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ENGINEERING NANOCELLULOSE-BASED MATRICES FOR 3D CELL CULTURES

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ABSTRACT

Cell and organoids culture in three-dimensions (3D) gel systems is important from a fundamental aspect for understanding the development and behaviour of body organs, and from a practical perspective for producing cells, tissues and even new organs for bio-medical applications. The cell culture requires a supportive network environment, biological or synthetic, which provides the suitable biological systems (proteins and co-factors), mechanical support (flexible morphology) and chemical composition for cells/organoids to grow, spread and migrate. Current naturally extracted matrices like, Matrigel and collagen, are expensive with poorly defined and variable composition; they are not reliable for common practice 3D organoids culture. To overcome issues with the naturally extracted matrices, researchers have been investigating and developing new synthetic and natural polymer gels as alternatives. Cellulose has emerged as an attractive matrix with strong potential for cell and organ culture in 2D and 3D networks. The inherent natural biocompatibility of cellulose fibres including non-toxicity, low cost, and their ability to form flexible gels, provide a compelling alternative to the current limited and expensive

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animal-based matrices. This review focuses on the recent development of cellulose nano fibres (CNF) based gel matrices for 3D cell and organoids culture. The review highlights how functionalisation of CNF optimizes the gel structure, visco-elastic properties and composition for supporting cell growth, interactions, spreading and migration. The state-of-the-art characterisation methods are discussed to monitor CNF stiffness, strength, morphology and composition, and furthermore, cell culture and their stability in the CNF network. The knowledge gained from this review aims at supporting bioengineers in further developing the potential of CNF gels for different 3D organs culture and tissue engineering applications.

Key words: Nanocellulose fibres (CNF), Organoids, Cell culture, Matrigel, Hydrogel

INTRODUCTION

Organoids are three-dimensional (3D) cellular structures capable of self-organizing and -regeneration 'on a dish'. Organoids are in vitro organs grown in a media that mimics the actual organs in the body [1]. These biological models resemble the physiological characteristics of developed organ and tissues, mimicking their behaviour and functionality [2]. Organoids have been used for a range of applications, highlighting drug development, screening and disease modelling. Indeed, biobanks of patient-derived organoids have allowed to predict the effects of anti-cancer treatments, matching lab results with clinical outcomes with nearly 85% accuracy and 91.97% specificity [3, 4]. Stem cells or tissue samples extracted from the body are used to construct organs [5] (Fig 1A). A 3D cell culture is better than a 2D culture as it permits investigation of complex 3D structure of organoids and also increases cell survival rate [6]. Researchers around the globe have grown and modelled organoids for brain [7], kidney [8], pancreas [9], intestine [10] and liver [11] with size ranging from micrometres to a fraction of a centimetre. Organoids studies have shown promising applications as therapeutic tools in understanding the complex functions of organs and exploring reasons and cure for different diseases developed in an organ [12]. Recent advances in tissue engineering and biomanufacturing approaches have prompted the emergence of complex, hybrid organoid systems [13] and high-throughput platforms [14].

Organoids are typically grown embedded or on the top of a biomaterial that recreates the extracellular matrix (ECM), and therefore, emulates cell-matrix interactions critical for cell proliferation and differentiation [15, 16]. There are

multiple commercial ECM gel networks on the market. Matrigel, MaxGel™ (MG), ExtraCel™ (EC), HydroMatrix™ (HM), PuraMatrix™ peptide (PM) are the main commercial hydrogel products for tissue culture and organoids. Despite this variety of ECM-like materials available for organoid cultures, the use of the laminin-enriched matrix, referred to as Matrigel, remains the 'gold standard' widely adopted by most laboratories worldwide. Matrigel is extracted from the basement membrane of Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains several ECM elements, such as collagen, laminin, entactin, nidogen and glycosaminoglycans [17, 18]. Matrigel provides a favourable chemical and mechanical environment to grow organoids of colon [19], liver [20], and pancreas [21].

However, this animal-derived matrix also presents a range of undefined proteins and peptides that can vary by up to 50 (wt) %. In addition, due to its animal source origin, Matrigel suffers from a critical batch-to-batch variability, which limits experimental reproducibility [22]. From a material's perspective, Matrigel is considered a 'soft' matrix with stiffness around 100 Pa, a value distant to those of human organs like breast and liver [23, 24]. The high cost and logistic challenges in the supply chain of Matrigel have hindered its reliance for organoid. Thus, alternative matrices of well-defined composition, high batch to batch consistency and low cost have been investigated and developed [22]. Kozlowski reviewed alternative materials like decellularized extracellular matrix (ECM), synthetic hydrogels, or gels with proteins to grow organoids in Matrigel free network[22] (Fig 1B).

