Final Report for the Tandem Forest Values Project: Renewing Biomedicine with Biopolymers: Engineering nanocellulose hydrogel scaffolds for delivery of bioactive cues in soft tissue engineering (BioforBio), TFV 2018-0029

Yury Brusentsev, Xiaoju Wang, Lars Wågberg, Chunlin Xu (Substitute PI to Stefan Willför, currently Vice-rector to Åbo Akademi University)

Åbo Akademi University (ÅAU)/Laboratory of Natural Materials Technology, Wood and Paper Chemistry Research Group

KTH, Royal Institute of Technology/Department of Fibre and Polymer Technology, Division of Fibre Technology

1. Description of the research that has been carried out

In the proposal for the project we aimed:

- To modify cellulose nanofibrils (CNF) with methacrylate groups via alkoxysilane chemistry in aqueous phase to tune the mechanical stiffness of the CNF hydrogel (**Task 1**)
- To increase biofunctionality of CNF hydrogels by grafting thermal-responsive polymers onto CNF for a controllable release of angiogenic growth factors (**Task 2**)
- To use wood hemicellulose derivatives as surface wettability modifier, as well as molecular anchor to CNF via physical affinity for enhanced cell attachment activity (**Tasks 3 and 5**)
- To optimize the CNF bioink formulation for successful 3D printing of hydrogel scaffolds *via* direct ink writing (DIW) (**Task 4**)

The full-time employed post-doctoral researcher Dr. Yury Brusentsev conducted the research during 1.1.2019-31.12.2020 as planned under the supervision by project manager Dr. Xiaoju Wang, Prof. Lars Wågberg (Co-PI), and Prof. Stefan Willför/Prof. Chunlin Xu (Substitute from 1.9.2019 to 31.12.2020) (PI). Research visit to KTH by Dr. Yury Brusentsev was carried out during 1.1.2020-31.7.2020. Research during the mobility period was carried out mostly as planned. Due to the influence of the pandemic, parts of the research tasks were not possible to be accomplished as proposed, including a major part of task 2 on grafting thermal-responsive polymers onto CNF and part of task 5 where cell tests for nanocellulose anchored with hemicellulose derivatives were planned. However, necessary changes were proposed to assist Dr. Yury Brusentsev to accomplish the tasks with analytical supports and thus relevant minor changes of the budget plan were approved in October 2020. It should be pointed out, though, that the unfinished tasks (task 2 and partial task 5) were taken forward by the research team in Åbo and will be accomplished outside the duration of this project. Thus, more specific description of research achievements is presented below and divided in four parts in correspondence to the objectives that are listed above:

1. Preparation and characterization of nanocellulose methacrylate (CNF-MA) hydrogel with tailored mechanical stiffness

2. Preliminary study of grafting thermal-responsive polymers onto CNF hydrogel for a controllable release of angiogenic growth factors
3. Optimization of ink formulation and assessment in light-assisted 3D printing mainly with extrusion-based Direct Ink Writing (DIW) and further with UV-light projector-driven Digital Light Processing (DLP)
4. Cell tests including cytotoxicity and cellular proliferation studies of the developed CNF hydrogel and cross-linked CNF-MA scaffolds with tailored mechanical stiffness using Human Dermal Fibroblasts (HDF) and HeLa cells lines

1.1. Preparation, and characterization of CNF-MA hydrogel with tailored mechanical stiffness

Chemical characterization of modified nanocellulose fibrils is a challenge. Thus, we first developed a method for characterization and quantification of the chemical composition of the modified cellulose hydrogel materials. For this purpose, we adopted the method of solid-state NMR analyses of cellulose materials via a short research visit to the University of Helsinki where an earlier developed method was adapted to our system and conditions (A. King at al. Biomacromolecules, 2018, 19, 7, pp. 2708-2720,). To manage this our PostDoc (Yury Brusentsev) made a short one-week visit to the Laboratory of Organic Chemistry at the University of Helsinki. Then the preparation of the ionic liquid for the dissolution of cellulose and optimization of the NMR parameters was conducted at ÅAU. This analytical method was then transferred to the KTH partner group during the research visit by Dr. Yury Brusentsev.

To prepare CNF-MA hydrogel, attempts were made to develop and evaluate several synthesis approaches.

