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## REVIEW

## The role of nanochitin in biologically-active matrices for Tissue Engineering-Where do we stand?

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Our regard to the use of chitin as a material has drastically changed since its discovery, 210 years ago. From an intractable material because of its insolubility in common solvents, it became one of the most important raw materials serving as a source of chitosan (its main derivative), and more recently, as source of nanometric forms: nanocrystals and nanofibers. Nanoscale chitin forms are remarkable high-value compounds for nanomaterials' development, due to their intrinsic biological and mechanical properties, as well as their potential as eco-friendly components to valorize the plentiful by-products of the seafood industry. Lately, these nanochitin forms have been widely used as nanofillers in polymer nanocomposites, and in particular, in natural biologically-active matrices for the development of biomaterials. The recent progresses achieved in the last two decades concerning the use of nanoscale chitin in biologically-active matrices for Tissue Engineering is highlighted in this review. First, an overview on the use of nanochitin in the different biomedical fields is presented and discussed. Then, the state-of-the-art regarding the development of biomaterials based on chitin nanocrystals or nanofibers is described in the context of the role of nanochitin in biologically-active matrices namely polysaccharides (chitin, chitosan, cellulose, hyaluronic acid, alginate), proteins (silk, collagen, gelatin) and others (lignin). Finally, major conclusions and perspectives on the use of nanochitin as an increasingly important raw material are described.

### 1. Introduction

Nowadays the trend on biomaterials for Tissue Engineering (TE) is the search of novel raw materials that mimic the key characteristics of the native extracellular matrix (ECM) namely the structure and biological function, since it provides the mechanical support and regulates cell behavior.<sup>1</sup> TE is an interdisciplinary field that applies the principles of chemistry, engineering, biology and medicine to fabricate tissue substitutes that could repair, maintain or enhance the function of a damaged tissue or organ.<sup>2,3</sup>

In this sense, several studies have been focused on the use of natural polymers (in particular, polysaccharides) which present appropriate structures and properties for promising applications in this field.<sup>4</sup> Among the different polysaccharides, chitin ranks second place after cellulose, being present in the

structure of many organisms both in oceans and land, such as fungi, algae, molluscs and a wide variety of arthropods (crab, shrimp, crayfish and insect).<sup>5</sup> Its great availability through the crustacean shells (mainly shrimp, crab and lobster, prawn and krill), makes the wastes of the fishing industry to be the main source for chitin production.<sup>6,7</sup>

Chitin, is considered a semicrystalline polymer-like nanofibrils. It is a high-molecular-weight linear polymer composed of N-acetyl-2-amino-2-deoxy-D-glucose units linked by  $\beta(1\rightarrow4)$  bonds (Fig. 1). Its structure is very analogous to cellulose, being the unique difference the replacement of the hydroxyl group in C2 by an acetamide group.

Indeed, both polymers play similar roles in the organisms providing the structural support and biological properties, but this acetamide group in chitin grant it some particular properties like antibacterial, wound-healing ability and non-toxicity, making chitin an interesting biopolymer to be studied for biomedical applications.<sup>4,8</sup> Three different crystalline polymorphic forms of chitin have been documented in function of how these chitin piles are stacked: polymorphs  $\alpha$ ,  $\beta$  and  $\gamma$ . In  $\alpha$ -chitin the piles of chitin chains are arranged in alternately antiparallel mod, in  $\beta$ -chitin the piles are all parallel and in  $\gamma$ -chitin is a mixture of  $\alpha$  and  $\beta$ , where there is a sequence of 2 piles parallel followed by 2 in antiparallel position.<sup>8,9</sup> Thus,  $\alpha$ -chitin possess strong inter and intra pile hydrogen bonds and is the most abundant being present where a structural support is

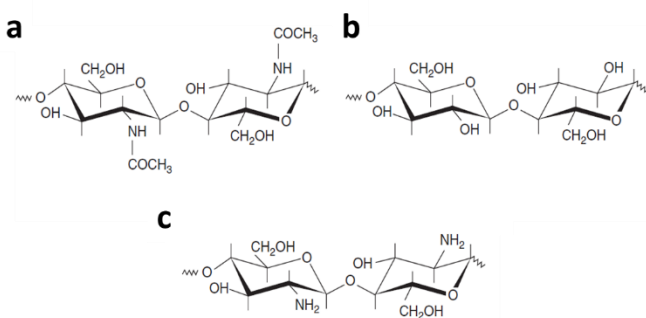
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**Fig. 1** Schematic representation of (a) completely acetylated chitin; (b) cellulose and (c) completely deacetylated chitin-chitosan. Reproduced from ref. 7 with permission from Elsevier.

needed (arthropods, fungal and yeast cell walls). On the other hand,  $\beta$ -chitin have a weak intra-pile hydrogen bonding (no intersheet hydrogen bonds) and can be found in more flexible structures like skeletal structures of cephalopods (*i.e.*, squid pens).  $\gamma$ -chitin is also present in flexible structures but is rarely found in nature, being reported in different cocoons and mushrooms.<sup>8,10–12</sup> Even if it is recognized that the type of polymorph can have an influence on the final properties of the biomaterials, in the majority of works this aspect is not mentioned. To date, most of the studies have been done using alpha-isoform. Regarding the biological properties, different studies have validated the relevant abilities of chitin: (i) to promote the cells adhesion, proliferation and differentiation,<sup>13,14</sup> (ii) to be degraded into non-toxic small molecular amino and polysaccharides being easily absorbed *in vivo*,<sup>15</sup> and (iii) to be treated as reliable carriers of drugs or cytokines.<sup>16,17</sup> These qualities, among others, confirm the biocompatible, biodegradable and chemical stability characteristic of chitin and makes understand its interest within the biomedical industry.

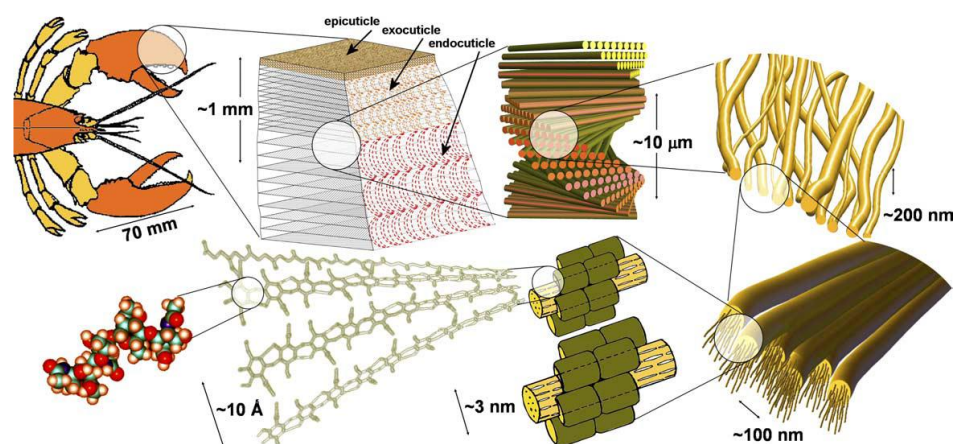
As a polymer-like nanofibrils, chitin presents a highly oriented structure from the nano- to the micrometer scale with crystalline and amorphous domains.<sup>5,18–20</sup> In Figure 2, a representative scheme of the hierarchical organization inside of the lobster shell is shown, where at molecular level there are long chains of chitin forming highly crystalline fibrils on the

nanometer scale. These fibrils (length,  $L \sim 30$  nm, Width,  $d \sim 3$  nm) are enveloped with proteins and assemble into nanofibers ( $d \sim 60$  nm), which in turn are assembled into bundles of chitin nanofibers. At the micrometer level, a network of bundles is formed, creating a twisted plywood structure (called “Bouligand structure”), which is embedded in protein and minerals (calcium carbonate and calcium phosphate).<sup>5,18,19</sup> This Bouligand structure pattern repeats to form the different main macroscopic levels that conform the shells; endocuticle, exocuticle and epicuticle.

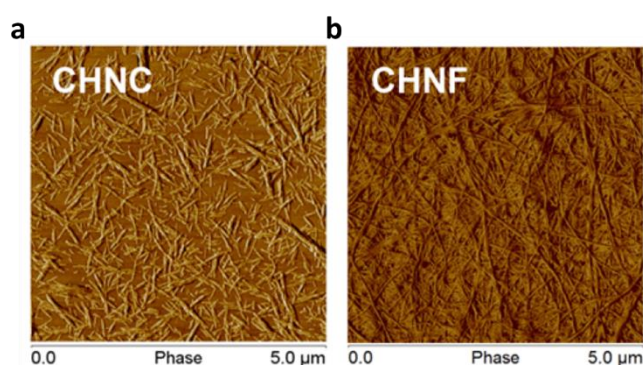
To obtain chitin nanofillers, first, chitin needs to be extracted from the raw material; and then, the isolation of chitin nanofillers can be done.<sup>6</sup> Regarding that, up to date, there have been many studies on how to isolate chitin nanofillers by using chemical, mechanical, physical treatments or a combination of different approaches.<sup>6,21–24</sup> The wide variety of chitin nanofillers that can be isolated in terms of size, shape, crystallinity, aspect ratio or morphology, depends on two factors such as: (i) the chitin source; or (ii) the isolation process.<sup>6,8</sup> The most common chitin nanofillers (altogether abbreviated here as nanochitin) are chitin nanocrystals (CHNC) and chitin nanofibers (CHNF).

