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Scaffolds for 3D Cell Culture and Cellular Agriculture Applications Derived From Non-animal Sources

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For decades, two-dimensional cell culture has been regarded as a major tool in cellular and molecular biology due to its simplicity, reproducibility and reliable nature. However, it is now recognized that 2D cell culture underrepresents the *in vivo* environment of living cells. The development and use of 3D scaffolds and biomaterials provide researchers an ability to more closely mimic the *in vivo* environment. However, many biomaterials are of animal origin, leading to variability, environmental and ethical concerns. Here we present three animal-free scaffolds: decellularized plant tissue, chitin/chitosan and recombinant collagen. Decellularized plant tissue provides a wide array of structures with varying biochemical, topographical and mechanical properties; chitin/chitosan-based scaffolds have shown synergistic bactericidal effects and improved cell-matrix interaction; and lastly, recombinant collagen has the potential to closely resemble native tissue, as opposed to the other two. These benefits, alongside potential scalability and tunability, open the door to applications beyond the biomedical realm, such as innovations in cellular agriculture and future food technologies.

Keywords: 3D cell culture, animal-free, cellulose, chitin, recombinant proteins, chitosan

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INTRODUCTION

Since the early 20th century, two-dimensional cell culture has been regarded as a reliable, simple and reproducible study of cellular behavior (Jedrzejczak-Silicka, 2017). However, a direct comparison between 2D and 3D cell culture is challenging due to dramatic differences in the cellular environment. *In viv*o, cells interact closely with other cells, a complex array of physical forces/stimuli, and biologically active extracellular matrix (ECM). In contrast, 2D cell culture is performed on a substrate with drastically different mechanical and biochemical properties (**Figure 1**). Comparisons between 2D and 3D cell culture have revealed significant differences in proliferation, differentiation, drug toxicity resistance, gene expression and protein synthesis (Huyck et al., 2012; Antoni et al., 2015; Ravi et al., 2015; Cavo et al., 2016; Fang and Eglen, 2017; Riedl et al., 2017). In order to overcome the gap between 2D cell culture and the 3D environment sensed by the cell *in vivo*, a plethora of natural and synthetic polymers, recombinant proteins, ceramics, and metal-composite scaffolds have been developed and reviewed previously (Carletti et al., 2011; O'Brien, 2011; Turnbull et al., 2018). Yet, in order to produce scaffolds with similar characteristics to those of the ECM, animal-derived polymers such as collagen are often considered

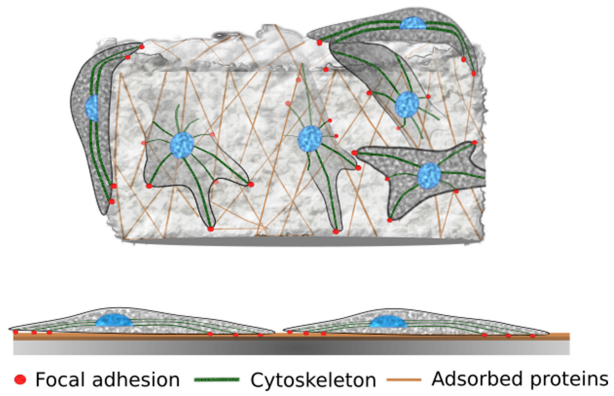

FIGURE 1 | Cells on 3D porous substrate vs. 2D substrate**.** Cells on 3D porous substrates can be found on the surface and interior, whereas cells on Petri dishes are bound to a 2D environment. Proteins naturally found in animal serum and those synthesized by cells adsorb to the surface of the material and facilitate cell adhesion. Through focal adhesion, adherent cells are able to interact with the substrate; therefore, the properties of the material (e.g., Stiffness) can influence the cell's behavior and morphology.

as the gold standard. However, the dependence on animals have made them undesirable due to variability (Shoseyov et al., 2013), environmental (Kraham, 2017), and ethical concerns (Verbeke and Viaene, 2000). Moreover, the scalability and consumer acceptance of cultured meat products will rely on a disconnect from animal sources. Research into animal-free scaffolds has emerged as a potential source for consistent, chemically defined and low-cost materials.