- Firstly, the method which was proposed in the application (attachment of the functionally substituted siloxane to the cellulose, M. Beaumont, at al. Molecules 2018, 23 (6), 1427) was examined for the TEMPO-oxidized CNF hydrogel. Moreover, the method for the preparation of the functionalized tri-ethoxy-silyl reagents with different functional groups (including azide and propargyl groups) was developed.
- A second method for direct functionalization of the nanocellulose hydrogels was then evaluated by carbodiimide-assisted amidation of carboxylic moieties on TEMPO-oxidized CNF. A challenge was identified in the aggregation caused by reduction of surface charge due to further derivatization of carboxylic groups.
- Lastly, a successful method of introducing methacrylate to CNF was developed in two steps (Figure 1): TEMPO-mediated oxidation was applied to activate the fiber surface prior to methacrylation and resulted in cellulose fibers with charge density of 0.91, 1.25, and 1.40 mmol/g, respectively. Following this, water was exchanged to DMF and further derivatization with methacrylic anhydride was performed. The oxidized fiber with charge density of 0.91 mmol/g achieved highest DS for MA, 0.03. A higher charge density had a negative impact on the DS for MA. Both unmodified CNF and CNF-MA were characterized with regards to NMR, dynamic light scattering (DLS), atomic force microscope (AFM) imaging, and transmission electron microscope (TEM) imaging to characterize their chemical functionality and morphology.

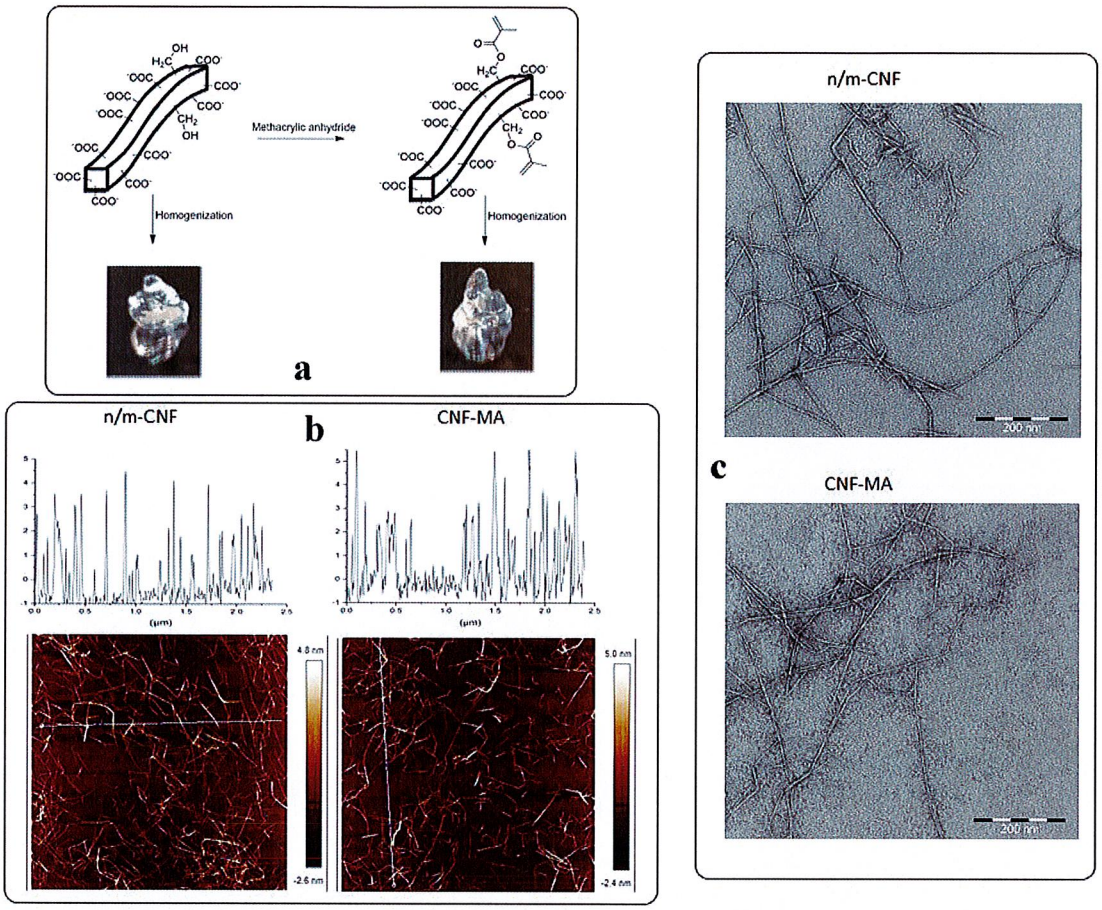

Figure 1. a) Illustration of the surface modification of methacryloyls on fiber with methacrylic anhydride in DMF, b) AFM visualization of spin-coated fibrils of TEMPO-CNF and CNF-MA; c) TEM images of TEMPO-CNF and CNF-MA.