CHNC are also known as chitin whiskers, chitin nanowhiskers or chitin nanoparticles. As the name suggests, the shape of these nanoforms are whiskers or rod-like shaped (Fig. 3a). It is a highly crystalline nanofiller (80–95%), with a very small size ( $d \sim 6$ –60 nm,  $L \sim 100$ –800 nm) and aspect ratio typically between 10 and 20.<sup>6</sup> Different techniques have been developed to get CHNCs from the degradation of the amorphous region of the chitin nanofibrils, including acid hydrolysis, ionic liquids<sup>25</sup> or tetramethylpiperidinoxy (TEMPO) mediated oxidation approaches.<sup>26</sup>

On the other hand, chitin nanofibers (CHNF) present a fibrillar morphology (Fig. 3b) with diameters between 10 and 100 nm and several microns in length presenting very high aspect ratios. CHNFs also present a high crystallinity index, but lower than CHNCs (at around 50–90%). The most popular CHNF isolation approach is related to mechanical treatment by using grinders, blenders or homogenizers; or physical techniques (ultrasonic), which facilitate fibrillation but do not eliminate the amorphous domains within chitin fibrils.<sup>6,27</sup> There is another approach to obtain chitin nanofibers by dissolving chitin and, then, electrospun it into nanofiber mats.



**Fig. 2** Scheme of the hierarchical organization in arthropods exoskeleton (*H. americanus*, American lobster), which reveals different structural levels. Reproduced from ref. 19 with permission from Elsevier.



**Fig. 3** Atomic Force Microscopy images of (a) CHNC isolated by acid hydrolysis, and (b) CHNF isolated by mechanical treatment. Magnification of  $5 \times 5 \mu\text{m}$ . Reproduced from ref. 13 with permission from American Chemical Society.

Electrospinning is a simple, cost-effectiveness and high production rate technique for the fabrication of nanofibers and highly porous scaffolds that mimic the natural extracellular matrix.<sup>28</sup>

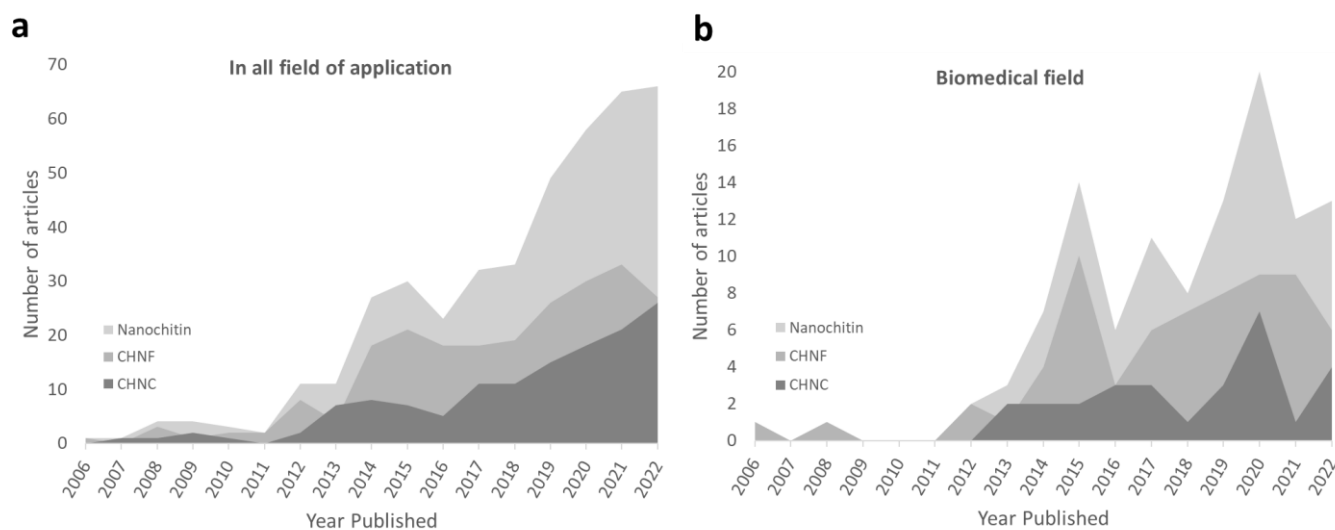
The fact that nanochitin exhibit unique mechanical properties combining strength and toughness and exceptional biological functions that interact with the surrounding environment, turns chitin nanoforms into very attractive raw materials. Thus, in the last decades, the use of chitin in Materials Science has been focused on its nanometric forms, either nanocrystals or nanofibers,<sup>29</sup> mainly alpha-isoform. They have been used as nanofiller agents in different types of matrices, such as chitosan,<sup>22,30,31</sup> starch,<sup>32,33</sup> polyurethane,<sup>34</sup> poly-methyl methacrylate,<sup>35</sup> PVA,<sup>36</sup> PLA,<sup>37,38</sup> cellulose<sup>39</sup> for a wide variety of applications such as agriculture,<sup>40,41</sup> food industry,<sup>31,42</sup> pharmaceutical,<sup>43</sup> and in particular biomedicine.<sup>44,45</sup>

According to the literature in the last two decades, the number of published articles concerning “nanochitin” has increased significantly (Fig. 4a). In particular, the major publications have

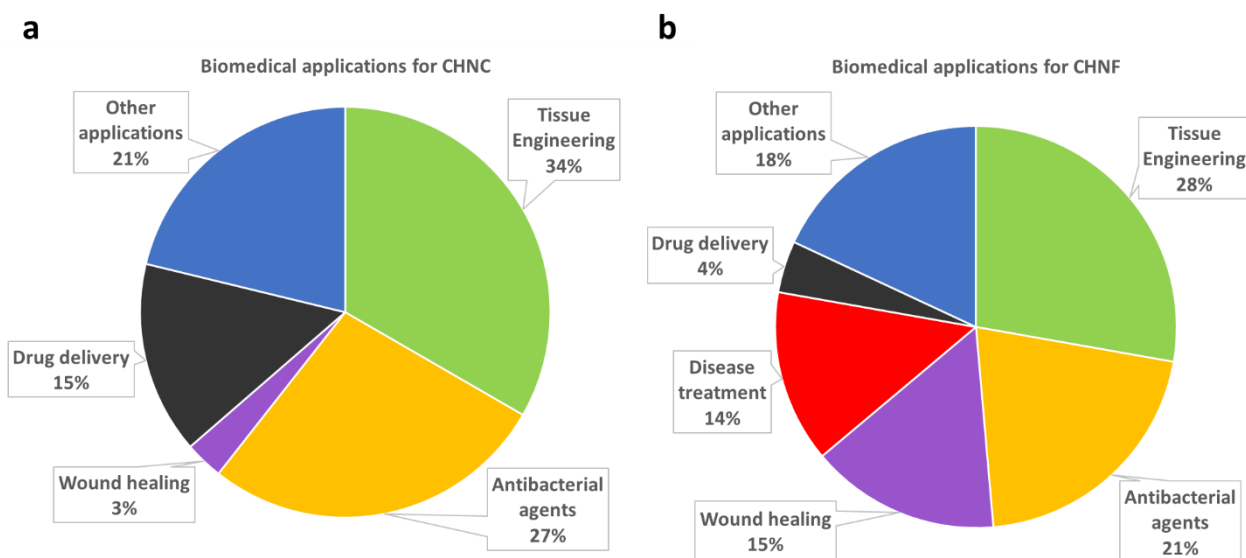
been in the last 5 years, with a profound increase between 2019 and 2021. Although both main chitin nanoforms have gained popularity, there can be seen a clear tendency in the use of CHNFs over CHNCs. Regarding the publications focused on the biomedical applications of nanochitin, there is also a general increasing trend but not in a homogeneous way (Fig. 4b). Consequently, the number of published articles regarding this field remains low, less than 20 articles per year for both nanoforms (unless in 2020 for nanochitin), which indicates that it is an area that is still in its infancy.

A huge number of reviews give detailed information on nanochitin isolation, characterization and modifications strategies,<sup>27,46–51</sup> as well as description of fabrication methods of biomaterials or composites derived from these nanoforms.<sup>5,8,12,23,45,52–57</sup> There has been a considerable increase in the number of publications in the last two decades about the use of nanochitin in biomaterials for biomedical applications, although is still a fairly new field of application.<sup>43,58–60</sup> Among biomedical fields, TE has recently gained great interest due to the need of new strategies to achieve the repair and regeneration of damaged tissues and organs. Nevertheless, despite the promising use of nanochitin in this field as nanofiller for different matrices, including natural biologically-active ones, today there is a gap in the literature concerning the role of the chitin nanoforms in biomaterials.

In this context, this review strives to describe the advances made in the use of nanochitin in biologically-active matrices with polysaccharides (chitin, chitosan, cellulose, hyaluronic acid, alginate), proteins (silk, collagen, gelatin) and others (lignin) for TE applications. The role of nanochitin in these matrices as biologically functional, structural or reinforcing agents is also discussed. The paper is concluded with a comparison between the role of both CHNC and CHNF in the different matrices and few perspectives.



**Fig. 4** Trend of research on nanochitin for the last 17 years in: (a) all field of application, and (b) focused in biomedical field. The number of articles was obtained using as following keywords: Nanochitin; Nannochitin\*Biomedical; Chitin nanocrystals\*Biomedical; Chitin nanowhiskers; Chitin nanowhyskers\*Biomedical; Chitin nanofibers; Chitin nanofibers\*Biomedical; Chitin nanofibrils; Chitin nanofibrils\*Biomedical.



**Fig. 5** Percentage attributed to each field within biomedical applications for (a) CHNC, and (b) CHNF. The percentage numbers were calculated by the authors taking in account the information from Fig. 4.

## 2. Overview on the use of nanochitin in biomedical applications

Chitin nanoforms have been used in a variety of biomedical applications such as TE, antimicrobial agents, wound healing, disease treatment, controlled drug delivery and others. Figure 5 displays the distribution in percentage of the different applications for CHNC (a) and CHNF (b), calculated from the previous selected articles in Figure 4.b. Interestingly, for both nanochitins TE is the main field of application followed by

antibacterial agents for both isoforms. The disease treatment is only present for CHNF.

