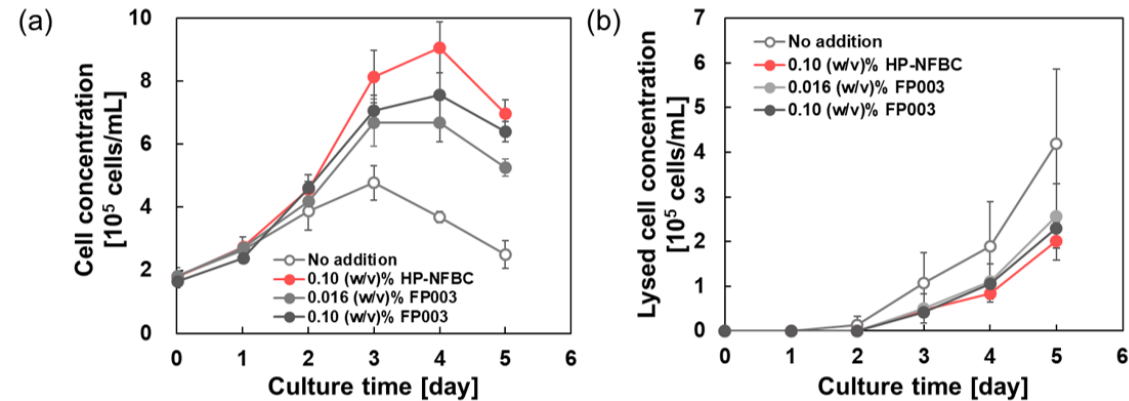


Fig. 3. Viable and lysed cell concentrations in the media with and without HP-NFBC and FP003 under stirring rate at 120 rpm. (a) Growth curves and (b) lysed cell concentration estimated by lactate dehydrogenase concentrations in the culture media. Experiments were performed at $n = 3$, and error bars are indicated by S.D.

Similar to FP003, HP-NFBC effectively inhibited cell death and increased cell numbers under rapid agitation. Both additives substantially augmented medium viscosity compared to the unsupplemented form, suggesting heightened viscosity as the main factor in protecting cells from hydrodynamic stress. The differences between HP-

NFBC and FP003 may lie in their dispersion states, molecular structures, or nanodispersions. While FP003 is anionic and can increase the number of chemical bridges containing divalent cations in the medium, HP-NFBC is non-ionic and forms a network by physical cross-linking due to fiber–fiber interactions (**Under review**). The viscoelastic state of this network is often associated with the crowding factor [47], which is influenced by fiber volume fraction and aspect ratio. Although NFBC fibers are considered stiff rods, their exceptional length and curvature complicate the direct application of this theory. Nonetheless, the elongated NFBC fibers likely contribute to increased viscosity through enhanced fiber–fiber interactions and cross-linking points. To discern whether thickening mechanism or medium physical properties exerted greater influence on hydrodynamic stress reduction, HP-NFBC was added to the medium to attain viscosity equivalent to FP003-supplemented medium (Fig. S4), followed by suspension culture testing (Fig. S5). Notably, no significant differences in cell proliferation or lysis were observed, indicating negligible impact of dispersion state difference under similar viscosity conditions. While increased medium viscosity is evidently crucial for hydrodynamic stress suppression, elucidating physical and chemical interactions between HP-NFBC and cells warrant further investigation.