In our endeavour to synthesize photo-reactive CNF-based hydrogel, the CNF-MA was copolymerized together with the presence of acrylamide monomer under the UV-irradiation at 365 nm. CNF-MA with 1 mmol/g surface charge and 0.02 methacrylate modification degree was selected, as an adequate level of surface ionization is essential to achieve more homogenized defibrillation of cellulose nanofibers in the final product, but an intermediate level of surface charge of $-COO^-$ has been proven to better support the proliferative activity of cell lines, such as fibroblasts in our previous study. To detect the effect of the photochemistry and the kinetics of the reaction, the storage modulus of G′ upon UV irradiation was registered on Modular Compact Rheometer (MCR) in an oscillatory mode with a constant strain of 1% and frequency of 1 s^{-1}. Photorheology of CNF-MA was studied by MCR equipped under UV curing by varying several parameters which include choice of initiator, concentration of initiator and monomers. As a result of the initial studies, 0.2 % Irgacure 2959 (initiator) and acrylamide as a crosslinking monomer was selected as optimal for further use, and this low concentration of initiator may minimize the

risk to cause cell toxicity in later stages. Optimization of the acrylamide crosslinker content via the photorheology tool was also performed with the CNF-MA content set as 1.1% and the acrylamide content varying from 0.25 to 4% to yield hydrogels with a broad range of mechanical strength.

In the targeted applications such as biomaterial matrix in supporting 3D cell culture, reswelling ability was evaluated by drying the CNF based hydrogels into films/aerogels followed by reswelling it in the applicable medium, with the hydrogels prepared by UV cross-linking of 1.1% CNF-MA+1% acrylamide formulation. An excellent reswelling capability of the dried film was observed. With the study on drying methods, i.e. air, vacuum, and freeze drying, it was noticed that both air-drying sample and freeze-drying sample have higher speeds of water uptake than the vacuum-dried sample. The reswelled hydrogel in PBS was further subjected to compression test to assess their mechanical properties.

1.2. Preliminary study of grafting thermal-responsive polymers onto CNF hydrogel for a controllable release of angiogenic growth factors

For this task, it was only a possible to perform a possible study due to the unrest caused by the pandemic. In order to introduce a thermo-responsive co-polymer to the material as function for targeting drug delivery such formulation as 1.1% CNF-MA + 1.5% N-isopropylacrylamide + 0.01% N,N'-methylenebisacrylamide +0.2% Irgaqure 2959 was developed and evaluated. With this formulation it was possible to form a thermo-responsive material by UV initiation and the material had a transition temperature at 36-38 °C. The thermo-responsive poly-N-isopropylacrylamide is formed by UV polymerization was either covalently bonded to CNF or entangled with the fibrils. With the preliminary result, the host research team is currently further developing hemicellulose-based thermo-responsive polymers that can fulfill the pre-determined tasks and thus will not be reported here.

1.3. Optimization of the ink formulation and assessment in light-assisted 3D printing mainly with DIW and further with digital light processing (DLP)

3D printability and shape fidelity of the developed formulations were evaluated by extrusion-based 3D printer with a pneumatic dispensing system and conical polyethylene nozzle. The printability was optimized for a nozzle diameter of 250 μm and printing speed of 600 mm/min. After the extrusion the scaffolds were cross-linked by the printer's integrated UV source. The formulations containing fixed amount of acrylamide (1%) and initiator Irgacure 2959 (0.2%) with CNF-MA concentration variations from 0.85 to 1.3% were evaluated for the printability. As the printing approach allows the cross-linking only after extrusion, the printed object is expected to keep its shape until the UV cross-linking is applied. Therefore, only formulations with CNF-MA content no less than 1% were suitable for the printing.