### 2.1. Tissue Engineering

Although the incorporation of nanochitin has led to successful applications in TE with a multitude of synthetic polymers,<sup>61–68</sup> herein the discussion throughout the next section (section 3) is focused only on natural polymers as biologically-active matrices. Table 1 summarizes the TE applications for biomaterials prepared using chitin nanocrystals or nanofibers into biologically-active matrices in the last years.

## 2.2. Antibacterial agents

The second most popular biomedical application for nanochitin is as antibacterial agent, which accounts 21-27% depending on the type of nanochitin (Fig. 5).

Several authors have tested antibacterial and antifungal activity of CHNFs as such,<sup>21,69,70</sup> and by adsorbing silver nanoparticles on their surface to enhance it.<sup>71-74</sup> On the other hand, CHNCs can improve the antibacterial activity of enzymes like lysozyme by acting as immobilization platforms,<sup>75</sup> and the antioxidant activity of immobilized phlorotannins.<sup>76</sup> Also, nanocomposites films based on chitosan,<sup>77</sup> carrageenan,<sup>78</sup> carboxymethyl cellulose,<sup>79</sup> methylcellulose,<sup>80</sup> cellulose nanofibers,<sup>39,81</sup> pectin,<sup>82</sup> nanolignin,<sup>83</sup> polylactic acid,<sup>38,84</sup> highly porous and spongy monolith cryogel of polyvinyl alcohol<sup>85</sup> and molybdenum disulfide quantum dots,<sup>86</sup> have been developed. Regarding antifungal activity, films have been developed using CHNFs alone,<sup>87</sup> in combination with chitosan<sup>88</sup> or by incubation in sodium hypochlorite solution.<sup>89</sup>

## 2.3. Wound healing

Chitin nanoforms have showed promising applications in wound healing since Mezzana *et al.*, firstly report a CHNF-based hydrogel with clinical efficacy.<sup>119</sup> Skin models have demonstrated that both CHNF and CHNC could be components of skin-protective formulations<sup>120,121</sup> and that surface deacetylation of the nanoforms are beneficial on wound healing process.<sup>122</sup>

Motivated by these promising results, different forms of wound healing biomaterials have been developed.  $\alpha$ -CHNC and CHNF casting films showed no toxicity to epithelial- and fibroblast-like cell lines<sup>123</sup> and CHNF-based hydrogels successfully encapsulated cells and induced bone marrow stromal cells (BMSCs) differentiation towards angiogenic cells and fibroblasts for the treatment of wounds with large defects.<sup>124</sup> Adipose-derived mesenchymal stem cell (ASCs)-loaded  $\beta$ -CHNF hydrogel also promoted wound healing in rats.<sup>125</sup> Another approach is to make electrospun nanomaterials with polyethylene oxide (PEO). Electrospinning CHNF/PEO has been combined successfully with chitosan<sup>126</sup> and lignin<sup>127</sup> as promising skin-friendly materials for wound dressing, and wound healing activity *in vivo* was demonstrated.<sup>128</sup>

**Table 1** TE applications of chitin nanocrystals or nanofibers in combination with biologically-active matrices.

Nanochitin	Tissue of application	Year	Reference		
Nanocrystal	Not specified	2007	90		
		2017	91		
		2019	92		
		2020	28,93		
		2022	94		
		Skin	2020	95,96	
			Cartilage	2020	14
				2015	97
		Bone	2016	98	
		Nanofiber	Not specified	2017	99
				2018	100-102
				2019	103
				2020	104
2021	105				
Skin	2006			106	
	2014			107	
	2019			108,109	
	2021			110	
Cartilage	2022			111	
	Tendons/Ligaments			2019	112
Vascular/Cardiac				2016	113
	2020			114	
	2015			115	
Bone	2017			116	
	2020	114,117			
	2017	118			

Surface-deacetylated CHNFs as biological adhesives in combination with carboxymethyl chitin derivatives allowed the inflammatory cells to migrate to the wound,<sup>129</sup> and in freeze-dried gel form showed potential for prednisolone release in wound healing.<sup>130</sup>

## 2.4. Disease treatment

CHNFs have been intensely used for therapeutic purposes in various diseases. Several works have studied the effects of oral administration of CHNFs, showing its effectivity increasing plasma metabolites such as adenosine triphosphate (ATP) or serotonin and stimulation of gut microbiota.<sup>131</sup> It was also demonstrated its use as potential food for patients with hypercholesterolemia because of its hypolipidemic effect,<sup>132,133</sup> as gastrointestinal-resistant biosorbent for cholesterol and bile salts,<sup>134</sup> and as useful agents preventing mucositis induced by anticancer drug 5-fluorouracil.<sup>135</sup> These CHNFs also present good results when administrated intravenously in the treatment of the ortoneurological pathology.<sup>136</sup> Goto *et al.*, have recently found that surface-deacetylated CHNFs exert anti-hepatic and antioxidative effects that could be beneficial in non-alcoholic steatohepatitis treatment.<sup>137</sup> This novel activity adds to the previously shown anti-inflammatory and anti-fibrosis effect on mice model, with application on inflammatory bowel disease,<sup>138</sup> and suppression of skin inflammation in atopic dermatitis-like lesions.<sup>139</sup> On the other hand, the combination of surface-deacetylated CHNF with magnetic nanoparticles as hydrogels, results in a promising tool for osteosarcoma therapy.<sup>140</sup>

## 2.5. Drug delivery

More recently, drug delivery systems in which nanochitin acts as filler of the main matrix were developed. The first system was presented by Tabuchi *et al.*, in which CHNFs were added to sulfoethyl ether  $\beta$ -cyclodextrin gel and used as controlled release carrier of prednisolone for the treatment of colitis and other inflammatory or oxidative diseases.<sup>141</sup> They have also designed systems based on self-assembly of positively charged partially deacetylated  $\alpha$ -CHNF and negatively charged TEMPO-oxidized cellulose nanofibers, for anticancer drug (5-fluorouracil) delivery<sup>142</sup> and with surrounding pH-dependent drug release capacity.<sup>143</sup> Between the systems that incorporate CHNC, we can find both stable emulsions even in the upper digestive system that transport vitamin D3 to the intestine,<sup>144</sup> as well as pH-sensitive hydrogels that are carriers of antibiotics for vaginal administration.<sup>145</sup> Also, partially deacetylated CHNCs demonstrated success as filler of drug delivery systems based on poly(vinyl alcohol),<sup>146</sup> alginate<sup>147</sup> and polyacrylamide/Zn<sup>2+</sup> hydrogels.<sup>148</sup>

## 2.6. Other applications

Due to high protein loading capacity of nanochitin, nanocrystalline chitin thin films have potential applications for enzyme immobilization,<sup>149</sup> such as lysine-immobilized chitin/carbon nanotube microspheres that are an efficient, safe and blood compatible adsorbent of bilirubin that can be employed for its removal from the human blood.<sup>150</sup> TEMPO-oxidized CHNC have been successfully labelled with a fluorescent imidazoisoquinolinone dye and carbohydrate ligands with lectins and bacteria recognition properties.<sup>151</sup> Recently, novel applications have arisen for the use of CHNF, such as surface-deacetylated CHNF/chitosan system that promotes hair growth *in vivo*,<sup>152</sup> CHNF/chitosan haemostatic material for effective haemorrhage arrest during surgery,<sup>153</sup> CHNF electrospay as skin care cosmetic product<sup>154</sup> and CHNF/alginate hydrogel microcapsules as a novel *in vitro* 3D platform for the assessment of nanoparticle toxicity.<sup>155</sup> Other types of biomaterial forms with biomedical applications for both chitin nanoforms are also addressed. Paper-like materials made of CHNF are used as a smart and easy-to-use optical (bio)sensing platform<sup>156</sup> and CHNC/cellulose fiber ones as glucose colorimetric analytical device.<sup>157</sup> Functional medical adhesive materials of methacrylic anhydride filled with CHNC<sup>158</sup> and CHNF-stabilized latex have also been developed.<sup>159</sup> There are also several authors who came to design promising advanced biobased materials with potential biocompatibility by using CHNCs<sup>160–162</sup> or CHNFs<sup>163–168</sup> as fillers, but which still don't have a developed and proven medical application.

## 3. Nanochitin in biologically-active matrices for TE

Incorporation of natural nanofillers, like nanochitin into natural polymeric matrices to obtain biomaterials with superior properties has become popular, as they are biocompatible,

bioactive, and bioresorbable agents.<sup>13,128,169,170</sup> The complementary effects of the nanofiller into the natural matrix includes changes in the mechanical behavior<sup>171,172</sup> and in the bioactivity,<sup>107,173,174</sup> but these changes depend greatly on the nanofiller shape, size and morphology, surface characteristics, and degree of dispersion.<sup>90</sup> Overall, chitin nanoforms represent promising nanofillers for the development of novel biologically-active matrices through the combination with diverse natural biopolymers,<sup>13,95,174–176</sup> by mimicking the nanofibrous structure of ECM components.<sup>177,178</sup>

This is due to their bioactivity, that is, the ability of a material to stimulate or induce a biological response at the boundary or surrounding of the material.<sup>173</sup> Since the concept of bioactive material was first termed in 1971,<sup>179</sup> it has evolved from been related exclusively to bone regeneration,<sup>180</sup> to be expanded to the rest of tissues. Thus, biologically-active matrices can be defined as those that exhibit bioactive properties such as biocompatibility, biodegradability, antimicrobial activity, anti-inflammatory or antioxidant properties, among others, and can activate *in vivo* mechanisms of tissue regeneration.<sup>181</sup> Although both synthetic and natural polymers are used as biologically active matrices, the demand for the use of natural ones have increased during the past decade because they are nontoxic and are widely available.<sup>169,182</sup> Additionally, natural polymers have greater advantages since their physical and chemical resemblance to human native tissue architecture, better overall interactions with various cell types and lack of immune rejection.<sup>183,184</sup> Nevertheless, they present some disadvantages such as inferior thermal and mechanical properties.<sup>95</sup>

In the next sub-sections, we will focus on the recent advances on the use and role of chitin nanocrystals and nanofibers in biologically-active matrices prepared with polysaccharides (chitin, chitosan, cellulose, hyaluronic acid, alginate), proteins (silk, collagen) and others (lignin) for TE applications.