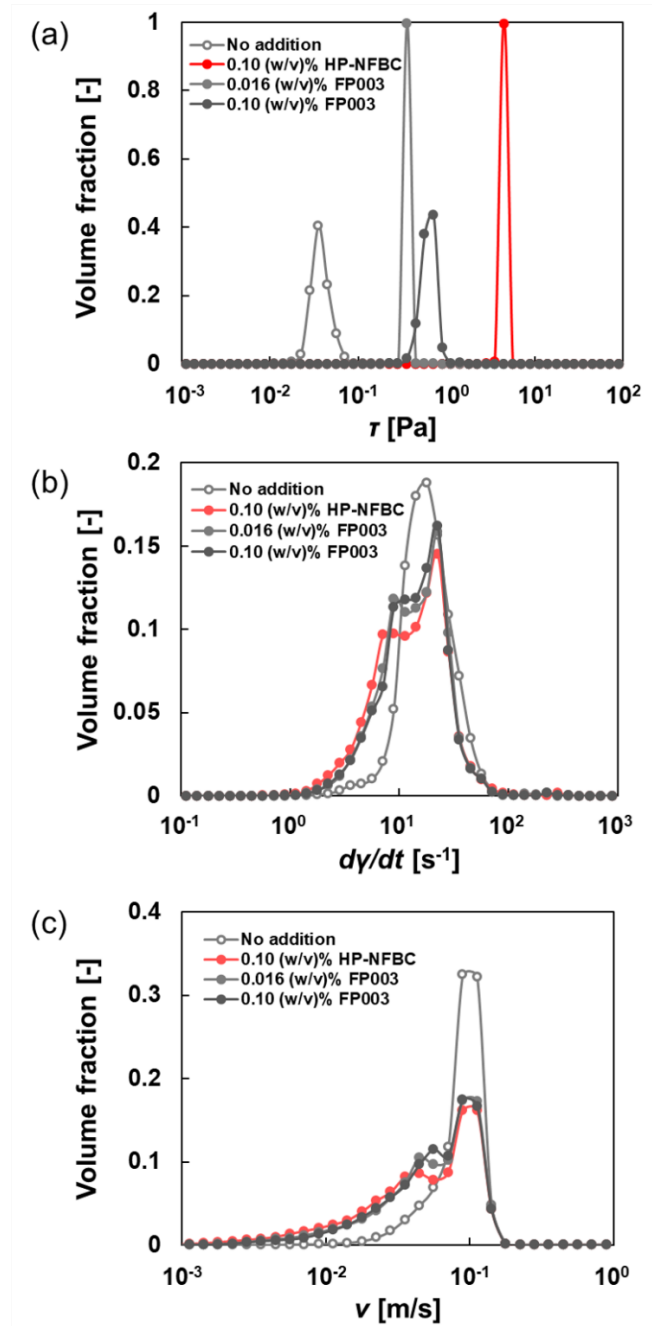
3.3 CFD simulation for suspension culture using media containing HP-NFBC

To elucidate the effect of HP-NFBC on fluid flow within the spinner flask and its role in mitigating hydrodynamic stress on cells, we examined the hydrodynamics within the spinner flask containing the media with and without additives using CFD simulations. Surprisingly, the shear stress was markedly increased with the addition of each additive (HP-NFBC or FP003) (Fig. 4(a)). This increase is attributed to the substantial elevation in viscosity upon additive inclusion, as shear stress was expressed as the product of this and the shear rate. Additionally, histograms of shear stress exhibited sharp uniformity under the additive conditions.

However, the frequency between 10-20 s^{-1} was smaller and shifted toward lower shear rates in the medium with additives compared to that without additives, and the shift was even greater in the medium with HP-NFBC (Fig. 4(b)). These shifts decreased the mean value of each parameter (Fig. S6(b)), which suppressed the hydrodynamic stress. Notably, the viscosities between 0.1-1 s^{-1} of the medium without additives could not be measured precisely because of its low viscosity (Fig. 2); however, the cumulative frequency from 0.1 to 1 s^{-1} in the unsupplemented condition was sufficiently small ($<10^{-4}$) that the effect of the lack of viscosity data in this range could be considered negligible. As shown in Fig. 4(c), the frequencies in flow velocity around 0.10 m/s decreased with adding these additives, and the mean value also decreased (Fig. S6(c)). However, no

293 significant differences existed in the flow velocity among the additives.

294



295

296 Fig. 4. Histogram of the hydrodynamic parameters of the media with and without HP-
 297 NFBC and FP003 under stirring rate at 120 rpm. (a) Shear stress, (b) shear rate, and (c)
 298 flow velocity.