Formulation "Ink-1.1%" with 1.1% of CNF-MA was determined to be most suitable for the printing conditions. Optimal strut diameter (0.45 mm) for the "Ink-1.1%" formulation was determined to extrude at 14 kPa pressure (Figure 2A). It was also shown that the material is suitable for printing the complex shape objects (Figure 2B). To further assess the resolution capacity of the ink formulation, UV cross-linking was applied during extrusion. The printed struts were clearly distinguished at distance over 1 mm when 250 μm cylindrical stainless steel nozzle, 240 mm/min speed and 100% dispensing rate were set to print the calibration grids with 1-5 mm mesh. It was observed that up to 10 layers could be printed on top of each other without significant fusing of the grid. In comparison to the previous studies where CNF hydrogel and such auxiliary

methacrylate components as gelatine and biopolymer methacrylates were formulated, the current CNF-MA ink can only be used in manufacturing relatively soft hydrogel matrix applications. Additionally, printability of the CNF-MA-based ink formulation was also assessed with digital light processing (DLP) printer.

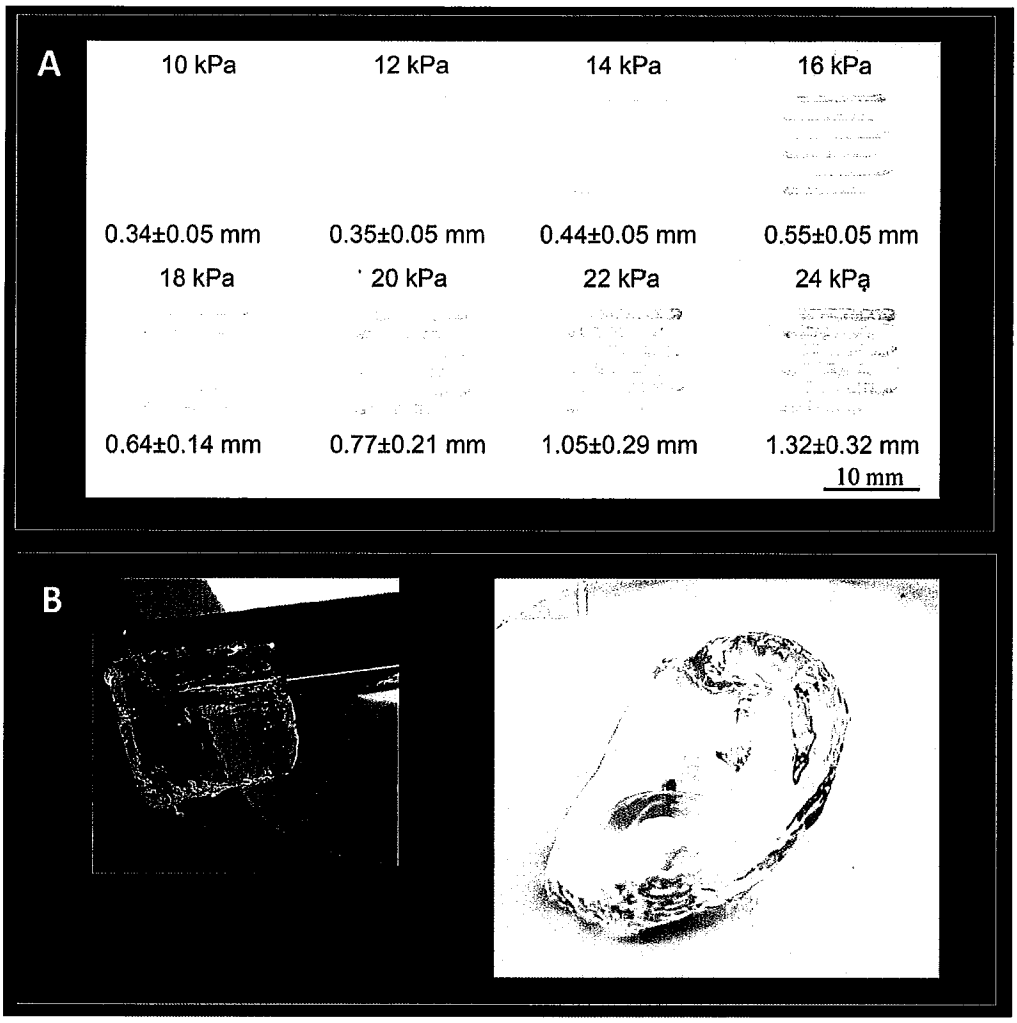

Figure 2. (A) Extrusion of filament using 1.1% CNF-MA + 1% Acrylamide under variant pressure in a pneumatic dispensing system and conical polyethylene nozzle (250 μm) with a printing speed of 600 mm/min. (B) Complex scaffolds 3D printed using pneumatic extrusion.