Cellulose has emerged as an attractive alternative material for developing novel biomaterials, bio-diagnostics, biosensors and other advanced biotechnologies. Cellulose is the most abundant polymer available on earth. It is a polysaccharide formed by the linear arrangement of glucose as monomeric unit [25]. In cellulose, the glucose units are linked by the β-1, 4 glyosidic bond into long chains, networked together into hierarchical structures by a network of hydrogen bonds [26]. These chain networks form cellulose nano- and micro-fibres which are the building blocks of plants, trees and some bacteria [27]. The nanoscale cellulose fibres (cellulose nanofibres; CNF) of diameter 5–20 nm and micrometre long have shown great advantages over their bulk counterparts. CNF can be extracted from raw pulp by mechanical, chemical or enzymatic treatments. CNF has found many applications as suspension, powder, gels [28], thin films [29, 30], foams [31] and other biofriendly advanced technologies. In particular, CNF hydrogel network was found to be suitable for sustaining biomolecules and their activity [32]. The cellulosic gel network provides the suitable micro- and nano-scale environment for biomolecules to survive while retaining their functionality.

This review focuses on the different pathways for engineering and using CNF-based hydrogel matrices for organoids culture. Different modifications and

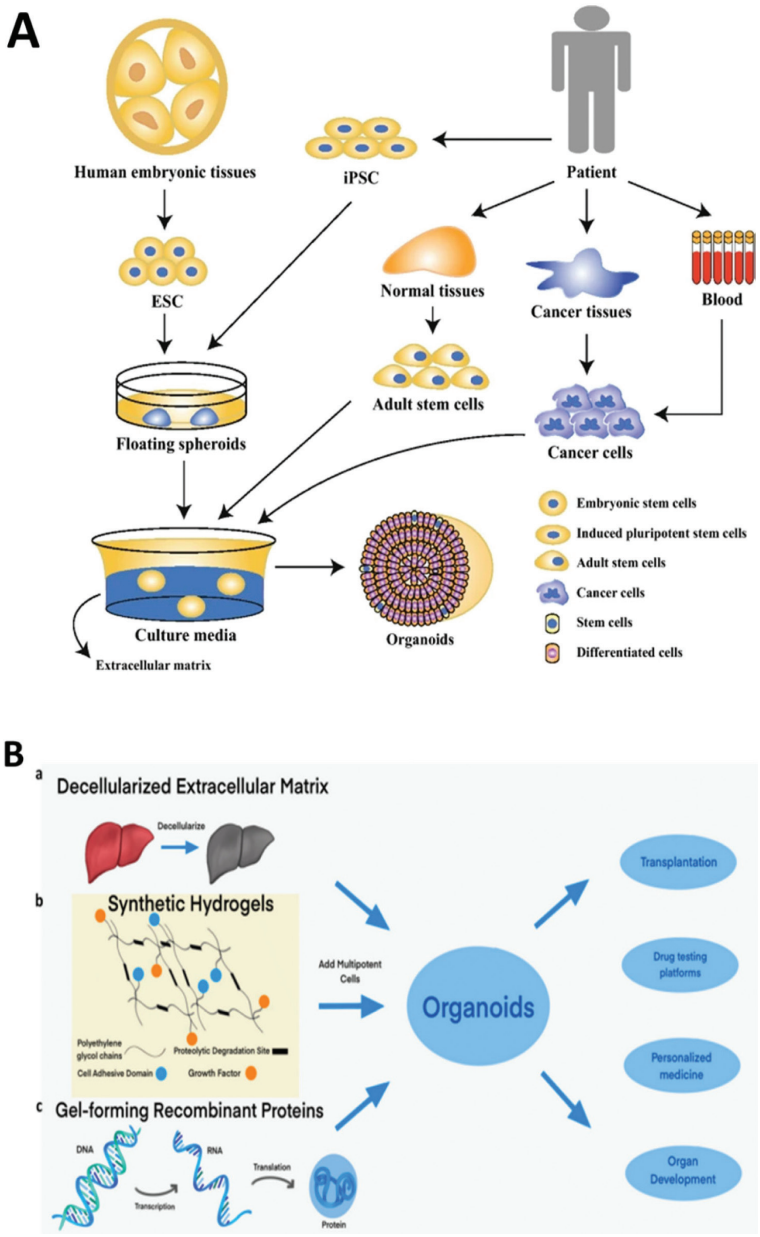

Figure 1. (A) Growth of 3D Organoids from tissue sample [33]. (B) Different methods and materials applied to grow organoids as an alternative to Matrigel [34].