### 3.1. Chitin nanocrystals in biologically-active matrices for TE

CHNCs are good candidates as nanofillers in biomaterials for TE, since their high surface area can facilitate their effective interaction with cells, proteins and other compounds, and improve their interaction with the matrix via hydrogen bonds, which further improve the structural and mechanical properties of the ensuing matrix<sup>92,98</sup> mimicking the native ECM that regulates cell behaviour.<sup>177,178</sup> In the next sub-sections a brief summary of state-of-the-art of the role of CHNCs in biologically-active matrices namely chitosan, cellulose, hyaluronic acid, alginate and silk is presented.

**3.1.1. Chitosan and its derivatives.** Chitosan, the deacetylated derivative of chitin, has been widely applied in many fields as a multi-functional material including biomedical materials because of its high biodegradability, low immunogenicity, antimicrobial activity, biocompatibility, solubility in weak acid aqueous solutions and film-forming ability.<sup>185,186</sup> Nonetheless, its applications may be limited because of its strong swelling in liquid media, poor mechanical properties and absence of biological response.<sup>14,174</sup> To overcome these limitations and

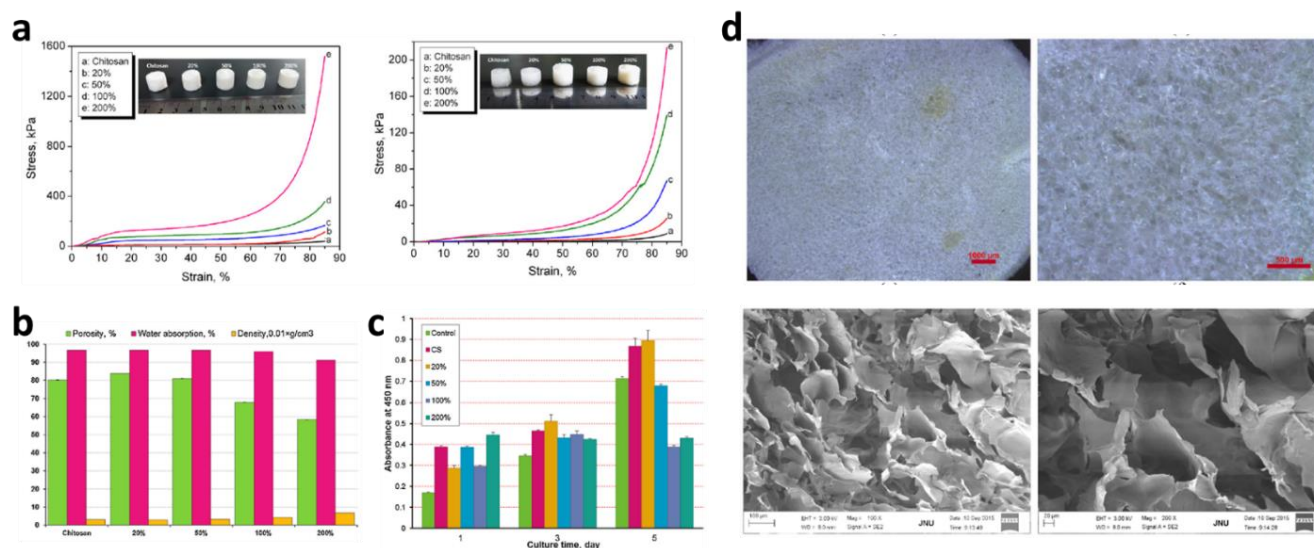
optimize the chitosan-based matrix properties, mineral or organic nano-sized fillers have been incorporated.<sup>187</sup> As both chitin and chitosan are compatible with human organisms, chitosan-based biomaterials reinforced with chitin nanofibers offer interesting prospects for biomedical applications as discussed below.<sup>91</sup>

Liu *et al.*, made a chitosan-chitin nanocrystal composite scaffold by dispersion-based freeze dry approach that permitted CHNCs to retain the crystal structure in the composite scaffold (Fig. 6).<sup>98</sup> Compared with pure chitosan, composite scaffolds prepared with CHNCs exhibited enhanced compressive strength and modulus, increased scaffold density and slightly decreased water swelling ratio (Fig. 6a and b). Chitosan/CHNC scaffolds showed excellent biocompatibility with MC3T3-E1 osteoblast cells line after 5 days of culture (Fig. 6c), and cell proliferation and adhesion were promoted by the increase in the surface roughness. Well-interconnected porous structure with high porosity (>80%) and pore size (100-200  $\mu\text{m}$ ) permitted a good cell distribution and penetration throughout the 3D porous scaffold (Fig. 6d).

Chitosan/CHNCs 3D scaffolds developed by Zubillaga *et al.*, is a promising template for articular cartilage repair, which is a specialized connective tissue with limited self-repair due to its avascular nature and limited cellularity.<sup>14</sup> The genipin-crosslinked chitosan 3D scaffolds exhibit improved mechanical and biological activities by the addition of CHNCs,<sup>13</sup> supported and favored chondral differentiation in hypoxic condition of hASCs seeded as spheroids.

Petrova *et al.*, developed a chitosan-alginate-CHNC bilayer electrospun scaffold with improved adhesive characteristics with potential biomedical applications.<sup>28</sup> The high porosity of the scaffold and the presence of CHNCs onto the surface of the chitosan-based scaffold, gave increased biocompatibility and promoted human mesenchymal stem cells (hMSCs) proliferation and adhesion. The hMSCs formed colonies in the shape of multicellular spheroidal aggregates on the chitosan nanofibers, which possesses a greater capacity for differentiation, but flatter colonies and monolayer on alginate nanofibers.

Another approach to construct chitosan-based hydrogels with diverse functions is via Schiff bases. They have been widely explored as biomaterials for haemostasis, drug delivery, and bioadhesives due to their good biocompatibility and biodegradability under physiological conditions.<sup>188</sup> It is known that cell behaviour can be controlled by changes in physico-chemical film properties like molecular mass, hydrophobicity, crosslinking degree, protonation degree and molecular structure. Kiroshka *et al.*, developed chitosan-based matrices doped with CHNCs that made bone marrow stromal cells (BMSCs) behaviour (shape, spreading area, cytoskeleton structure and the dynamics of proliferation) similar to polystyrene tissue culture plates.<sup>91</sup> Addition of CHNCs in chitosan-based film reduced viscosity by destruction of the weak interlocking network by the adsorption of a certain portion of polymer molecules onto the nanoparticle surface, and decreased the swelling ratio by the disturbance of the system of intermolecular bonds in the chitosan matrix.



**Fig. 6** (a) Compressive stress-strain curves of chitosan/chitin nanocrystals (CS/CHNCs) composite scaffolds in dry and wet state; (b) Porosity, water absorption and density of CS/CHNCs composite scaffolds; (c) Viability assay of MC3T3-E1 cells; and (d) SEM and stereoscopic microscope micrographs of 50% CS/CHNCs composite scaffolds. Reproduced from ref. 98 with permission from Elsevier.

Additionally, the introduction of CHNCs promoted the formation of macropores, whose size increases with increasing CHNC content. The experimental data lead to the conclusion that the molecular structure of polymers is the crucial factor that determines the interaction between polymers and BMSCs, with promising applications in TE.

Schiff base crosslinking hydrogels were reported, for the first time, by blending carboxymethyl chitosan (CMCS) and dextran dialdehyde (DDA) and with addition of CHNCs.<sup>96</sup> Carboxymethyl chitosan was used in order to improve the solubility of chitosan without losing its desirable properties like biocompatibility, biodegradability and bioactivities, while oxidation of the

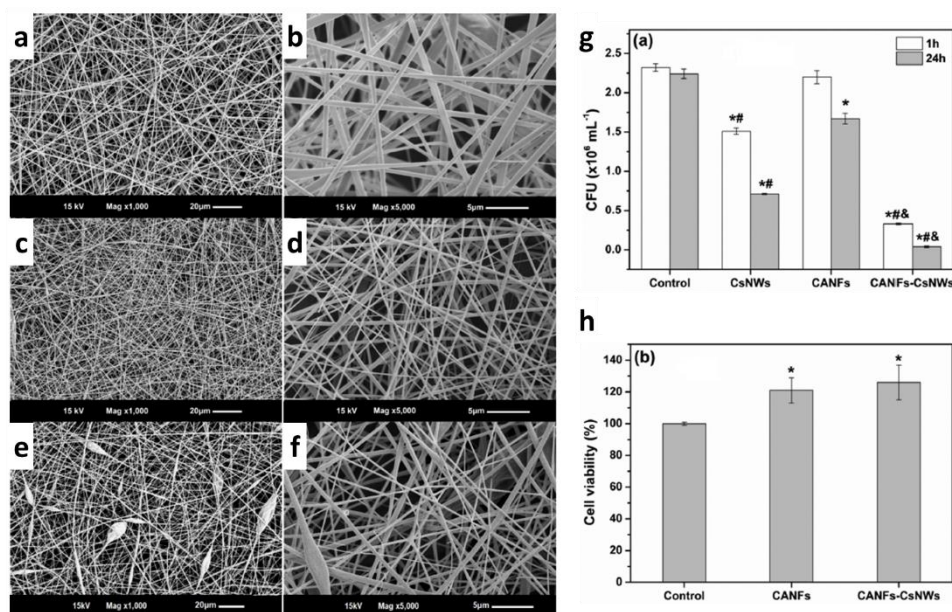


aldehyde groups in dextran can be introduced and react with carboxymethyl cellulose to form hydrogels. Addition of CHNCs improved compressive stress (1.87 fold) and adhesive strength (1.51 times) on porcine skin and also reinforced hydrogels mechanically in terms of gelation time. The composite exhibited biodegradability, biocompatibility and optimum antibacterial and haemostatic capacities. *In vivo* implantation studies showed that the hydrogel was degradable without long-term inflammatory responses. Additionally, desirable efficacy was demonstrated in rat liver injury model, where injectable hydrogel reduced inflammation and undesired tissue adhesion and promote wound repair in a superior way to commercial 3 M™ vetbond™ tissue adhesive.