1.4. Cell tests including cytotoxicity and cellular proliferation studies of the developed CNF hydrogel and cross-linked CNF-MA scaffolds with tailored mechanical stiffness using HDF and HeLa cells lines

The cyto-compatibility of the cross-linked CNF-MA+polyacrylamide (PAA) hydrogels as the cell culture matrix was preliminarily evaluated in the cell culture of HDF and HeLa cells lines. With the cell culture mock as control, three types of CNF-MA+PAA hydrogels (cross-linked from 1.1% CNF-MA and monomeric AA of varied concentration of 0.25%, 0.5%, and 1%) were compared in terms of the cytotoxicity and the cell proliferation behaviors. In the culture of HDF and HeLa cells, all three groups of CNF-MA+PAA hydrogels demonstrated satisfactory cell viability compared to the culture on two-dimensional (2D)-Mock. Moreover, the CNF-MA+PAA hydrogels supported the cell proliferation after longer incubation periods. The cell proliferation was quantitatively evaluated at time points of day 1(D1), D2, D3, and D5 as shown in Figure 3a for HDF and in Figure 3b for HeLa cells. At D1 and D2, the cell growth was inhibited in comparison to 2D-Mock. After 72h, both HDF cells and HeLa Cells recovered high proliferation speed and the proliferation rate showed no significant deviation from the 2D-Mock control. Meanwhile, the cell proliferation made no distinguished variation among the three groups of CNF-MA+PAA hydrogels, which indicates that the content of polyacrylamide resulted in the hydrogels plays a minor role in affecting the gel's compatibility in supporting the cell proliferation. Featured with low dosage of monomeric AA in resulting sufficient crosslinking, ease of fabrication, and satisfactory cyto-compatibility, the CNF-MA+PAA hydrogels have a great potential as a novel matrix system in creating stiffness gradient hydrogels with a patterned or moving photomasks for UV photopolymerization.

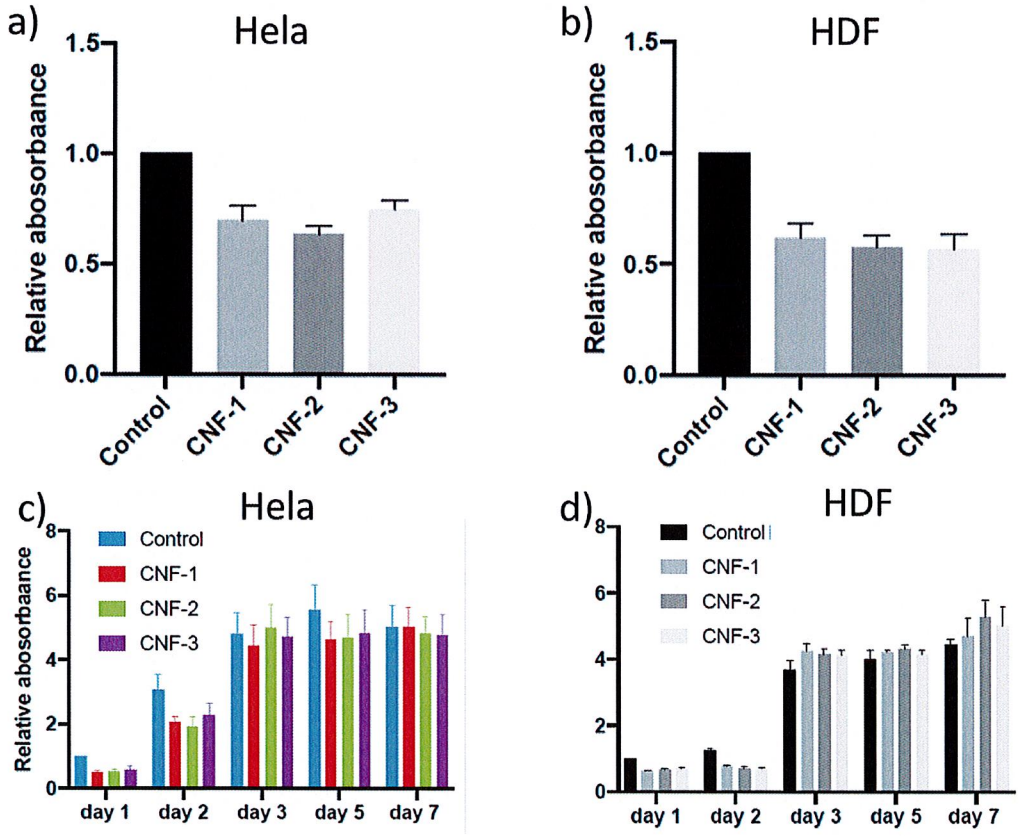

Figure 3. Cell viability tests of Hela and HDF cell lines and proliferation tests of Hela HDF cell lines in 6 days of incubation time.