299

300 In the heat map of the flow velocities shown in Fig. 5, the planes of the impeller
301 and the axis of the magnetic stirrer bar are perpendicular to each other. Higher velocities
302 were observed above and below the impeller and magnetic stirrer bar in media without
303 additives than with HP-NFBC or FP003 additives. Vortex-like streamlines were observed
304 in media without additives but not in media with additives (Fig. S7). The
305 counterdirectional flow observed at the center of the vortex was expected to cause
306 significant hydrodynamic stress on the cells, suggesting that the suppression of vortex
307 formation suppressed hydrodynamic stress.

308 Detailed discussion of these results follows later.

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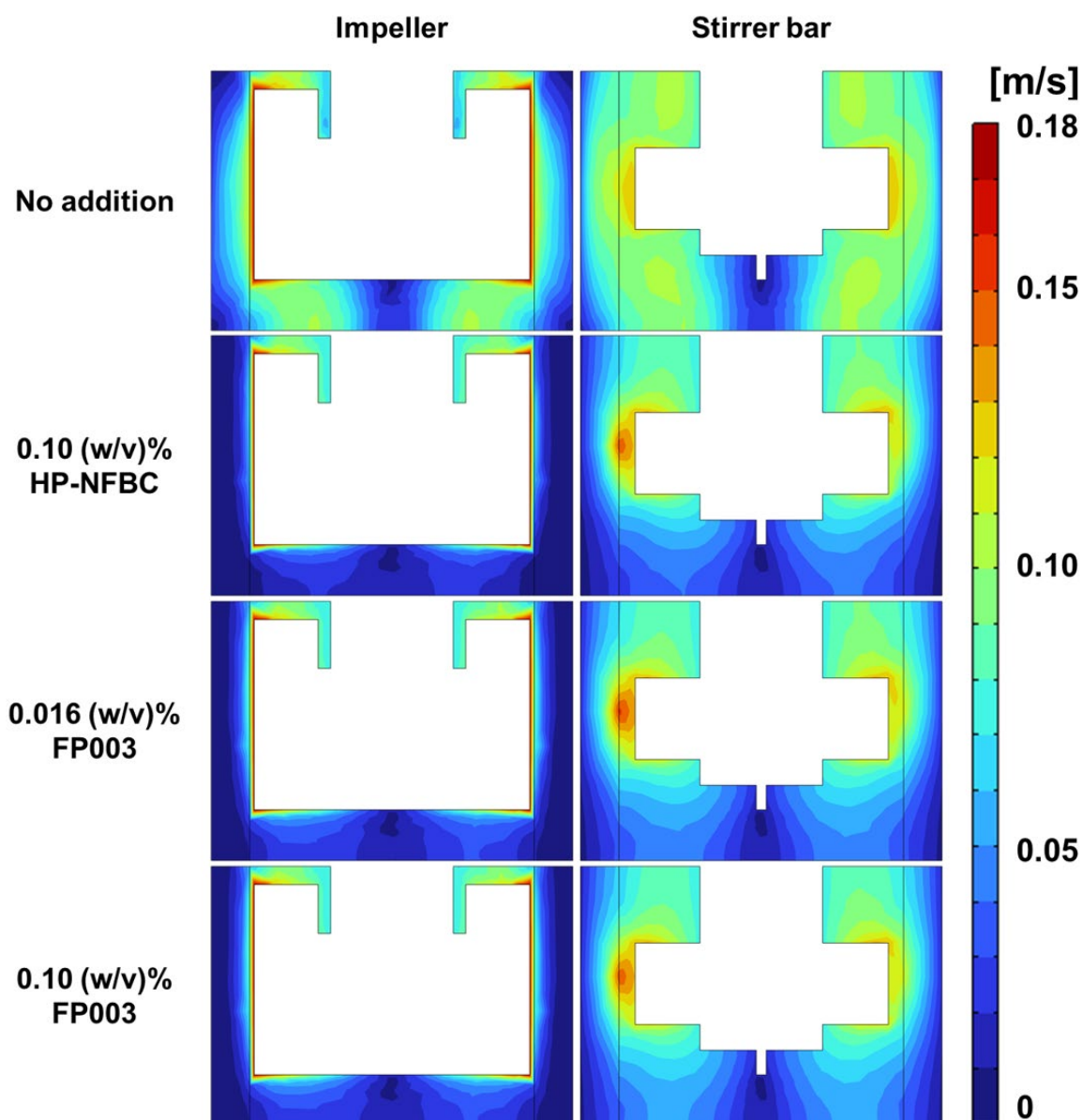


Fig. 5. Heatmap of the flow velocity within spinner flask filled with the media with and without HP-NFBC and FP003 under stirring rate at 120 rpm.