Synthetic or natural animal-free polymers such as cellulose (Huber et al., 2012; Hickey et al., 2018), chitin/chitosan (Jayakumar et al., 2011), alginate (Lee and Mooney, 2012), recombinant silk (Widhe et al., 2010), PLA (Serra et al., 2013), and PCL (Li et al., 2017) provide low cost, consistent and tunable scaffolds. In this concise review we have chosen to focus on chitin/chitosan, cellulose (bacterial and plant), and recombinant collagen and their use in tissue engineering and potential applications in cellular agriculture. The biomaterials chosen here meet the criteria for cellular agriculture applications, such as animal-free, abundant, biocompatible, versatile, provide nutritional benefits, and are already part of commonly consumed products. However, we recognize that many other biomaterials and scaffolding approaches do exist and, as above, we refer the reader to other topical reviews for a deeper examination of those strategies (Carletti et al., 2011; O'Brien, 2011; Derakhshanfar et al., 2018).

As tissue engineering and regenerative medicine continues to expand with promising results, the potential for novel food applications has arisen due to the similarity in techniques and approaches. Although meat/tissue has proven to be difficult to replicate *in vitro* due to its complex composition (muscle, nerve, water, minerals, growth factors, hormones, and Extracellular matrix proteins) (Listrat et al., 2016), the native structure of foods such as mushroom (Jo-Feeney et al., 2014) and jackfruit (John et al., 1992) have the potential to contribute the expected palatable properties of meat. In addition to rheological properties, these foods contribute nutritional benefits, such as insoluble fiber (McDougall et al., 1996; Cheung, 2013).

DECELLULARIZED PLANT TISSUE AND BACTERIAL CELLULOSE

As the most abundant polymer in nature (1.5×10^{12} tons of total biomass) and the main component of plants, cellulose, a 1-4β D-glucose polymer has shown great potential as scaffolding material due its low cost, versatility and overall biocompatibility (O'Sullivan, 1997; Klemm et al., 2005). Cellulose hydrogels (Isobe et al., 2018), composites (Huber et al., 2012; Johns et al., 2018), functionalized plant cellulose (Modulevsky et al., 2014; Fontana et al., 2017) and decellularized plant tissue (Modulevsky et al., 2014; Gershlak et al., 2017) have been developed. This in turn shows the versatility of cellulose. Moreover, cellulose and its derivatives (e.g., Methylcellulose and 6-carboxycellulose) have been functionalized and blended with other materials to improve its mechanical, biological and chemical properties (Novotna et al., 2013; Thirumala et al., 2013; Fontana et al., 2017). Cellulose as a biomaterial has been extensively reviewed previously (Kalia et al., 2011; Hickey and Pelling, 2019). This section will emphasize on decellularized plant tissue and bacterial cellulose.

It was shown that decellularized apple hypanthium (**Figure 2**) can be used as a substrate for 3D cell culture. HeLa cells, 3T3 fibroblast, and C2C12 murine myoblast proliferated throughout the 3D matrix (Modulevsky et al., 2014). In order to decellularized the tissue, a surfactant, in this case SDS, was used to create pores in the plant cell membrane, leading to the release of cellular components (Brown and Audet, 2008; Modulevsky et al., 2014). The mechanical properties of these scaffolds which are known to influence cell behavior, have also been altered through functionalization and crosslinking (Modulevsky et al., 2014) and further shown to resemble skeletal (Modulevsky et al., 2014; Hickey et al., 2018) and cardiac muscle tissue (Gershlak et al., 2017). However, cellulose based scaffolds do lack a wide array of mammalian biochemical cues; thus, biofunctionalization or coating with functional surface proteins may be required for specific cell lines, especially in a serum-free environment (Hayman et al., 1985; Courtenay et al., 2017; Johns et al., 2018). It was noted, however, that the viability of C2C12 cells was not affected by the bare cellulose scaffold when compared to collagen and gelatin coating (Hickey et al., 2018). Nonetheless, seeding efficiency has been shown to be greatly improved with surface coating and functionalization (Modulevsky et al., 2014; Fontana et al., 2017; Hickey et al., 2018).