2. If the work has resulted in publications – attach a reference list.

The work has brought up results for one drafted manuscript and two conference presentations that are under planning.

- The development of the CNF-MA and the UV-cross-linkable formulation is described in the manuscript "*Methacrylated cellulose nanofiber-based photocrosslinkable hydrogel ink for extrusion-based 3D printing*". The manuscript is currently proofing by co-authors and will be submitted to the journal "Advanced Functionalized Materials" shortly in the spring of 2021.
- One conference abstract is under preparation and will be submitted to the 7th International Polysaccharides Conference that will take place in Nantes, France on October 11-15, 2021.
- Another conference on renewable materials (e.g. International Conference on Nanotechnology for Renewable Materials) will be identified for early 2022 when traveling is enabled.

3. Description of how the grant has contributed to competence building that will facilitate and strengthen long term collaboration between Finland and Sweden.

During the research exchange of the hired postdoc researcher to KTH partner, the chemical approach of introducing methacrylate to nanocellulose was found an important approach for ongoing other activities. Thus, PhD candidate Maria Cortes Ruiz supervised by Co-PI Prof. Lars Wågberg has adopted the methacrylation approach to investigate fibril-fibril interactions with protonated or ion-cross-linked nanocelluloses. During the visit to KTH our Post Doc candidate also was able to acquire all the knowledge in the KTH team about fibre modification and CNF preparation. The use of the UV-equipped rheometer at KTH also contributed a lot to the characterization of the crosslinking process.

It is also worth to mention that quantification of surface functionality of nanocellulose has been a challenge. To tackle this challenge, Yury Brusentsev visited the Organic Chemistry Research Group led by Prof. Ilkka Kilpeläinen at the University of Helsinki, who was also operating a research project granted by KSLA. The method was later on transferred to KTH host research group during the research visit by Yury Brusentsev.

The project outcome is a natural part of bioeconomy. It further promotes valorization of materials from trees in 3D printing and biomedical treatments. In the long term, this will certainly benefit both Finnish and Swedish enterprises, in a value chain from forest companies to biomedical entrepreneurs. As an example, Swedish bioconvergence company Cellink and Finnish biomaterial company UPM Biomedicals are committed to biomaterials for life sciences. The current research is well line with the technologies that support their business. In fact, there was a joint project between ÅA partner and UPM Biomedicals to look into formulation of different grades of nanocellulose for 3D printing in 2020. There is a long list of Finnish and Swedish industries (e.g. Orion Oy, CH Bioforce Oy, and Cellutech AB) who can potentially utilize the knowledge that is developed by this project.

Considering the intensive and broad collaborative activities between ÅAU and research organizations in Sweden and other Nordic countries, ÅAU has emphasized in its new strategy for year 2021-2030 to increase Nordic research collaboration and together to promote sustainable development and contribute to the global change. Thus on a general level, the TFV program outcomes make a solid base for further cooperation between ÅAU and Swedish partners and companies in the bioeconomy area.

4. Description of research areas being started or strengthened at the departments in Finland and Sweden.

Modification of nanocellulose has been a challenging topic. Here we developed an approach to anchor methacrylate moiety to cellulose fibres before the preparation of the nanofibrils. Thus, photo-crosslinkable nanocellulose hydrogel has been obtained and can be applied in 3D printing and surface coating, both of which are relevant research areas highly focused in Finland and Sweden.

5. Description of how the grant has contributed to strengthening the forest sector in Finland and in Sweden.

Photo-crosslinkable nanocellulose hydrogel has been assessed in direct ink writing 3D printing and their cytotoxicity has also been assessed. Applications in 3D printing will have a dramatic impact on the forest bioeconomy, especially the form of valuable biomedical solutions. 3D printing produces personalized devices, implants, and drug dosage forms based on individual demands, which is extremely interesting from a medical and therapeutic perspective. In a short term, the developed products can find clinical applications in cell culture and drug screening. In a long term, applications will be extended to implant and pharmaceutical applications. The value application of nanocellulose will most certainly benefit both Finnish and Swedish enterprises, in a value chain from forest companies to biomedical entrepreneurs. As not that many of the established forest companies yet have moved into the biomedical area (with at least UPM making an exception), this will give a valuable insight for them for how to apply e.g. wood-based polymers in tissue engineering and similar areas. This also provides more solutions for ink formulations, for example, in line with CellInk's business.