**3.1.2. Cellulose and its derivatives.** Cellulose is the most abundant structural biopolymer.<sup>189</sup> This natural polysaccharide has been exhaustively used because of its abundance, crystallinity and biocompatibility, among others.<sup>186</sup> Tyshkunova *et al.*, recently developed cellulose cryogels with phosphoric acid as solvent and were able not only to enhance the mechanical properties but also to modify the intrinsic biological properties of the cellulose with the incorporation of CHNCs, leading to a more biocompatible and suitable scaffold for its use in combination with hMSCs.<sup>94</sup> Methylcellulose is a nontoxic derivative of cellulose that exhibits a thermo-reversible sol-gel transition by the hydrophobic interaction of methoxy groups in aqueous solution. However, as its low mechanical strength limits its application, cuttlefish bone extracted  $\beta$ -CHNC have been used as reinforcing fillers.<sup>20</sup> Gelation rate and mechanical strength of resulting hydrogels increased even at low content of  $\beta$ -CHNC, maybe due to additional polar interaction by hydrogen bonding. Although no biocompatibility studies were performed, given the intrinsic non-toxic behaviour of the materials that is composed this nanocomposite has potential application as TE scaffold or injectable hydrogel. Other interesting derivative of cellulose is cellulose acetate, which is a soluble esterified-derivative that can be mixed with a large number of polymers and compounds and is easily electrospun into nanofiber mats.<sup>190,191</sup>

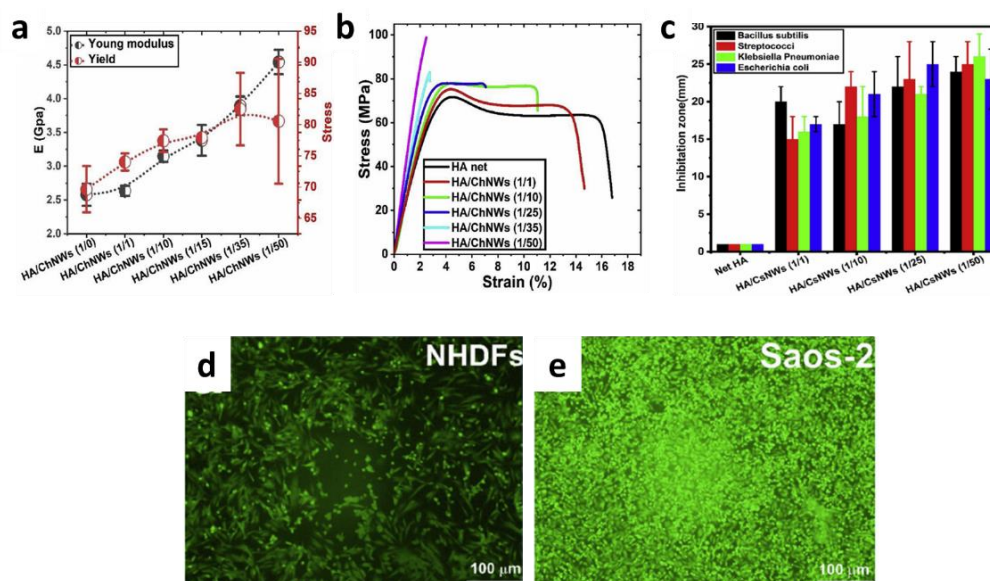
Pereira *et al.*, made electrospun cellulose acetate nanofibers (CANFs) with surface charge modification by adsorbed CHNCs.<sup>93</sup> The negatively charged nature of cellulose acetate permits the deposition of positive species by physical adsorption.<sup>192</sup> Consequently, addition of CHNCs reduced fiber diameters (Fig. 7a-f) favoring the electrical conductivity of the composite solution. The presence of the CHNCs in the nanofibers surface, also resulted effective against Gram-negative *E. coli* (Fig. 7g) and exhibited no cytotoxicity to Vero cells after 72 h of incubation (Fig. 7h). This enhanced biological activity induced by the adsorption of CHNCs on cellulose acetate was also demonstrated by Goetz *et al.*<sup>193</sup> They showed that the antibacterial activity exhibited by the addition of CHNCs was similar to other electrospun cellulose acetate that contained conventional antimicrobial agents like metal nanoparticles and metal oxides.

**3.1.3. Hyaluronic acid.** Hyaluronic acid (HA) is an anionic polysaccharide named glycosaminoglycan, composed of glucuronic acid and N-acetylglucosamine disaccharide units with a carboxyl group on each disaccharide unit.<sup>194</sup> HA is widely used due to its biocompatibility, non-toxicity and healing properties, but showed poor mechanical stability, rapid degradation, and no antimicrobial properties which are indispensable for avoiding infection in healing process.<sup>195</sup> These limitations have been overcome by adding partially deacetylated CHNCs (Fig. 8),<sup>95</sup> which acted as reinforcing agents by enhancing Young's modulus and strength of the HA composite biofilms (Fig. 8a and b). They also conferred significant antibacterial activity against different types of Gram positive and Gram-negative bacteria, including *B. subtilis*, *Streptococci* and *E. coli*, that increased by increasing the concentration of CHNCs in the biofilm matrix from 1 to 50% (Fig. 8c).



**Fig. 7** SEM images of (a,b) CANFs, (c,d) CANFs-0.5wt%CHNCs and (e,f) CANFs-2.5wt%CHNCs; (g) antibacterial activity against *E. coli* and (h) cytotoxicity assay with Vero cells. Reproduced from ref. 93 with permission from Elsevier.

Additionally, HA/CHNC nanocomposite films exhibited good biocompatibility to normal primary human dermal fibroblasts (NHDFs) and human osteogenic sarcoma (Saos-2 cells) (Fig. 8d and e), making this biofilm appropriate for skin regeneration application.



**Fig. 8** (a) Mechanical properties in relative units as a function of chitin nanowhiskers (ChNWs) volume fraction. (b) Stress-strain curves of nanocomposites biofilms with different content of ChNWs in HA matrix. (c) Antibacterial properties of nanocomposite biofilm against different types of bacteria. (d, e) Fluorescent microscope images of NHDFs and Saos-2 cells in nanocomposite biofilms. Reproduced from ref. 95 with permission from Elsevier.

**3.1.5. Silk.** Silks are protein polymers that are spun into fibers by some lepidoptera larvae, like *B. mori* silkworm ones that have been the primary silk-like material used in biomedical applications as sutures.<sup>196</sup> Silk fibers and regenerated silk fibroin films exhibit comparable biocompatibility *in vitro* and *in vivo* with other commonly used biomaterials such as polylactic acid and collagen.<sup>197</sup> Regenerated silk fibroin is also

biodegradable,<sup>198</sup> and presents high cytocompatibility to a number of mammalian cell types,<sup>199</sup> what makes silk fibroin an interesting scaffolding biomaterial.

The incorporation of CHNCs into the silk fibroin matrix was found to solve one of the major limitations of silk, its poor dimensional stability.<sup>90</sup> Moreover, the reinforced silk/CHNC sponge enhanced the compression strength and exhibited

interconnected pore network with an average pore size of 150  $\mu\text{m}$  that promoted cell spreading, compared to the neat silk sponge. Thus, all of these results might indicate the potential utility of this nanocomposite system for further exploration as a TE scaffolding material.

Table 2 summarises the state-of-the-art of the biologically-active matrices prepared with CHNCs, the role of CHNCs in the biomaterial and the TE application.