3.4 Effect of the concentration of HP-NFBC on the viscosity of the medium

As shown in 3.1 to 3.3., adding HP-NFBC to the medium increased the viscosity, successfully inhibited cell death, and maintained high cell concentrations, similar to the addition of FP003. However, high viscosity inhibits mass transport, such as oxygen and nutrient delivery, and metabolite diffusion, consequently affecting cell proliferation. Therefore, we varied the concentration of HP-NFBC in the range of 0.01–0.20 (w/v)% to test it at different viscosities and examined its effect on rheological properties and cell proliferation in suspension culture.

The viscosity of the medium increased with the concentration of HP-NFBC added (Fig. 6); at 0.01 (w/v)%, the viscosity above 100 s^{-1} was similar to that of the medium without HP-NFBC, and the viscosity curve was flat, indicating that the network structure of the HP-NFBC fibers was broken and oriented in the flow direction. However, when 0.05 (w/v)% or more was added, a linear decrease in viscosity was observed in all regions, indicating that the network structure was maintained even at high shear rates.

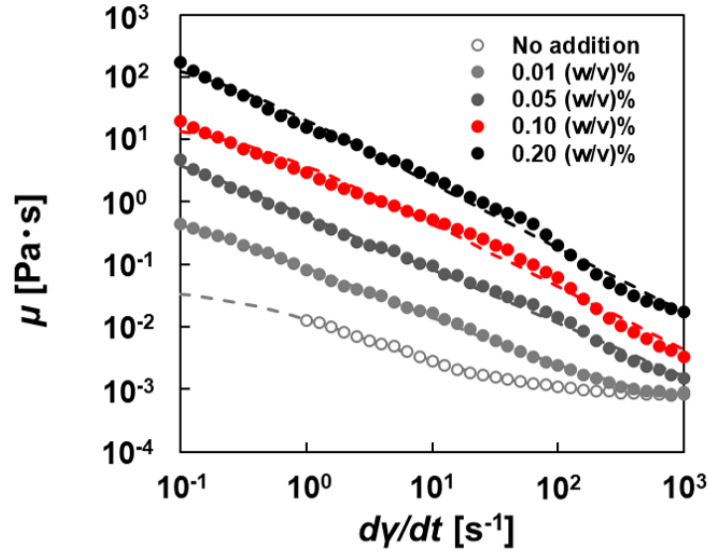


Fig. 6. Shear viscosity of the media with various concentrations of HP-NFBC. Dotted lines indicate calculated value of the Cross model (Eq. (1)). Constants for each medium are shown in Table S2.

3.5 Effect of the concentration of HP-NFBC on suspension culture

Next, the effect of the HP-NFBC concentration on the CHO cell suspension culture was examined. As shown in Fig. 7(a), the cell concentrations increased with increasing amounts of HP-NFBC. The cell concentration in the medium with 0.01 (w/v)% HP-NFBC increased as well as with 0.05 (w/v)% HP-NFBC until day 3, but decreased from day 4, in contrast to the high cell concentration maintained at 0.05 (w/v)% and above. The maximum cell concentration was obtained at 0.10 (w/v)% (9.07×10^5 cells/mL) on day 4. Notably, the cell concentration at 0.20 (w/v)% was higher than 0.10 (w/v)% only on day 5 (7.97 and 7.03×10^5 cells/mL, respectively), suggesting a longer stationary phase

as the concentration of HP-NFBC increased. We also investigated the relationship between the integral viable cell concentration (IVCC), which was calculated by the trapezoidal integration of growth curves, and the HP-NFBC concentration, which was related to the viscosity of each medium. As shown in Fig. S8., IVCC exhibited an increase with concentration up to 0.10 (w/v)%, while no increase was observed by 0.20 (w/v)%.