functionalisation of CNF chains can enhance the formation of the hydrogel network environment used to promote the cell culture, movement and their migration in the network. The mechanical, visco-elastic, chemical and structural properties and characterisation required for growing organoids in CNF gel matricx are analyzed. We hope this review will enable bio-engineers to develop biodegradable, low cost and high strength CNF gels for a variety of specific 3D organs culture, diagnostics, drug delivery, biosensors and tissue engineering applications.

NANOCELLULOSE MATRICES FOR 3D ORGANOIDS CULTURE

The use of CNF for tissue generation has received considerable attention in the recent years. CNF forms hydrogel at low concentrations (< 1 wt%) by a combination of entanglement of fibres, ionic interactions and hydrogen bonding[35]. It has the similar inherent properties of collagen. CNF is used as a hydrogel to grow organs which can help in replacing the diseased or damaged organ [36]. It is an effort to engineer CNF hydrogel to mimic ECM and Matrigel matrices. To support the culture of organoids and spheroids, cellulose-based hydrogels must form a porous 3D structure absent of cytotoxic elements. For example, residual oxidizing reactants would injury cellular structures and cause cell death. In addition, it is essential that cellulosic matrices are swollen in physiological buffers, such as phosphate saline, that mimic the isotonic microenvironment that surrounds cells in the human body.

To recreate a microenvironment that supports cell growth, proliferation and differentiation, ECM-like biomaterials must recapitulate the essential signals derived from the cell-matrix interactions [16]. In this context, nanocellulose has been functionalized with cell adhesive sequences and full ECM proteins that activate and regulate integrin-associated signalling pathways [37–39]. Carboxylated CNF was grafted with fibronectin-derived sequences (RGD peptides) and formed into a hydrogel for the growth of intestinal organoids (Fig 2A) [37]. Glycine was used to balance the osmolality of this hydrogel (295 mOsmol/kg) whilst preserving the colloidal stability of the matrix. In order to achieve the required mechanical properties, CNF fibres were physically crosslinked with Ca^{2+} ions. The clear and reversible shear thinning hydrogels developed reached a storage modulus equivalent to that of Matrigel. Intestinal crypts were cultured during 4 days and formed organoids with cell viability as high as 80%. Organoids were stained for the expression of proliferative signals (Ki67 marker) and showed stable levels of metabolic activity. Transcriptome sequencing revealed a good correlation to organoid grown in the hydrogel and Matrigel. However, as observed for many other matrices [40], the organoids could only be cultured in CNF hydrogels after being firstly established in Matrigel.

In another approach used to assess its compatibility with full ECM proteins, nanocellulose was blended with type I collagen [38]. This thermo-responsive matrix presented sol-gel transition and viscoelastic behaviour equivalent to those of Matrigel. Intestinal organoids embedded in collagen-nanocellulose maintained their epithelial integrity and a major living area during 3 days in culture. The expression of the stem cell marker OLFM4 suggested that collagen-nanocellulose could sustain the self-renewability of organoids and allow its continuous passaging.

Recently, cellulose nanocrystals (CNC) blended with gelatine was used as a matrix for the culture of breast cancer organoids [41]. The storage modulus of CNC-gelatine hydrogels could reach up to 1 kPa, based on the concentration of CNC added. Organoids were formed and could be passaged up to 4 times, whilst showed the expression of proliferative signals (Ki67 marker). Organoids grown in this hydrogel showed a response to treatments with chemotherapeutics similar to those cultured in Matrigel. These reports present the potential of functionalized nanocellulose hydrogels as fully engineerable matrices for the culture of a variety of different organoids, each with their specific requirements. Here, nanocellulose provides the mechanical support and chemical system that is fully compatible and independent of the biological system.