**Table 2** The role of CHNCs in biologically-active matrices and TE applications

Matrix	Chitin Origin & Isoform	Additives	Biomaterial	Role of CHNCs	TE Application	Ref.
Chitosan	-	-	3D porous scaffold	Enhance mechanical properties Enhance biocompatible to MC3T3-E1 cells	Bone	98
	Shrimp Alpha isoform	-	3D porous scaffold	Enhance mechanical properties Improve biological activities Support & favor chondral differentiation of hASCs	Cartilage	14
	-	Alginate	Bilayer electrospun scaffolds	Enhance adhesive properties Increase biocompatibility Promote hMSCs proliferation & adhesion	Biomedical application*	28
	Alpha isoform	-	Films	Decrease swelling ratio Promote macropores formation Control BMSCs behavior	Biomedical application*	91
Carboxymethyl chitosan	Alpha isoform	Dextran dialdehyde	Hydrogels	Enhanced mechanical properties Improve adhesive strength	Tissue adhesive Biomedical application*	96
Cellulose	Crab Alpha isoform	-	Cryogels	Enhance mechanical properties Increase biocompatibility with hMSCs	Biomedical application*	94
Methylcellulose	Cuttlefish bone Beta isoform	-	Injectable hydrogel	Enhanced mechanical properties Higher gelation rate	Biomedical application*	92
Cellulose acetate	Crab Alpha isoform	-	Electrospun nanofiber mats	Increase electrical conductivity Introduce antibacterial activity ( <i>E. coli</i> ) Enhance biological activity	Biomedical application*	93
Hyaluronic acid	Shrimp Alpha isoform	-	Composite films	Enhance mechanical properties Promote antibacterial activity against Gram-negative and Gram-positive bacteria	Skin	95
Alginate	-	-	Hydrogels	Enhance mechanical properties Promote cell adhesion/proliferation	Bone	97
Silk fibroin	Shrimp Alpha isoform	-	Matrix	Improve dimensional stability Enhance mechanical properties Promote L929 cell spreading	Biomedical application*	90

\*Not specified

### 3.2. Chitin nanofibers in biologically-active matrices for TE

CHNFs, as CHNCs, exhibit outstanding mechanical properties, wide-availability, sustainability, biocompatibility and

biodegradability.<sup>200</sup> However, the use of CHNFs confer new structures and properties to the developed biomaterials due to the nanoscale effects of the chitin fibers. Thus, biopolymer nanofibril-based materials exhibit architectures closer to those found in natural material systems and mimic ECM texture

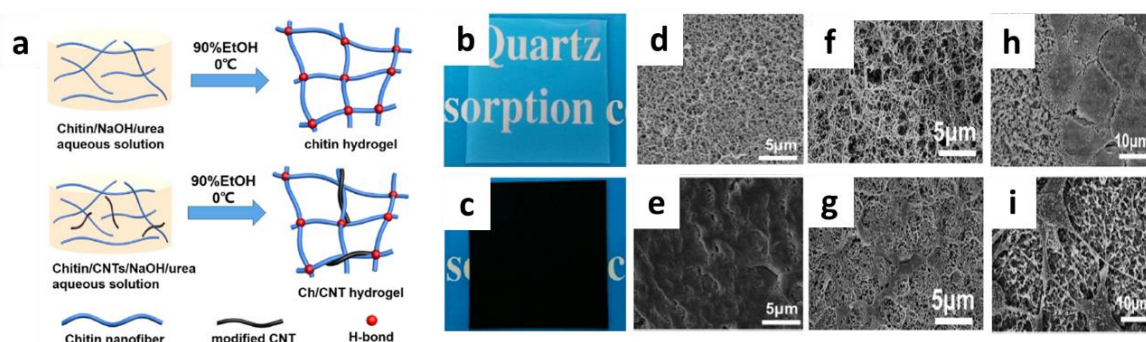
favoring cell behaviors mediated or influenced by topography such as cell adhesion, proliferation and differentiation.<sup>55</sup>

**3.2.1. Chitin.** Although chitin has poor solubility, its application in biomedical field is of interest due to its distinctive biological properties that include good biocompatibility, biodegradability, and wound healing effects.<sup>27</sup> The first TE approach using CHNFs was made by Noh *et al.*, with electrospinning technique, to synthesize chitin nanofibrous matrices containing CHNFs.<sup>106</sup> The nanofibrous matrices exhibited cytocompatibility and good cell attachment and spreading of normal human keratinocytes and fibroblasts *in vitro*. *In vivo* assay showed that biodegradation was faster than commercially available CHNFs, and there was no inflammation after the chitin nanofibrous matrix was implanted into rat subcutaneous tissue. Additionally, the nanofibrous matrices could be modified using different ECM proteins such as type I collagen, fibronectin or laminin, by adsorption onto the chitin matrices as substrates. Cell behavior evaluation showed that collagen type I presented best interactions with both cell lines. Results indicated that this chitin nanofibrous matrix could be useful for wound healing and regeneration of skin and oral mucosa. Subsequent studies have highlighted the role of the degree of deacetylation of CHNF's surface on cell behavior, showing that an increase in the surface-deacetylated CHNF contributes to a better mouse fibroblast adhesion and proliferation<sup>104</sup> and that they support rat cardiac myoblast and mouse bone osteoblast viability and proliferation after one-week contact cell culture.<sup>114</sup>

Later, chitin hydrogel was developed for nerve TE by CHNF and carbon nanotube (CNT) addition (Fig. 9).<sup>118</sup> As CNTs possess excellent mechanical, chemical and electrical properties, they have been suggested for promoting neuronal cell growth, viability and differentiation,<sup>201</sup> but biomedical applications of

CNTs is limited due to their potential cytotoxicity. Thus, in this work they proved the effectivity of mixing CNTs with a highly biocompatible matrix such as chitin, to avoid their toxicity. CNTs dispersed homogeneously in the composite hydrogels, based on chitin and combined with CHNF to form a net nanofibrous network with improved mechanical properties (Fig. 9a-g). *In vitro* assay verified that CHNF/CNT hydrogels possessed good hemocompatibility, biodegradation and biocompatibility to PC12 neuronal cells and RSC96 Schwann cells, but also exhibited significant enhancement of the neuronal cell adhesion, proliferation and neurite growth of neuronal cells (Fig. 9i). This hydrogel was proposed as a good neuronal growth substrate for the potential application in nerve regeneration.

CHNF-based composites have been also employed for the development of bone mimetic structures. Nanofibrous biocompatible polymers and hydroxyapatite (HAP) have been extensively demonstrated to be successful strategy for mimicking the natural bone hierarchical nanostructure, as the native non-collagenous proteins are key in the nucleation process of HAP crystals within the collagen matrix.<sup>202</sup> Similarly, natural shells of marine animals contain a mineralized nanofibrous chitin and these chitin nanofibers are thought to play key roles as organic templates for the mineralized architecture formation.<sup>203,204</sup> Based on this, *in situ* HAP mineralization approach was developed to obtain a bone scaffolding material by coating HAP crystals on nanofibrous chitin microspheres.<sup>116</sup> Microsphere scaffolds exhibited biocompatibility and promoted *in vitro* cell adhesion of MC3T3-E1 osteoblasts cells. *In vivo* rabbit radius defect repair demonstrated an appropriate biodegradability and a high-inducing osteoconduction, as the provided structure permitted cell invasion and proliferation resulting in an almost completely healing within three months.



**Fig. 9** (a) Schematic representation of chitin and chitin/carbon nanotube (ChCNT) hydrogels construction. (b,c) Photographs of wet chitin and Ch/CNT hydrogels with 3wt% CNTs (Ch/CNT3). SEM images of the (d, e) surface and (f, g) inner parts of the lyophilized chitin and Ch/CNT3 hydrogels. (h, i) SEM Images of PC12 cells cultured on wet chitin and Ch/CNT hydrogels for 24h. Reproduced from ref. 118 with permission from Elsevier.

Recently, has emerged enzymatic mineralization as a novel biomimetic approach for a more homogeneous mineralization of the bulk material.<sup>205</sup> This method employs homogeneously

dispersed enzymes which induce mineral formation by a pH change or that convert precursor molecules into active mineralization compounds.<sup>206</sup> Yao *et al.*, employed alkaline phosphatase to create highly mineralized hybrid materials based on controlled mineralization of calcium phosphate (CaP)

in polysaccharide nanofiber material made by anionically charged cellulose nanofibers (CNFs) and cationically charged CHNFs building blocks (Fig. 10a).<sup>117</sup> Results showed that anionic cellulose nanofibers lead to a more homogeneous mineralization with high mineral contents up to 70 wt %, while CHNFs lead to the formation of rod-like crystals and initial amorphous calcium phosphate deposits that during prolonged mineralization transform into crystalline HAp structures (Fig. 10b).

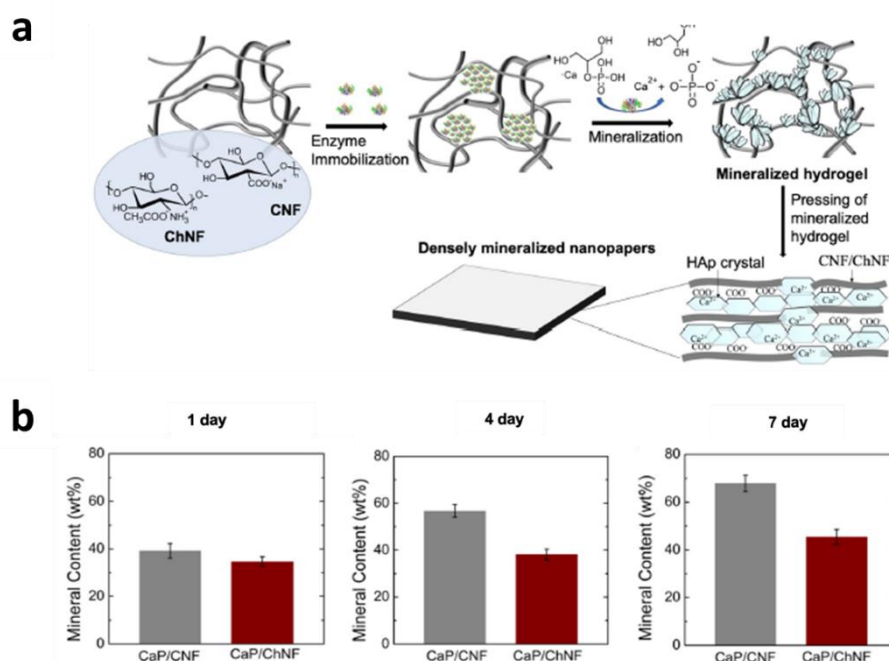
The incorporation of HAp on the surface of genipin-crosslinked CHNF hydrogel/cryogels has also resulted in an interesting strategy for its use in cartilage TE, since it showed good biocompatibility with chondrocytes and improved their adhesion and growth.<sup>111</sup>