The lysed cell concentrations estimated from the released LDH activity are shown in Fig. 7(b). The lysed cell concentrations decreased as the amount of HP-NFBC added increased. In particular, the accumulation of lysed cells observed on day 5 was reduced by approximately 28 % at 0.05 (w/v)%, 56 % at 0.10 (w/v)%, and 71 % at 0.20 (w/v)% compared to the medium without HP-NFBC addition. In the condition of 0.20 (w/v)% HP-NFBC, cell proliferation did not exceed that with 0.10 (w/v)% HP-NFBC (Fig. 7(a)), but cell death was inhibited more than with 0.10 (w/v)% HP-NFBC (Fig. 7 (b)).

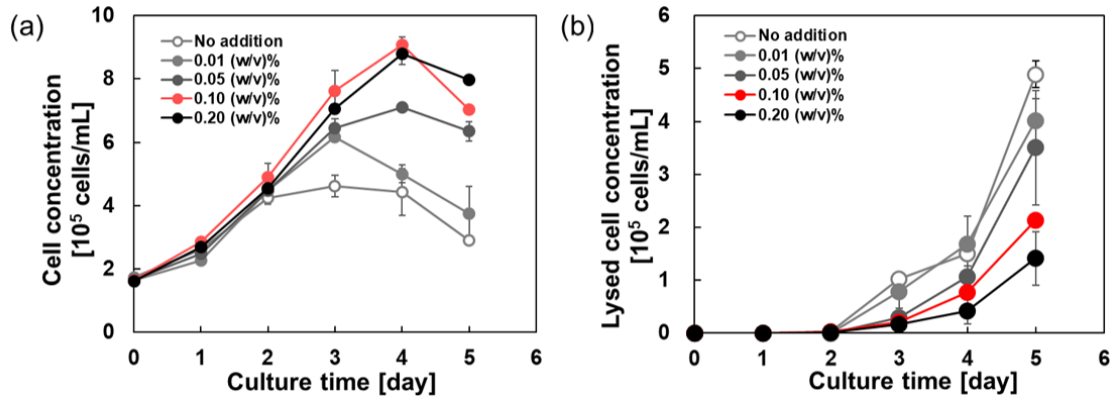
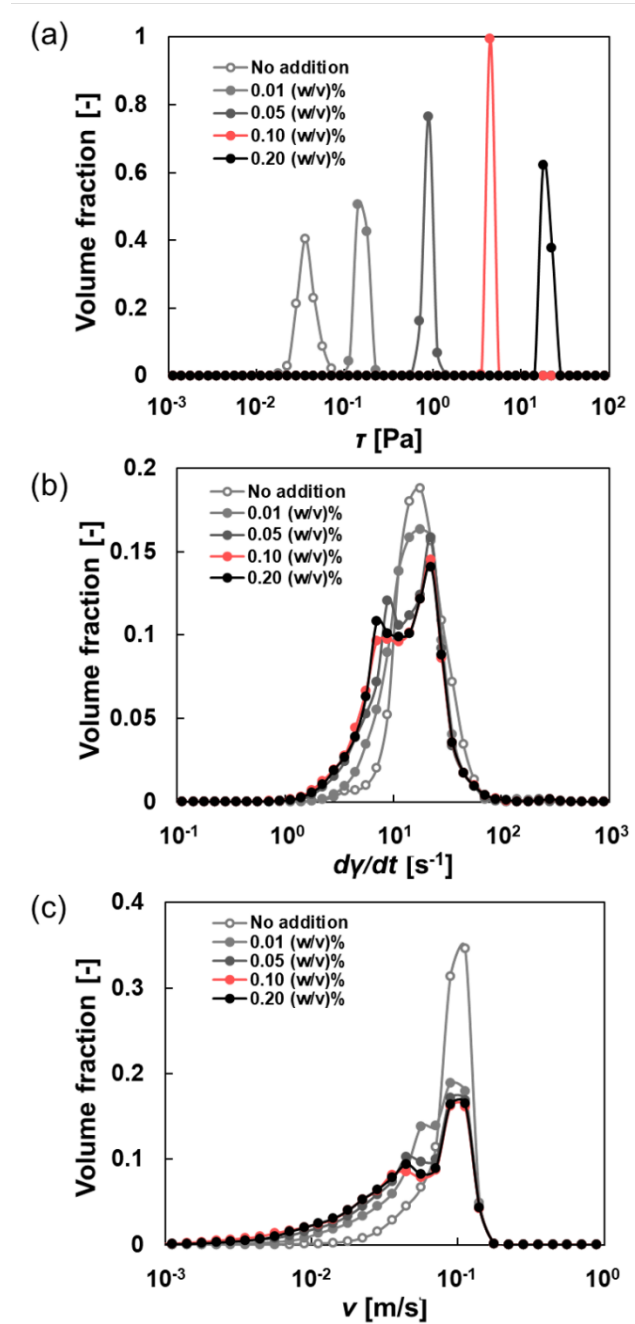