An advantage of decellularized plant tissue is the wide array of natural topographies that can be used to study cellular behavior and potentially mimic *in vivo* conditions without long and costly processing (Modulevsky et al., 2014, 2016; Fontana et al., 2017). By utilizing the topographical cues present in the vascularization of stems and leaves, guided cell alignment was noted (Fontana et al., 2017). In this case, cell alignment was likely due to the confinement of cells within the vascularization channels. Alignment in cell culture is a highly desirable characteristic,

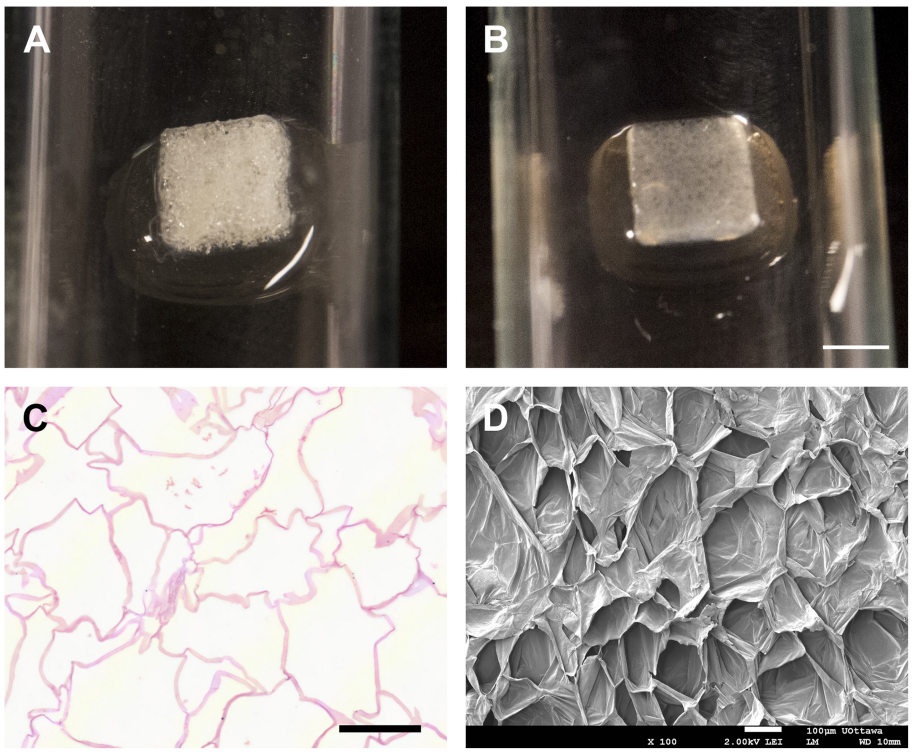

FIGURE 2 | Preparation of cellulose scaffold. Macroscopic appearance of a freshly cut apple hypanthium tissue **(A)** and the translucent scaffold biomaterial post decellularization and absent of all native apple cells or cell debris **(B)**. H & E staining of cross sectioned decellularized cellulose scaffold **(C)**. The cell walls thickness and the absence of native apple cells following decellularization are shown. The 3D acellular highly porous cellulose scaffold architecture clearly revealed by SEM **(D)**. Scale bar: A–B = 2 mm, C–D = 100 μm. Reprinted from Modulevsky et al. (2016).

especially in musculoskeletal research. By inducing alignment, researchers try to mimic the physiological state of myoblast and myotubes (Zhao et al., 2009; Bettadapur et al., 2016). In comparison to synthetic microchannel development techniques, such as 3D printing (Tijore et al., 2018), soft lithography (Glawe et al., 2005) and photolithography (Lee et al., 2006), the decellularized vascular bundle of plants depict a low cost, highly accessible and easy to use material.

The vascularization of a decellularized spinach leaf was postulated as a way to overcome the 100–200 μm diffusion limitations of 3D scaffolds (Gershlak et al., 2017). Yet, it's still unclear if cells growing outside of the vascularization tracts can benefit from the circulation of nutrients. However, as of now, the need for vascularization in decellularized plant is not a requirement. Cells are able to grow throughout the porous decellularized apple hypanthium without developing a necrotic center (Modulevsky et al., 2014; Hickey et al., 2018). Yet, a necrotic center is likely to develop in very large scaffolds which may possibly be required in food applications. The porosity of the apple also supported angiogenesis when implanted *in vivo* (Modulevsky et al., 2016). This observation will not necessarily extrapolate to other decellularized plant scaffolds due to their underlying native tissue geometry which makes plant species/tissue choice important (Gershlak et al., 2017). Although decellularization is depicted as a simple biomaterial development method, it lacks the customizability of "bottom-up" approaches, such as that of cellulose nanofibril scaffolds and cellulose composites with varying porosity, biological, and mechanical characteristics (Khan et al., 2016; Courtenay et al., 2017; Courtenay et al., 2018).