6. Discription of communication with relevant stakeholders and end users.

With the outcomes of the research, the team members have attended several internal and external seminars and networks:

- The hired Postdoc researcher attended the kick-off seminar organized by the Tandem Forest Values programme and it is planned the research outcome will be presented on final seminar.
- Both Substitute PI Professor Chunlin Xu and project manager Xiaoju Wang have been active in networking, for example, being representing Åbo Akademi University at Tissue Cure Alliance Finland, where most industrial members are directly from medical areas.
- Project manager Dr. Xiaoju Wang held an oral presentation with the title of '3D printing of nanocellulose scaffold as culture platform and tissue mimics' on the International

Conference on Nanotechnology for Renewable Materials 2019 in Chiba, Japan on 3-7.6.2019.

- Project manager Dr. Xiaoju Wang was invited to have presented 3D printing of nanocellulose on the virtual workshop of 3D Bioprinting in Finland on 11.3.2021 (http://www.tissuecure.fi/3d-bioprinting-in-finland/, Xiaoju made presentation). There were more than 100 participants from Finland and abroad.
- Participation of several other workshops was planned however was cancelled due to pandemic.

The public dissemination has attracted attention of industries. For example:

- UPM Biomedicals approached us and contract research on ink formulation of UV-crosslinkable biopolymers was conducted in 2019.
- Currently, another contracted research project with Bayer on ink development for drug delivery solutions is ongoing.

7. Financial accounting

The financial report is appended. Some detailed description on the use of the funding and adjustment is explained as follows.

- There are two parts due to the project coding system was changed at Åbo Akademi University. Thus, the first part is costs of 75485.96 € for 2019, with the old project number 28002009K1. The second part with new number 28002222K1 for the period of 01.2020-03.2021 turned out costs of 113802.51 €. The total cost sums up as 189288.47 EUR.
- There was amending in the costs in 2020 to realize the planned research and tackle the challenges of disturbances that were caused by the pandemic.
    - Chemicals booked to the project 2020 was 9406 €.
    - A small laboratory equipment light-assisted fabrication device (DLP) was purchased from Makex and 50% of the cost (approximately 3000 €) + customs fee + vat 1290 € was booked to the project. 3D printing of developed nanocellulose hydrogel was preliminarily investigated with this instrument.
    - In the last two months of project, when the PI noticed the challenge of accomplishing all planned research tasks due to the disturbances of pandemic, the PI requested to KSLA foundation with Eva Ronquist to amending the use of the remaining budget. The application was approved and the PI was informed by Eva Ronquist. The requested changes were stated in the email on 18 October 2020 addressed to Eva Ronquist and approval to the request of change was confirmed by Eva Ronquist on 30 October 2020. The request is documented as following: 'We have to take proper actions to make sure that we are able to accomplish as much as possible the planned research tasks including, use of more manpower in supporting analytical experts (e.g. Ekaterina Korotkova, Jarl Hemming, in the original budget plan solely to own financing) to provide analytical support, and balancing the budget between travels, materials, equipment costs, and bought services. Moreover, the Åbo Akademi University Mobility Programme financially co-supported the research mobility to KTH. These actions will not alter the employment period of postdoc candidate (1.1.2019-31.12.2020). It will only change the allocation of budget as described above. The specific changes will result in: 9827 EUR that was

part of planned budget for postdoc candidate salary to analytical support experts (Ekaterina Korotkova, Jarl Hemming) and about 10000 EUR that was planned to use for supporting mobility to materials, equipment costs, and bought services. In summary, this requested change will not reduce the resources of Postdoc, but change other supporting costs including, analytical support, materials, equipment, travel, and services.'

Date: 20. March.2021

Signatures:

Prof. Chunlin Xu (Substitute PI to Stefan Willför), PI, Åbo Akademi University

Prof. Lars Wågberg, PI, KTH Royal Institute of Technology

Stefan Willför
Vice Rector
Åbo Akademi University

Prof. Stefan Willför, Vice Rector, Åbo Akademi University