Fig. 7. Viable and lysed cell concentrations in the media with various concentrations of HP-NFBC under stirring rate at 120 rpm. (a) Growth curves and (b) lysed cell concentration estimated by lactate dehydrogenase concentrations in the culture media. Experiments were performed at $n = 2$, and error bars indicate the minimum and maximum values.

3.6 CFD simulation for suspension culture using media containing HP-NFBC at various concentrations

To understand the effects of HP-NFBC concentration on the fluid flow in the spinner flask and how the hydrodynamic stress on the cells was suppressed, we examined the hydrodynamics in the spinner flask containing media with different concentrations of HP-NFBC and media without HP-NFBC using CFD simulations. Fig. 8 shows the histograms of the shear stress, shear rate, and flow velocity, and a similar trend was observed, as shown in Fig. 4, where HP-NFBC and FP003 were compared to the medium without HP-NFBC. Shear stress increased with an increase in the concentration of HP-NFBC.



375

376 Fig. 8. Histogram of the hydrodynamic parameters of the media with various
 377 concentrations of HP-NFBC under stirring rate at 120 rpm. (a) Shear stress, (b) shear rate,
 378 and (c) flow velocity.

379

The heat map of the flow velocity for each medium (Fig. 9) and the histogram of the flow velocity (Fig. 8(c)) showed that the flow velocity for each medium decreased as the concentration of HP-NFBC increased. The frequencies of higher flow velocity and shear rate values decreased, alongside a reduction in average values (Fig. S9). Vortex-like streamlines were not observed at a concentration of 0.05 (w/v)% or higher (Fig. S10).