Cellulose is not only found in the plant kingdom, but is also produced by certain strains of bacteria, such as *Acetobacter*spp. (Schramm and Hestrin, 1954; Jonas and Farah, 1998). Although plant and bacterial cellulose share an identical α-cellulose structure, bacterial cellulose possess greater crystallinity, degree of polymerization and water holding capacity (Esa et al., 2014; Moniri et al., 2017). These attributes have been invaluable in a wide array of applications, including medical (Petersen and Gatenholm, 2011; Fürsatz et al., 2018), cosmetics (Pacheco et al., 2018) and food (Shi et al., 2014). The food applications include cultural desserts such as *Nata de coco*; and functional properties such as gelling agent, stabilizer and thickener (Shi et al., 2014). Moreover, bacterial cellulose has been used to incur juiciness and chewiness in emulsified meats (Lin and Lin, 2004).

An advantage of bacterial cellulose is the wide array of carbohydrate-rich by-products that have been used for its production (e.g., Wheat thin stillage, waste fiber sludge, pullulan fermentation waste water, beer culture broth) (Ha et al., 2008; Cavka et al., 2013; Revin et al., 2018; Zhao et al., 2018) and the wide array of chemical modifications that can be introduced

to further improve biocompatibility and mechanical properties (Kurniawan et al., 2012; Saska et al., 2012; Lopes et al., 2014; Ostadhossein et al., 2015). The biocompatibility, low cost and nutritional attributes make this material a potential candidate for *in vitro* meat production.

CHITIN AND CHITOSAN

As the second most abundant polymer in nature, chitin is found in the exoskeleton of arthropods (e.g., crab and shrimp) and fungi (Percot et al., 2003; Deguchi et al., 2015). In this review, fungal chitin is of interest due to the animal-free nature. Although the chitin sources referenced throughout this section are either not disclosed or declared to be animal derived (likely due to abundance) there is currently no reason to believe that it can't be replaced with fungi chitin (Bierhalz et al., 2016).

Through alkaline deacetylation, chitin is turned into chitosan (Rodríguez-Vázquez et al., 2015). The degree of deacetylation of chitin leads to physical, chemical, and biological changes, such as interaction with cells directly or with glycoproteins and proteoglycans through ionic complexes. In addition to the interaction with glycoproteins and proteoglycans, chitosan's resemblance to glycosaminoglycans has the potential of regulating and modulating bioactive factors (Madihally and Matthew, 1999; Yang, 2011; Chicatun et al., 2013). Moreover, it was also shown that chitosan can be blended with other polymers to further improve the mechanical properties with the aim of resembling native tissue (Zakhem et al., 2012; Hajiabbas et al., 2015).

Chitosan has shown to be a desirable material in tissue engineering due to its biocompatibility (Tamura et al., 2011; Croisier and Jérôme, 2013), antibacterial properties (Benhabiles et al., 2012), and accelerated healing rate on skin wounds (Tchemtchoua et al., 2011). It has been shown that chitosan and chitosan oligosaccharides provide a synergistic bactericidal effect on planktonic bacteria and biofilms when combined with antibiotics such cloxacillin (Decker et al., 2005; Breser et al., 2018) and sulfamethoxazole (Tin et al., 2009). Chitin and chitosan alone portrayed a bacteriostatic effect on gram negative bacteria, *Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae,* and *Bacteroides fragilis* (Benhabiles et al., 2012). The synergistic effect with antibiotics and overall bacteriostatic properties are a desirable attribute for applications in cellular agriculture; as antibiotic use decreases social acceptability (Karavolias et al., 2018), has the potential to cause long term health problems, and increase the development of antimicrobial resistance (Thanner et al., 2016). However, to our knowledge, the antimicrobial potential of these compounds in long term cell mammalian cell culture has yet to be tested or verified.