**3.2.2. Chitosan.** Intrinsic antibacterial activity makes chitosan a good matrix to develop materials for dermal or skin tissue engineering. However, there is the need to use a nanofiller to overcome the poor mechanical properties and the absence of biological responses. Kiroshka *et al.*, got chitosan-based films with improved strength and deformation properties, increased elasticity modulus and plastic limit by the addition of CHNFs as filler agents.<sup>107</sup> The subsequent reduction on the deacetylation rate and local decrease in surface charge density, promoted adhesion and proliferation of keratinocytes and fibroblasts in

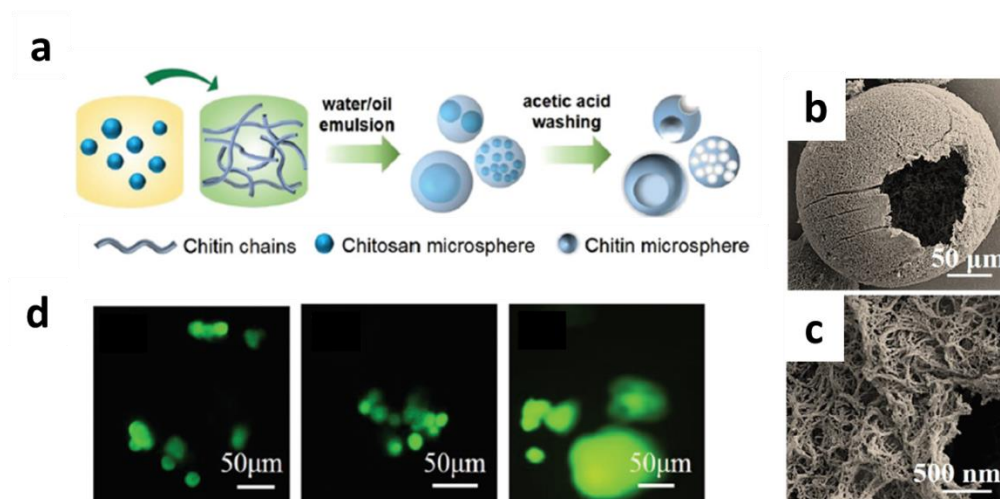
comparison with pure chitosan matrix. These results are in accordance with those showed in a previous work<sup>207</sup> where was demonstrated the dependence of adhesion and proliferation of both type of cells on deacetylation degree of chitosan films.

Thus, this matrix can be used in TE to control the growth of specific cell types, and in regenerative medicine due to its capacity to stimulate cell adhesion and proliferation in the damaged areas. Another group demonstrated that 5 % wt. CHNFs was sufficient as reinforcing agent to improve mechanical, physicochemical, specific conductivity and bioactive properties of chitosan-based film matrix for its use in combination with fibroblasts on dermal TE.<sup>110,174</sup>

The use of CHNFs on chitosan-based materials has been also found to be useful in 3D cell culture (Fig. 11).<sup>208</sup> Macroporous chitin microspheres were constructed via a “bottom-up” approach based on chitosan microspheres coated with chitin solution (Fig. 11a). Resulting material exhibited good mechanical properties due to the existence of nanofibrous network (Fig. 11b and c). This structure provided open 3D spaces that supported growth, attachment and migration of monkey kidney fibroblasts (COS-7), normal human hepatocytes (L02) and human pancreatic cancer cell line (PANC-1) (Fig. 11d), and maintained multi-lineage differentiation capacity of seeded human embryonic stem cells (hESCs) (Fig. 11e).



**Fig. 10** (a) Schematic representation of enzymatic mineralization of chitin and cellulose nanofibril networks toward mineralized nanopapers. (b) Mineral content of CaP/CNF and CaP/ChNF after 1, 4 and 7 days of calcification. Reproduced from ref. 117 with permission from American Chemical Society.



**Fig. 11** (a) Scheme of the porous chitin microspheres (PCMS) preparation procedure. SEM images of (b) PCMS and (c) the nanofibrous structure of PCMS. (d) Live/dead assay fluorescent images of PANC-1, L02 and COS-7 cells cultured on PCMS. Reproduced from ref. 208 with permission from Royal Society of Chemistry.

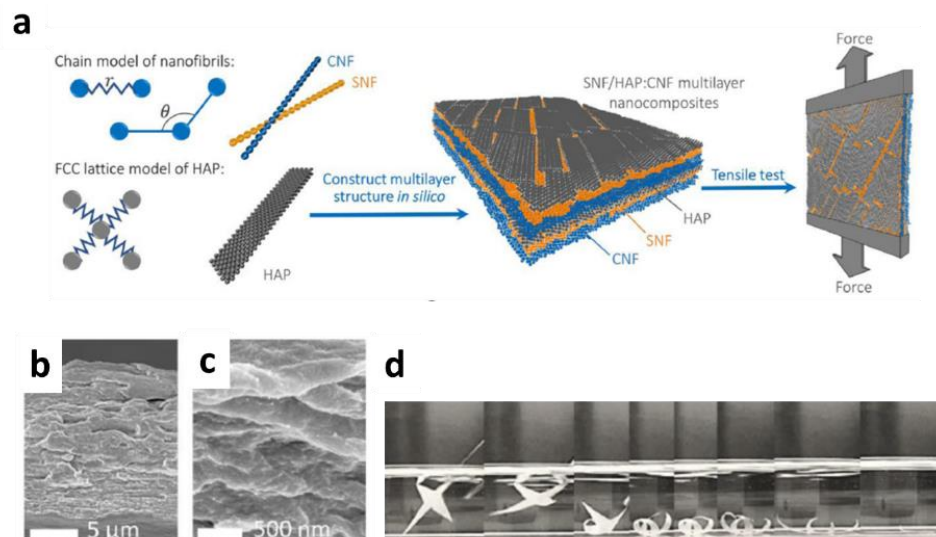
**3.2.3. Cellulose.** Torres-Rendon *et al.*, developed a mathematically defined gyroidal hydrogel scaffold based on cellulose nanofibers and CHNFs.<sup>115</sup> This novel approach allows to define millimetrically the architecture of the material to obtain advanced and customized materials for TE with a biomimetic structure that controls and induces the cellular behavior of interest. In this case, the cellulose nanofiber/CHNF hydrogel exhibited promising applicability in both soft and hard TE, as the designed material can support fibroblast growth and viability and induce hMSCs differentiation toward osteogenic lineage when a collagen-mimetic coating is deposited on the surface of the hydrogel.

**3.2.4. Silk.** Nacre is composed of biopolymers and aragonites, which are organized into a hierarchical structure to optimize mutual interactions and to combine properties towards longer length scales.<sup>209</sup> Therefore, biomimetic approaches like “assembly-and-mineralization” methods that combine preformed laminated matrices and *in situ* mineralization techniques, are focused on the production of materials with structural and mechanical characteristics similar to natural ones.<sup>210</sup>

A biomimetic material was developed by *in silico* design,<sup>100</sup> in which mechanical enhanced multilayer nanocomposites was obtained with controlled assembly of silk nanofibers (SNFs), HAp and CHNFs (Fig. 12a). This nacre-like composite showed a biomimetic structure (Fig. 12b and c) with enhanced mechanical properties due to the presence of CHNFs, and presented self-bonding function (Fig. 12d). Thus, it is found a potential approach in the design of novel third-generation biomaterials for potential clinical applications.

**3.2.5. Collagen.** Collagen is the major insoluble fibrous protein in the ECM of mammalian animals, mainly located in connective tissues. It has been widely used in biomedical applications due to its biodegradability, low antigenicity and ability to enhance cell attachment and proliferation.<sup>211</sup> However, like other ECM-derived biomaterials, collagen by itself is not enough to obtain solid materials with good mechanical properties.<sup>212</sup> CHNFs are found to be promising nanofillers that improve mechanical strength of extruded collagen fibers comparable to post-crosslinked ones.<sup>102</sup> Moreover, increasing CHNF concentration to 15% wt. CHNFs, increases tensile strength and modulus of collagen fiber to highest values of 506.6 MPa and 12109.5 MPa, respectively. These mechanically tough collagen fibers exhibited biocompatibility towards fibroblasts and induced their alignment on the rough surface of the fibers, showing potential application as orientation scaffold for blood vessels, muscle and neural repair. The work of Barbalinardo *et al.*, more focused on the biocompatibility, has shown that the availability of  $\beta$ -CHNFs after its combination with collagen plays an important role in promoting cell viability due to the carbohydrate-protein interactions that mediate cell adhesion. Thus, the homogeneous dispersion of the CHNFs in the matrix improves the performance of the resulting scaffold for its application in TE.<sup>105</sup>

**3.2.6. Gelatin and its derivatives.** Gelatin is a denatured form of collagen widely used in biomedical field due to its biocompatibility, biodegradability and ability to form a hydrogel at low temperatures. However, final mechanical properties of gelatin-based materials are poor and limit its applications.<sup>213</sup> To address this limitation, nanoscale chitin fillers have been proposed as reinforcing agents.<sup>214</sup>



**Fig. 12** (a) Representative *in silico* design of mechanical-enhanced silk nanofibril/hydroxyapatite/chitin nanofibril (SNF/HAP:CNF) bionanocomposite. (b,c) SEM images of 10:10 SNF/HAP:CNF membrane. (d) Self-bonding behaviour of the 10:10 SNF/HAP:CNF nanocomposite. Reproduced from ref. 100 with permission from Wiley.

CHNF/gelatin composite film developed with high transparency and viscosity exhibited fine nanofiber structure.<sup>99</sup> The composite film stimulated fibroblast proliferation *in vitro* and did not induce severe inflammation *in vivo*, indicating high biocompatibility. Surface-deacetylated CHNFs have been also employed to improve mechanical properties of both gelatin-based nanocomposites<sup>101</sup> and hydrogels,<sup>112</sup> with promising use as biomaterials in biomedical applications.