Gehlen et al developed granular CNF hydrogels for 3D cell culture [36]. The granular CNF hydrogel sustains a high biocompatibility, porous network to support cell adhesion, spreading and migration. The hydrogels were fabricated by pre-crosslinking of TEMPO-oxidized CNF using calcium ions, followed by mechanical crushing through a 10-micrometer sized nylon mesh. Later, the granular CNF hydrogel were mixed with fibroblast to crosslink with the culture matrix. The granular CNF hydrogels show lower stiffness which supports a better distribution of the mixed fibroblast compared to the non-granular CNF hydrogel. The authors claim the method is cost effective and can be automated and upscaled. Further, the granular CNF hydrogel is promising in replacing the expensive Matrigel. Bhattacharya et al used CNF hydrogel for promoting 3D liver hepatocytes cell culture without using any bioactive molecules [42]. The CNF hydrogel culture is mixed with Hepatic cells; HepaRg and HepG2 (HepG) from liver tumours. The CNF hydrogels concentration ranging between 0.1–1.2 wt% successfully allows the growth of 3D spheroid formations of HepaRG (after 30 days) and HepG2 (after 5 days) cells. There was no cytotoxicity detected during growth of cells. By comparison, the CNF hydrogels mimic the properties and environment of the commercial gels of MaxGel™ ECM (MG), Extracel™ hydrogel (EC), 0.25% HydroMatrix™ peptide cell culture scaffold (HM) and 0.25% PuraMatrix™ peptide hydrogel (PM).

Xing et al used gelatine crosslinked cellulose fibres to make scaffolds for growing 3D brain and human mesenchymal stem cells in vitro [43]. The cellulose

based gelatine network provides a support 3–8 times stronger than gelatine alone, with improved porosity and superior tensile strength for improved cell culture. The 70% porosity with large pore size is suitable for the transport of the nutrients required for efficient cell growth in the network. The incorporation of cellulose fibres allows cells to culture in a separate pathway rather than forming the aggregates and clumps observed in the pure gelatine network.

Besides its nanoscale dimensions, another advantage of the CNF is the possibility to functionalize their surface with a variety of chemical groups via physical and chemical interactions [44, 45]. The presence of a high concentration of negative hydroxyl (OH^-) groups at the nanocellulose surface allows to functionalize the CNF surface with carboxyl (COO^-) [45], azide [46] and phenolic esters [47], as well as biomolecules such as RGD (Arg-Gly-Asp) [48], protein IKVAV (Ile-Lys-Val-Ala-Val) [49], xyloglucan-RGD [50] and growth factors such as VEGF [51]. Mendoza et al functionalized CNC by grafting biosourced and biodegradable phenolic esters (diethyl ferulate) using the click chemistry based on the copper-catalyzed azide/alkyne cycloaddition reaction. These phenolic functionalized CNC were blended in polyvinyl alcohol (PVA) network [47]. The obtained transparent nanocellulose-diethyl ferulate showed excellent protection against the harmful effects of UV radiation. The surface functional groups allow specific selectivity and functionality to the nanocellulose to interact with a specific biomolecule, polymer chains or organic/inorganic nanoparticles.

Curvello et al functionalized TEMPO oxidised CNF with the RGD cell adhesive peptides and cross-linked the fibrils with cationic Ca^{2+} and Mg^{2+} to form hydrogel networks for culturing 3D intestine organoids [39]. The organoids were successfully cultured in the CNF matrices for 4 days and recovered for passaging and RNA extraction (Fig 2B). On the 4^{th} day, the organoids show signals of budding and maintain a high viability RGD-CNF hydrogel comparable to Matrigel, the current gold standard. Rheology measurements showed a smaller value of storage modulus 47 Pa for $CaCl_2$ ionic crosslinked RGD-CNF hydrogel, which is slightly smaller than the Matrigel storage modulus of 66 Pa. Mendoza et al found the storage modules of 60 Pa for collagen to provide a good support for the 3D growth of organoids [52]. In comparison, the granular hydrogels formed by Gehen et al showed a significantly higher value of storage module of 746 Pa [36]. Bodin et al functionalised bacterial CNF with xyloglucan (XG) and XG-RGD pentapeptide to monitor the Endothelial cells adhesion onto the cellulosic surface. A higher level of cell adhesion was observed on the XG-RGD modified CNF which was linked to the RGD epitope[50]. In another study, bacterial CNF functionalized with the peptide IKVAV showed improved attachment with the neuronal and mesenchymal cells [49]. Leppiniemi et al functionalised anionic CNF by conjugation with avidin (Avd) to prepare hydrogel for 3D culture of Fibroblasts [53]. The Avd is covalently bonded to the carboxylic group of