Often materials for medical applications are segregated into permanent or temporary. The degradation of chitosan by lysozymes found in the body can be controlled through the degree of deacetylation (Muzzarelli, 1997; Tomihata and Ikada, 1997; Rodríguez-Vázquez et al., 2015). Degradability is not necessarily an undesirable characteristic, as degradation rates can be controlled; and the by-products have the potential to provide neuroprotective (Pangestuti and Kim, 2010) and anti-inflammatory properties (Azuma et al., 2015; Kim, 2018). Furthermore, biodegradable hydrogels with controlled degradation rates are expected to be a temporary matrix for adherent cells. The objective is to match matrix deposition by cells with the degradation rate of the scaffold (Berthod et al., 2006; Bitar and Zakhem, 2014; Ren et al., 2018). This not only applies to medical applications, but also to potential applications for *in vitro* meat production. A temporary matrix can allow for cellular ECM deposition with the end of goal of obtaining a scaffold with characteristics similar to that of native tissue.

The structure of certain types of mushroom provide a mouthfeel and umami flavor which resembles that of meat, often perceived as a vegan alternative (Jo-Feeney et al., 2014). Moreover, the cell wall components of mushroom contain chitin, 1-3-alpha-D-gucans and mannans, which confer nutritional benefits, such as dietary fiber (Cheung, 2013; Fernandes et al., 2015). The antimicrobial and nutritional properties, alongside its animal-free nature and abundancy, make chitin/chitosan-based scaffolds a potential substrate for cellular agriculture applications.

RECOMBINANT COLLAGEN

The well-known and extensively studied extracellular matrix protein, collagen, is often derived from bovine (Chan et al., 2016), porcine (Smith et al., 2000), and murine (Isobe et al., 2012) sources. Collagen type I, a fibrillar heterotrimeric protein composed of two α1(I) chains and one α2(I) chain, has been produced in numerous forms, including porous hydrogels, composites and a number of substrates with topographical cues and varying mechanical properties (Rich and Crick, 1955, 1961; Vernon et al., 2005; Stein et al., 2009; Antoine et al., 2014; Wang et al., 2016; Wu et al., 2018). Yet, variability (e.g., age and physiological state of donor), potential pathogen transmission, and contaminants including cytokines and growth factors have been a concern for this animal derived product (BANFIELD, 1956; Kohn and Rollerson, 1960; Keefe et al., 1992; Badylak and Gilbert, 2008; Shoseyov et al., 2013).

In order to overcome these issues, genetic engineering has led to the development of transgenic organisms capable of synthesizing the desired amino acid repeats. Through the insertion of COL1A1 and COL1A2 genes, the repeating amino acid sequence, Gly-X-Y, can be translated and transcribed. In this case, the X and Y often correspond to proline and hydroxyproline, respectively (Stein et al., 2009; An et al., 2014; Shoseyov et al., 2014). The repeating amino acid sequence leads to the triple helical conformation and specific thermal stability of collagen (Rich and Crick, 1955; Bella et al., 1995; An et al., 2014).

The production of procollagen through recombinant methods has been observed in bacteria (An et al., 2014), mammalian cells (Geddis and Prockop, 1993), insect cell culture (Myllyharju et al., 1997), yeast (Olsen et al., 2001), and plants (Stein et al., 2009; Xu et al., 2011). The introduction of COL1A1 and COL1A2 genes encode for the amino acid sequence. Yet, the post translational modifications are fundamental in the production of collagen with

similar mechanical and biochemical properties to that of native collagen found *in vivo*. Procollagen for *in vivo* and *in vitro* use has been produced in a tobacco plant capable of expressing COL1A1 and COL1A2 proteins, alongside post translational modification proteins localized in the vacuole: Prolyl 4-hydrolysase (PH-4) alpha, PH-4beta and Lysine hydroxylase (LH1-3). P4H acts on the proline residues leading to directionality and thermal stability, whereas LH1-3 plays a role in collagen fibril formation and stabilization (Pihlajaniemi et al., 1991; Ruotsalainen et al., 2006; Shoseyov et al., 2013).