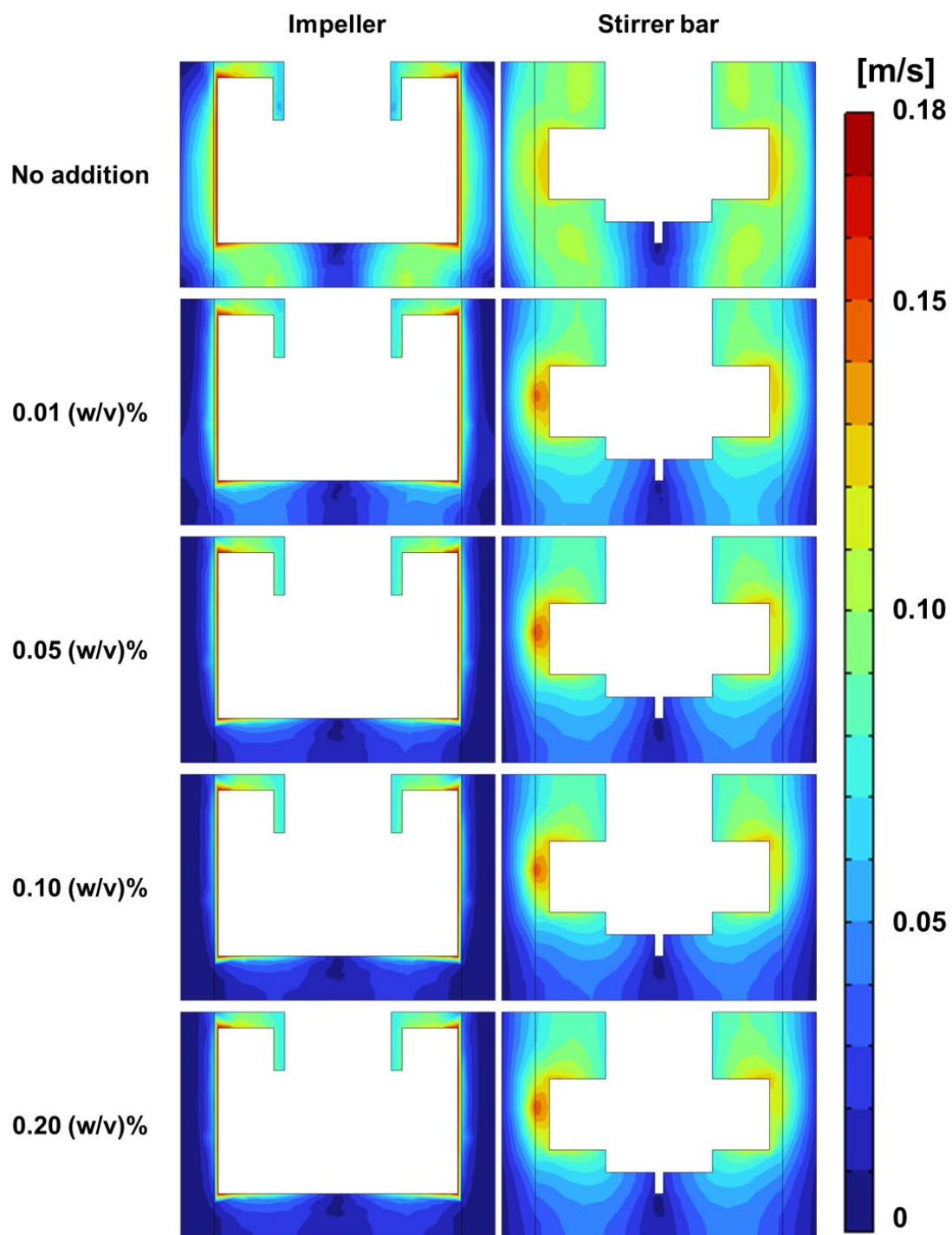


Fig. 9. Heatmap of the flow velocity within spinner flask filled with the media containing various concentrations of HP-NFBC under stirring rate at 120 rpm.

CFD analysis showed a decrease in the shear rate and flow velocity from 0 (w/v)% to 0.05 (w/v)%, whereas these parameters changed slightly above 0.05 (w/v)% (Fig. S9(b) and (c)). The incubation results indicated that at HP-NFBC concentrations below 0.10 (w/v)%, the viable cell concentration increased with increasing HP-NFBC concentrations (Fig. 7(a)), indicating that the CFD analysis explained the culture results well at concentrations below 0.05 (w/v)%.

Many fluid dynamic parameters, such as shear stress [48,49], energy dissipation rate [50], and Kolmogorov length scale [51], were considered as factors of hydrodynamic stress, and their relationship with cell viability was discussed. Shear rate is a factor of cell damage [52], and in this study, it was found to decrease when HP-NFBC and FP003 were added to the medium (Figs. 4(b) and 8(b)). Since the shear rate can be expressed as the strain applied per unit distance in a unit of time, a decrease in the shear rate implies that less strain is applied to the cells, resulting in less stress. Furthermore, under the additive conditions of HP-NFBC or FP003, the average flow velocity in the spinner flask decreased, and a low-flow velocity region appeared (Figs. 4(c), 5, 8(c), and 9). First, the decrease in the flow velocity was thought to have resulted in a decrease in the shear rate and, consequently, a decrease in the hydrodynamic stress.