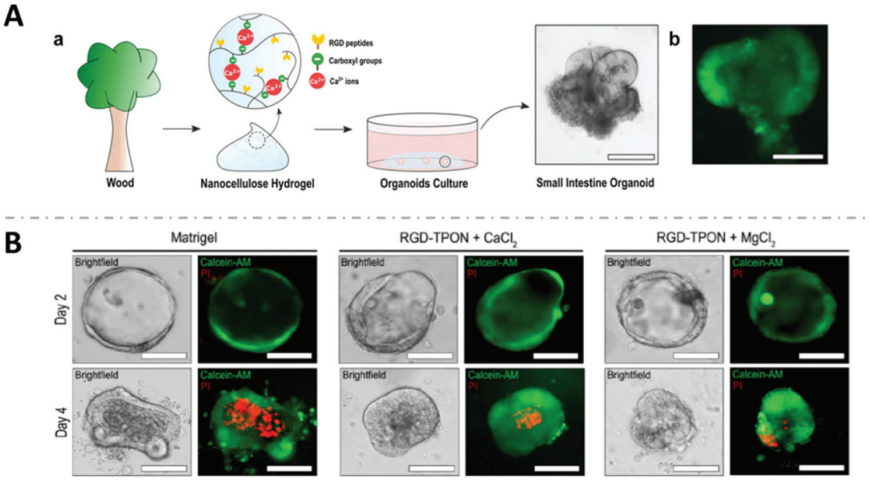

Figure 2. (A) Carboxylated CNF was functionalized with RGD peptides to form a surface that mimics the cell-matrix interactions for organoids culture. Organoids were formed after 4 days and had a major living area (green). Scale bar = 100 μM [37]. (B) Intestinal organoids seeded in Matrigel and RGD-CNF hydrogels cross-linked with $CaCl_2$ and $MgCl_2$ and observed culture for 2nd and 4th day. On the second day of culture, cystic organoids present a major viable area. On the fourth day, organoids in Matrigel and RGD-CNF hydrogels show signals of budding and maintain a high viability with a minor population of apoptotic cells. Scale bars: 100 μm [39]. Reprinted with permission from Curvello et al [39]. Copyright 2021 American Chemical Society.

CNFs by the classical EDC/NHS-chemistry. The Avd-CNF conjugate prepared exhibits shear thinning and gel-forming properties. Further functionalization of Avd-CNF with biotinylated fibronectin (B-FN) allows 3D culture of mouse embryonic fibroblasts. Higher cell proliferation rates were also observed in the B-FN-AvD-CNF. The B-FN-AvD-CNF hydrogel shows 13.7 times higher cell growth after 7 days compared to the AvD-CNF. Thus, CNF surface functionalisation can significantly affect the viscoelastic, chemical and structural properties of gel and make the gel suitable for tissue culture. However, despite being classified as biocompatible, plant-based nanocelluloses are still isolated by harsh reactions, such as TEMPO-mediated oxidation. These reactions require safety procedures to prevent health issues related to the exposure of hazardous chemicals and the management of toxic wastes. Therefore, it is critical that novel green, non-hazardous protocols to isolate cellulose nanofibres and form nanocellulose hydrogels are introduced.

CHARACTERISATION OF CELLULOSIC HYDROGELS

Characterisation of the organoid morphology, the hydrogel matrices structure and mechanical properties, and the interactions between hydrogel network and organoids are challenging. Obtaining such information is important in understanding and co-relating the initial and final phases of the organoid's growth, spread and migration in their matrices. The mechanical and structural properties of the CNF hydrogel network depend on the crosslinking density, fibre dimension, stiffness, viscoelasticity, mesh size and chemical composition. Cellulose hydrogels may contain fibres of different widths and lengths as well as the presence of bundles. These variable dimensions affect the mechanical properties of the matrix, hence modulating the cell behaviour. In addition, larger fibres or bundles would decrease the hydrogel transparency and prevent the imaging of 3D cell structures. Therefore, it remains critical that cellulose-based gels are formed by nanofibres of dimensions similar to those of extracellular matrix proteins, such as collagens (2 nm diameter, several microns length). Different direct imaging and indirect scattering characterising methods such as: Rheology, Atomic force microscopy (AFM), Dynamic light scattering (DLS), Small angle X-ray scattering (SAXS), Scanning electron microscopy (SEM), phosphorescence lifetime imaging microscopy, Radiometric methods, microfluidic devices, and optical microscopy have been applied to quantify and investigate the structure and viscoelastic properties of hydrogel network and the morphology of the organoids cultured [2, 54, 55].