Although the production of recombinant collagen has proven to be difficult in part due to the need for post translational modification machinery natively found in mammalian cells (Werkmeister and Ramshaw, 2012; An et al., 2014), a fibrillar protein with a similar melting point and overall chemical structure to collagen has been observed and isolated in microbes, such as *Streptococcus pyogenes*. The protein's properties have been attributed to the presence of collagen like proteins, *scl 1* and *scl 2* (Lukomski et al., 2000, 2001; Yu et al., 2014). The production of this collagen like protein lacks the different biochemical cues found *in vivo* due to the lack of post translational modification; yet, the "blank slate" and gene customizability can be an attractive property for customization (Peng et al., 2010; An et al., 2014; Yu et al., 2014).

In order to fulfill the demand for recombinant collagen, yield optimization has been a major target. Standardized comparison has been difficult to accomplish due to the properties of the final product which are influenced by the level and presence of post translational modification proteins. Collagen production in plants, more specifically, tobacco, has been considered to be the most promising. Production of up to 200 mg of recombinant human type I procollagen per kg of fresh leaves (20 g/L reported by Werkmeister and Ramshaw, 2012) has been achieved through the vacuole targeted enzymes and genes (Stein et al., 2009). The biocompatibility of procollagen from transgenic tobacco plants was shown *in vitro* and *in vivo* (Shilo et al., 2013; Willard et al., 2013). *In vitro*, an increase in cell proliferation of human epidermal keratinocytes was noted when compared to bovine collagen (Willard et al., 2013). Bacteria collagen (Peng et al., 2010) and recombinant collagen produced in yeast (Liu et al., 2007;2008) have also shown *in vivo* and *in vitro* biocompatibility.

Collagen type I is not the only ECM protein that has been produced recombinantly. Other types of collagen (e.g., Type II and III) (Myllyharju et al., 2000; Pakkanen et al., 2003; Ruottinen et al., 2008), tropoelastin (Martin et al., 1995), and fibronectin (Staunton et al., 2009) fragments have also been produced recombinantly.

Collagen production in transgenic tobacco plants, yeast and/or bacteria has the potential to alleviate issues encountered through the use of animal derived biomaterials. Subsequently, the animal-free nature and similarity to native collagen can be a major step forward in the development of *in-vitro* meat, especially if producers wish to replicate the characteristics of native tissue.

CONCLUSION

Here we present three biomaterials that have shown promising results in tissue engineering and that can be translated to cellular agriculture applications in large part due to their abundance, animal-free nature and current food applications. Moreover, the wide array of natural topographies and dietary fiber found in plants, alongside the antimicrobial and rheological properties of chitin/chitosan further extend their potential in cell culture and cellular agriculture. However, these materials do lack the biochemical cues found in native mammalian extracellular matrices, leading to a need for functionalization. This need further increases the complexity of the process, reducing the scalability potential. On the other hand, the emergence of recombinant collagen extracted from plants has important advantages as a scaffold in its own right or if used to functionalize the surfaces of the materials above. Furthermore, these materials have been modified, either as microspheres or bulk, to possess the porosity necessary for diffusion of nutrients through dynamic or static bioreactors (Oh et al., 2009; Wu et al., 2011; García Cruz et al., 2012; Varley et al., 2017; Huang et al., 2018; Specht et al., 2018). In order to scale an animal-free product with similarities to that of native animal tissue, the need for fetal bovine serum, cost-effective engineering processes, antibiotic dependence, scaffold development, and cell line(immortalized vs. primary and cell co-culture) needs to be addressed (Specht et al., 2018; Stephens et al., 2018; Lynch and Pierrehumbert, 2019). It's currently believed that scaffolding will play a crucial role in the scalability of cultured meat. Therefore, the aim of this review was to summarize three animal-free materials with properties (e.g., rheological, nutritional and biological) that will likely be desirable in scaffolding for cultured meat applications. Yet, we wish to remind the reader that scaffolding is only one component of a much larger endeavor; and the scalability potential of the methods presented here is still unknown, and for some unlikely. Readers are encouraged to refer to Stephens et al. (2018) and Specht et al. (2018) for an overview on cellular agriculture and the major challenges.

AUTHOR CONTRIBUTIONS

SC wrote, revised, and edited manuscript. AP revised and edited manuscript.

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Conflict of Interest Statement: SC and AP are inventors on a patent application concerning the use of plant-derived cellulose for tissue engineering applications filed by the University of Ottawa.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.