Liu. et al. reported that the shear stress on particles and the frequency of

encountering high shear stress rise with increasing stirring rate due to enhanced access near the impeller [49]. Based on this finding, the frequency of high hydrodynamic stress decreased with decreasing flow velocity by adding HP-NFBC or FP003 to the medium. Consequently, cells can restore homeostasis before the next higher hydrodynamic stress is induced, and the accumulated hydrodynamic stress is reduced.

However, this explanation, depending on the CFD results, was insufficient to clarify concentrations above 0.05 (w/v)%, implying the existence of mechanisms suppressing hydrodynamic stress that were not considered in the CFD analyses. The cells were expected to experience various forces at different time scales, such as compression, shear, and tension; however, only shear was considered in this study. Tajima et al. reported that HP-NFBC suspensions have viscoelastic properties and exhibit gel-like behavior under cyclic stress [Under review], and the viscoelastic properties of HP-NFBC suspension may contribute to the inhibition of cell damage.

This study has several limitations. First, it did not consider the microscopic interactions between cells and HP-NFBC or FP003 in the flow field, which are crucial for understanding the underlying mechanism. We formulated three hypotheses: first, that the networks of HP-NFBC or FP003 buffer the shear force; second, that the plasma membrane is reinforced by adsorbing FP003 or HP-NFBC, such as Pluronic F68, a widely

used shear protectant; and third, that a high-viscous boundary between the cells surface and non-Newtonian fluid protects cells, as proposed by Horiguchi et al. [42]. Direct verification of these hypotheses using macroscopic parameters, such as viscosity or live/dead cell concentration in the spinner flasks, may be challenging. Thus, unraveling the microscopic interaction of suspended cells with additives will be essential for confirming these hypotheses.

Second, the study did not address the potential side effects of extremely high-viscosity media with HP-NFBC on CHO cells in suspension culture. Increased viscosity of culture media could reduce oxygen supply; however, changes in dissolved oxygen concentration and their effect on CHO cells could not be determined owing to the small scale of spinner flasks (20 mL) used in this study. Scaling-up is one of the most effective approaches to examine the effect of HP-NFBC on both oxygen supply and CHO cells, providing a basis for achieving industrial-scale cultivation.

Third, the effect of HP-NFBC as a shear protectant was evaluated using only CHO cells. It would be desirable to assess the effects of HP-NFBC on other spheroid-forming cells, such as induced pluripotent stem cells and mesenchymal stem cells. The suspension culture of these spheroid-forming cells plays a crucial role in drug screening and regenerative medicine. Therefore, reducing the negative impact of shear stress on

these cells with NFBC would be industrially beneficial. Furthermore, if HP-NFBC can enhance spheroid formation or reduce size variability in these cells, it would broaden its applications in cell suspension cultures.

4 CONCLUSION

A suspension culture of CHO cells treated with HP-NFBC exhibited a substantial reduction in cell death and a viscosity-dependent increase in cell number, similar to FP003. Specifically, a 0.10 (w/v)% concentration of HP-NFBC resulted in the highest number cell concentration in this study. This marks the first instance of cellulose nanofibers being applied to a stirred suspension culture of CHO cells in a dispersed state. Numerical simulations showed that the shear rate and flow velocity were decreased, which appeared to enhance cell viability. This study showcases the potential of NFBC as a shear protectant in suspension cultures of mammalian cells and would contribute to increasing their productivity. Future research aims to explore its applications in cell-friendly biomaterials, regenerative medicine, and mammalian cell engineering.

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DECLARATION OF INTERESTS

The authors declare that they have no financial or personal relationships that may be considered potential competing interests.

DATA AVAILABILITY

Data will be made available on request.

Author contributions: CRediT

Eiichiro Kaneko: Formal analysis, Investigation, Methodology, and Writing – Original draft. **Haruto Tsujisaki:** Investigation. **Masashi Fujiwara:** Supervision, and

477 Writing – Review and Editing. **Hidegori Ando**: Supervision. **Yasushi Sato**: Supervision.
478 **Tatsuhiro Ishida**: Supervision. **Hirofumi Tani**: Supervision, and Writing – Review and
479 Editing. **Kenji Tajima**: Conceptualization, Funding acquisition, Methodology, Project
480 administration, Supervision, and Writing – Review and Editing.
481

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