Rheology is often used for the mechanical characterisation of the gel network. In a previous study, we have quantified by rheology the effect grafting peptide (RGD)-Glycin to nanocellulose (CNF) has on the properties of hydrogels engineered for organoids culture. The rheology results showed the RGD-GLY hydrogels to have similar shear thinning and viscoelastic properties as Matrigel [37]. Krüger et al also used rheology to compare the Matrigel viscoelastic properties with the CNF hydrogels [56]. The low-cost CNF hydrogel exhibits the properties required in replacing Matrigel. They used different amplitude and frequency sweeps, alternating strain, and dynamic viscosity measurements for the mechanical characterisation of hydrogels. The alternating strain measurement shows the high resistance to strain of CNF hydrogels which is important in its recovery without losing properties. The G' and G'' in the frequency sweep measurements reveal the strong internal networking of the CNF fibres. In contrast, Matrigel shows weak resistance to strain, poor viscous response and does not show restore capability (self-healing) of its structure as CNF hydrogel does.

Magno et al provided a guide of different polymer hydrogels for organoid cultures with their advantages and limitations [57, 58]. They applied AFM to study the microscale mechanical properties of kidney-derived ECM fibrillated hydrogels in the media with variable concentration of Ficoll400. The elastic

moduli evaluated from AFM shows a decrease in the stiffness of the hydrogels from 391 Pa at standard conditions to 22 Pa at higher concentration of 25mg/mL of Ficoll400. The SEM study of these samples shows an increase in the fibre diameter as increases the Ficoll400 concentration.

Confocal microscopy has emerged as a promising direct method to visualise the growth and interaction of 3D organoids and spheroids in all three dimensions. Lou et al used confocal microscopy to visualize the 3D spheroids of Human embryonic stem cell (hESCs) and induced pluripotent stem cells (iPSCs) cultured in a nanocellulose hydrogel network [59]. The images clarify the formation of 3D spheroids in the 0.5 wt% CNF hydrogels after 3 days. However, increasing CNF concentration to 1 wt% hinders the growth of spheroids. The 0.5 wt% CNF gel has a lower viscosity which is favourable to the 3D spheroid formation. The CNF concentration dependent mechanical properties of hydrogel allow controlling the growth of the cells in the CNF network. In another study, Magno et al used immunofluorescence stained confocal microscopy to track the macromolecular crowding effect on the retention and distribution of ECM components in Kidney-derived scaffolds [57]. The images confirm the incorporation of ECM components in the fibrillar networks.

Microfluidic technologies are gaining interest in characterizing the movement or migration of cells, drugs and proteins during organoids growth [60, 61]. Anguiono et al used a microfluidic platform to determine the migration of H1299 lung adenocarcinoma cancer cells from the collagen and collagen-Matrigel hydrogel network [62]. They reported the cancer cells were migrating faster in the collagen Matrigel compared to collagen alone. This is due to the high crosslinking density, higher stiffness and large pore size of the collagen-Matrigel compared to the collagen gel alone. They concluded that microfluidic devices combined with the imaging methods is useful for developing tumoral invasion approaches in different environments created in the microfluidics.

CONCLUSION AND PERSPECTIVES

The recent development of cellulose nanofibres (CNF) gel matrices show strong potential in mimicking the expensive and unreliable commercial Matrigel for 3D organoids and cell cultures. The CNF surface functionalisation and tunable structure promote encapsulation, growth, spread and migration of cells in the CNF network. Different functionalisation strategies of CNF by peptides, polymers and specific ions can provide the favourable specific environment required for cells to grow with their sustained functionality. The mechanical and structural properties of CNF hydrogels mimic those of commercial Matrigel and collagen; CNF functionalization can also provide a similar chemical environment and stability. The

developed CNF gel networks have been successfully applied in the culture of different types of cells and growing different 3D organoids.

There has been enormous advancement in the engineering of nanocellulose hydrogels for cell culture. Among the next frontiers are the challenges in probing the dynamics, theoretical aspects, atomic and nanoscale changes to be resolved at both the spatial and temporal resolution. Further progress is required to engineer cellulosic network hydrogels having fully tunable viscoelastic, mechanical and thermal responsive properties over a wider range. A controlled and flexible structure of the CNF 3D mesh is required to populate single and multiple cell growth. Advance in nanoscale characterisation methods is needed to track the early stages of cell culture and their respective changes in the gel network. The biocompatibility and hydrophilicity of CNF and its flexible functionalisation have risen the CNF hydrogel matrice as a performant and promising material for tissue engineering, cell culture and growing 3D organs to replace diseased and damaged organs.