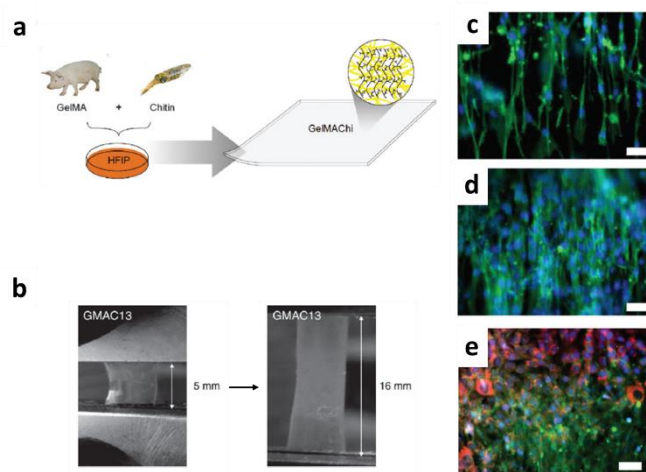
Advantageous properties of CHNFs as fillers have been also reported for gelatin methacrylate (GelMA), a gelatin derivative synthesized by the direct reaction of gelatin with methacrylic anhydride (Fig. 13).<sup>113</sup> The micropatterned GelMA/CHNF hydrogel (Fig. 13a) exhibited increased elastic modulus to 1000-fold and strain-to-failure over 200% (Fig. 13b and c). Moreover, the hydrogel permitted proliferation and alignment of human umbilical vein endothelial cells (HUVECs)/hMSCs co-culture (Fig. 13d and e), and also induced differentiation of hMSCs into smooth muscle cells and vascular network formation (Fig. 13f). This resulted in a novel platform for studying vasculogenesis with better mechanical properties than collagen-based hydrogels.<sup>215</sup>

**3.2.7. Lignin.** Lignin is an organic compound with a complex structure, which is highly available since it is present in all wood along with cellulose. On the nanoscale, lignin structure and purity can be controlled, resulting in several interesting properties such as antibacterial, anti-inflammatory, cicatrizing and anti-ageing effectiveness.<sup>216,217</sup> In addition, nanolignin shows potential application for improvement of mechanical properties of polymer composites, as lignin is responsible for the strength and rigid structure of the cell walls in plants.<sup>218</sup>

Danti *et al.*, developed a cytocompatible microcapsule-like system with anti-inflammatory activity combining CHNFs and nanolignin, loaded with glycyrrhetic acid as a bioactive

molecule for skin regeneration.<sup>108</sup> This approach is based on previous work where electropositive and electronegative nanoforms were combined to entrap both hydrophilic and lipophilic molecules such as vitamins, microelements, anti-inflammatory drugs, antioxidants, anti-ageing substances, immunomodulating agents and enzymes.<sup>216</sup>

Table 3 summarises the state-of-the-art of the biologically-active matrices prepared with CHNFs, their role in the biomaterial and the TE application.



**Fig. 13** (a) Scheme of the self-assembly process of GelMA/chitin (GelMAChI) films dissolved in hexafluoroisopropanol (HFIP). (b) Optical image of chitin nanofibers-gelatin methacryloyl 1:3 weight ratio (GMAC13) before load application and under tensile load in mechanical tensile tester. (c,d) Fluorescent images of Actin/Dapi stained cell orientation cultured for (c) 3 and (d) 5 days on the patterned GMAC13. (e) Immunostaining of vascular markers for HUVECs/hMSCs cultured for 5 days on GMAC13. Scale bar represents 50  $\mu\text{m}$ . Reproduced from ref. 113 with permission from Royal Society of Chemistry.

**Table 3** The role of CHNFs in biologically-active matrices and TE applications.

Matrix	Chitin Origin & Isoform	Additives	Biomaterials	Role of CHNFs	TE Application	Ref
Chitin	-	-	Electrospun nanofiber mats	No-inflammation <i>in vivo</i> Improve cell attachment and spreading	Oral mucosa Skin	106
	Alpha isoform	-	Nanofiber mat	Surface deacetylation increase cell adhesion and proliferation	Biomedical material*	104
	Crab Alpha isoform	-	Wet-spun microfibers	High water sorption capacity Support proliferation of rat cardiac myoblasts and mouse bone osteoblasts	Cardiac Bone	114
	-	Carbon nanotubes	Hydrogels	Enhance mechanical properties Hemocompatible Biocompatible to neuronal and Schwann cells Improve neuronal cell behavior	Nerve	118
	Alpha isoform	Hydroxyapatite crystals	Microsphere scaffolds	Promote cell adhesion <i>In vivo</i> bone healing	Bone	116
	-	Alkaline phosphatase	Mineralized nanopaper	Enhance mechanical properties Homogeneous and spatial controlled mineralization	Bone	117
	Alpha isoform	Hydroxyapatite	Hydrogel/Cryogel	Enhance mechanical properties	Cartilage	111
Chitosan	-	-	Films	Enhance mechanical properties Promote cell adhesion and proliferation	Skin	107
	-	-	Films	Enhance mechanical properties Improve biological activities	Dermal tissue	109
	Alpha isoform	-	Porous microspheres	Enhance mechanical properties Allow hESCs multi-lineage differentiation	3D cell culture	103
	-	-	Films	Enhance mechanical properties Orientation of structural elements Increase specific conductivity Promote skin fibroblasts adhesion, viability and proliferation	Skin	110
Cellulose	-	-	Hydrogel	Induce hMSCs osteogenic differentiation	Bone	115
Silk	-	Hydroxyapatite	Multilayer films/membranes	Enhance mechanical properties Self-bonding function	Biomedical material*	100
Collagen	-	-	Microfibers composite	Enhance mechanical properties Induce cell alignment	Vascular Muscle Neural	102
	Beta isoform	-	Films	Complement collagen biocompatibility on fibroblast growth	Biomedical material*	105
Gelatin	Crab Alpha isoform	-	Films	Enhance wound healing process	Biomedical material*	99
	Crab Alpha isoform	-	Nanocomposite films	Enhance mechanical properties	Biomedical material*	101
	Crab Alpha isoform	-	Hydrogels	Enhance mechanical properties	Tendons Ligaments	112
	-	-	Hydrogels	Enhance mechanical properties	Vascular	113
Gelatin methacrylate	-	-	Hydrogels	Enhance mechanical properties	Vascular	113
Lignin	Alpha isoform	-	Microcapsule-like system	Biocompatible Anti-inflammatory activity	Skin	108



\*Not specified

## Conclusions

In the last two decades, chitin's research has been focused not only on its main derivative, chitosan, but also on its nanometric forms, named chitin nanocrystals (CHNC) and chitin nanofibers (CHNF). Specifically, there has been an increase in the number of articles published in the last 17 years in which chitin nanoforms have been used for the design and development of new biomaterials for biomedical applications, being Tissue Engineering the field of greater interest.

Herein, we have done comprehensive summary of the state-of-the-art of both CHNC and CHNF in combination with biologically-active matrices such as polysaccharides (chitin, chitosan, cellulose, hyaluronic acid, alginate), proteins (silk, collagen, gelatin) and others (lignin); of their role in those matrices; the type of biomaterial developed and the TE application.

Particularly, chitin nanoforms have been used as natural nanofillers to obtain biomaterials with superior properties, since they cause changes in the properties of the natural matrix, mainly improvement of the mechanical properties. Additionally, they can improve the bioactivity of the matrix since CHNC facilitate effective interaction with cells, proteins or other compounds, while CHNF confer a fibrous structure that better mimics the texture of the ECM, and thus favoring topography-dependent cell behavior. CHNC have been mainly used in combination with polysaccharide matrices and there is a tendency to develop hydrogel-like biomaterials. On contrast, CHNF have been equally combined with polysaccharide and protein matrices, mostly in the shape of a film followed by hydrogels. Regarding the application tissue, CHNF have a predisposition to soft tissues (cartilage, skin, vascular, etc) and bone, but there is no marked preference when using CHNC. With all this, CHNF have been more studied because they offer greater versatility to be combined with biologically-active matrices and even biomaterials can be developed only with nanofibers.

The use of nanochitin in combination with biologically-active matrices for TE applications is still in its infancy and the number of publications in this area is expected to continue to increase for both nanoforms. With the bases established, it is expected that the combination of nanochitin with the different biologically-active matrices will give rise to increasingly complex matrices, and that the use of CHNC will be extended to the level of CHNF. Although great progress has been achieved to develop these distinct chitin nanoforms into biomaterials for TE, many aspects still need to be taken into consideration and analyzed. For instance, initiatives and efforts to mention and understand the effects of chitin origin, type of isoform, molecular weight, degree of acetylation and chemical or enzymatic modification of chitin nanoforms on the final physicochemical properties and versatile functions and applications of the biomaterials.

Future developments should be carried out in the study of the suitability of the effect produced by the incorporation of each

type of nanoform, CHNC or CHNF, on the biological properties or bioactivity of the resulting biomaterial, depending on the origin of the matrix. Furthermore, the use of these nanoforms in the creation of biomimetic soft architectures through 3D and 4D assembly methods is limited and needs to be addressed more meaningfully in future studies. In addition, a larger number of studies are required to assess the suitability of biomaterials developed with a greater variety of cell types, in order to broaden their application to different specific tissues. Likewise, it is key to take a step forward and increase the number of *in vivo* studies that support the applicability of these new biomaterials, since their compatibility and real efficacy are unknown.

## Author Contributions

Conceptualization: S.O. and S.C.M.F.; Writing - original draft: S.O.; Writing – review & editing: S.O., A.M.S., A.A-V., A.S., S.C.M.F.; Supervision: A.A-V and S.C.M.F.; Validation: S.O., A.M.S., A.A-V., A.S., S.C.M.F.; Funding acquisition: A.A-V and S.C.M.F..

## Conflicts of interest

There are no conflicts to declare.

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