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ENGINEERING NANOCELLULOSE-BASED MATRICES FOR 3D CELL CULTURES

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So you answered my last question with your conclusion which is good, but my first question I had was, now that you can use plant-based material to form a gel, you are not limited to wood-based cellulose. What is the optimal plant-based material that maybe would be used for this purpose? How would we go about figuring that out and do you think there is something better than cellulose?

Gil Garnier

Very good point, I still believe that nanocellulose is the best because it is just the simplest form of plant-based material. We could go with chitin or chitosan but this is animal based. I think we can do a lot with cellulose because it is a material that's easy to engineer. Let's think about what we are trying to do. Basically, the gel we are discussing is made of about 99.9% water, so all I do is to take my organoids and cells and immobilise them in a position and these small organs like to be pampered like a baby; that is not to be wrapped too tightly or too loosely. The organoids are the same, so what we want to do is to wrap a kind of structural network around them so that can expand as they grow but still provide enough elastic response as support. It is like if you have socks, you need a bit of compression on the leg: not too much, but just enough, and depending on the type of person you need to adjust

this tension. So I think the nanocellulose provides a fantastic opportunity because you are going to suspend organoids and hold them separate. Also, there is a lot of biological inhibition, and if you can keep the organoids separated, because they secrete biomarkers which affect each other, that is a benefit. And then if you just suspend organoids in a fibrous network, then you can flow the nutrient around them to improve growth. We do need more knowledge, because what I realise is that the biology component is a bit like black magic. There is a secret recipe with 15 or so ingredients that you add in a defined order and in a precise sequence, so I do think that we need to improve our knowledge. But if we rely on a simple fibrous network it is easier to control, and I think the nanocellulose does a great job for that. Here we use eucalyptus but we can have same rheology with radiata pine and long fibres, and I know the UPM is using beech fibres. It doesn't matter because they have same diameter and relatively the same aspect ratio, and I think we can get the same gel conformation and support. So, I may be wrong, but that is my current understanding and that is the approach I am investigating.

Lars Wågberg KTH Fibre and Polymer Technology

I have a bit of problem with your gel definitions here, so you have a physical gel and not a covalently cross-linked gel in the system you describe. I can buy that to some extent but then you claim that it is self-healing. In the essence of this term, I wonder if it really is self-healing?

Gil Garnier

You asked a very good question, I will maintain that yes, the gel is self-healing. What we did was to measure gel rheology under the cycle of shearing followed by relaxation, while we were measuring the rheology. We achieved fairly constant rheology (G″ and G″) after each cycle of relaxation.

Lars Wågberg

If you make an experiment where you perform a stress relaxation at increasing strains, I wonder if you get a self-healing at all strain? In my experience you get a final break-down when you pass a certain strain limit in these types of systems. Is there not a strain-limit?

Gil Garnier

I am sure there is a strain limit but if we shear at constant strain, we will be able to do 4 or 5 cycles and achieve fairly constant rheology. However, I do not know the limits as you increase strain.

Lars Wågberg

You are not just stretching the segments of gel, you are also stretching the definition! You also showed so much data that I had trouble in getting it all in. You showed data from high resolution scattering techniques, which showed that the fibrils had dimensions of around 3 nanometres, then you showed an inter-fibrillar distance and then the mesh size of the gels. How do you differentiate between the inter-fibril distance and the mesh size? How are they different?

Gil Garnier

We need to have Vikram here. Basically, we look at the intensity – the Q-1 relationship of the SANS curves, and analyse the different regions of the curves, and especially the peaks, by using various models (rod–rod or rod–sphere) to calculate the fibre diameter and fibre -fibre distance as well as aggregates by curve fitting.

Karin Zojer Graz University of Technology

And I would like to follow up a little bit the question of agar if I may, and you might help with some insight to broaden my perspective, maybe a little bit. What is your take? What does a matrix material have to bring to the table to enable 3-dimensional growth, some real 3-dimensional growth? As opposed, for instance, to agar, which is also 99.999% water but it appears to prevent the 3-dimensional growth?

Gil Garnier

That is a good question as Agar has been a gold standard for so long. However, it is a water soluble carbohydrate, while nanocellulose is a fibrous gel which presents key advantages in terms of mechanical strength and transparency (to observe cells and organoids). I wanted to see what we can do with nanocellulose, but I expected to fail. I knew that UPM was growing cells in nanocellulose gels, and they might have done organoids by now, so I knew it was possible and biologically compatible. But I never thought about matching the properties of Matriogel which is the best material. All I wanted to see is how far we can push nanocellulose for biological applications. I have always been fascinated by gels and the fundamental work of De Gennes, so I would say it was just curiosity. I do not claim nanocellulose gels are the best, but I think we can make it work to grow organoids and cells.

Karin Zojer

I like the example, because I have seen also a group which was able to cultivate lung tissue in 3-dimensional fashion in paper, so possibly the fibre aspect is also quite interesting.

Discussion

Alexander Bismarck University of Vienna

Lots of people are interested in organoids and most of bioengineers tend to use alginate to grow organoids, and it does work too. Where is cellulose better than alginate, also a polysaccharide, and before you answer, may I have another one? You use silver and monomers that are not so nice for tissue engineering applications, how do you purify your gel to get rid of every trace of your thermoresponsive nanocellulose in order not to kill your organoids?

Gil Garnier

I will start with your first question which is easiest. Why did we decidd to go with nanocellulose and not alginate? It is simple, our biomedical group at Monash works on cancer and is buying Matrigel, which is the gold standard and best gel to grow organoids. They currently spend thousands and thousands of dollars to test the effects of their new drugs and cancer organoids and my friend was very frustrated with having to pay $10,000 to get 10 flasks and test 10 conditions on patients. So, for them it was Matrigel or nothing. I have never heard anybody at the Cancer Institutes mention alginate. So, for better or worse, I decided to investigate nanocellulose gels as they could be so much cheaper. The raw materials are around 1 million times cheaper.

I was ignorant; that is the real answer, and I decided to continue in my ignorance and went from ignorance to a bit of success, so that is how we started with the nanocullulose gel and we adopted an engineering perspective.

I listened to what were the requirements the bio-engineers were looking for in their gels, and we had a few hypotheses. Our organoids cultures worked and eventually matched the properties of Matrigel – the best material. I do not say that nanocellulose gel is better than alginate – as I have never tried it. I would like to measure the ionic content of alginate because the salt content is such a big issue. Curiosity, naivety and a simple approach is the best way to start. Now I am a little bit less ignorant to start to be competent. So, nanocellulose has three hydroxyl groups and if you are not happy with one hydroxyl group, you can react it into carboxylate groups, so they are very flexible. I wanted to see how far we can go with the nanocellulose and I think we can still develop the material much further. The best proof of that is that UPM has built a business on the concept, so that is the first and easy part of the question.

The second question is did I remove the silver catalyst used to graft NIPAM on nanocellulose to make them thermosensitive? Here, we simply added the Silver catalyst as initiator, and then the NIPAM monomers until the level of polymerisation needed. We then cleaned the fibres with water by dialysis for a few days to hopefully remove monomers and catalyst. Last, we made and characterised gels.

Silver is difficult to remove. However XPS analysis did not show the Ag peak. For biological applications, sterilisation is critical. Here, we sterilised all gels using UV before any organoids experiments.

Alexander Bismarck

I was a bit surprised to see that Matrigel, the commercial gel to grow organoids, consists almost completely of collagen but in the sample that you produced out of pure collagen the cells were dead after 4 days?

Gil Garnier

Well, mostly. Commercial Matrigel is grown from tumours inflicted on laboratory mice; so that is a problem. We do not know how many elements and biomolecules are also present and in which concentration, nor the type of collagen. For our experiments, we used type 1 because it is the most common and most affordable. However, we do not have all the critical growth factors of Matrigel and that is one of the problems that we soon discovered here. Currently, growing organoids in gels is really biologically driven and the science in biology is not similar to physics; it is not as vigorous. There is a recipe that works and is used religiously. I do not like to use religious concepts in science. I like to understand why we use each element and how each affects the mechanism. However, we are not quite there yet.

Alexander Bismarck

What make you think that magnesium is better than calcium to crosslink nanocullulose cells? You showed it grows but why should now the gene expression be different?

Gil Garnier

For whatever reasons, calcium inhibits some critical genes in Colon Organoids, as we have measured by RNA analysis of the organoids. Magnesium does not have this genetic effect.