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Review

Chitosan-Based Nanomaterials for Skin Regeneration

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Abstract: Chitosan (CS) is a renewable polysaccharide widely used for the preparation of biomaterials due to its special properties such as its biodegradability and biocompatibility, mucoadhesive behavior, and antibacterial and anti-inflammatory effects. These features are very important for biomedical applications, especially for tissue engineering and skin regeneration. From the clinical point of view, there is an increasing demand for the development of new materials/scaffolds with dual roles: the promotion of skin tissue repair and the simultaneous exertion of potent antimicrobial effects. Nanotechnology has been extensively employed in several pharmacological applications, including cutaneous tissue repair and antimicrobial treatments. The combination of CS and nanotechnology might create new avenues in tissue engineering. In this sense, this review presents and discusses recent advantages and challenges in the design of CS-based nanomaterials (in the form of nanofibers, composite nanoparticles, and nanogels) for cutaneous tissue regeneration. The combination of CS-based nanomaterials with other polymers, active drugs and metallic nanoparticles is also discussed from the viewpoint of designing suitable platforms for regenerative medicine and tissue engineering.

Keywords: chitosan; nanoparticles; nanofibrous materials; nanocomposites; nanogels; skin regeneration

1. Introduction: Skin Regeneration

Skin is composed of several layers essential to its function and response to injury: epidermis,

dermis and hypodermis. Upon injury, damaged skin tissue naturally initiates a repair process, named wound healing [1,2]. Wound healing is a spontaneous and dynamic repair process in the injured tissue consisting of hemostasis, inflammation, proliferation and remodeling (or scar tissue formation) phases [3,4]. The early phase after skin trauma is hemostasis, and hemorrhage is one of the leading causes of early death [4]. Wound healing aims to repair the damaged tissue by re-establishing the integrity of the injured tissue and replacing lost tissue [5].

The inflammatory phase (1–3 days after wounding) is characterized by a high cellularity due to the migration of neutrophils, macrophages and lymphocytes to the wound site. This phase functions to clean the wound bed by attracting inflammatory cells to the wound site and promoting hemostasis. The next phase of wound healing is the proliferative granulation tissue formation phase (4–14 days after wounding). This phase is characterized by the proliferation of fibroblasts on the wound site and the production of important extracellular matrix components (EMC), such as elastin, collagen and glycosaminoglycans. The remodeling (or scar tissue formation) phase is the last phase of wound healing and it is characterized by the replacement of immature type III collagen with mature type I collagen. In this phase, the tissue becomes less vascularized, finally leading to complete skin tissue repair [6].

The remodeling phase is the phase that is most responsible for differences in the quality of scars and the formation of keloids. Keloids and hypertrophic scars are characterized by abnormal growth of the tissue due to extensive production of extracellular matrix, especially collagen, which is not confined to the original wound site and invades adjacent dermal tissues [7,8]. The main cellular components in the keloid process are fibroblasts, keratinocytes, melanocytes and mast cells. Myofibroblasts are a different form of fibroblast that seem to be absent, or minimally present, in keloid scars, in contrast to the situation in hypertrophic scars [7,8].

The aim of the wound repair process is to reach a rapid and efficient skin tissue repair with maximal function and minimal scarring, and without the formation of keloids. However, successful skin tissue repair still remains a biomedical challenge and a major healthcare issue. In particular, chronic wounds, such as diabetic and ischemic wounds, usually result in loss of functional ability, decreased quality of life and increased pain and infections [6]. Therefore, an enormous effort is being made to develop new and efficient approaches that can efficiently promote full skin tissue recovery with minimum side effects and costs [9]. To this end, biopolymers, including chitosan, have been extensively employed in tissue engineering and regenerative medicine in the form of several versatile biomaterials used to restore, maintain or improve damaged skin tissues.

2. Chitosan (CS) as A Promising Renewable Polymer in Biomedical Applications

The cationic polysaccharide chitosan (CS) is one of the most commonly employed biopolymers in biomedical applications due to its biocompatibility and biodegradability, and its mucoadhesive and hemostatic properties [4,10,11]. CS is obtained by the partial deacetylation of chitin, a natural

polymer found in the exoskeletons of insects, fungi and crustaceans [10]. Although several papers describe the use of chitin as a scaffold in biomedical applications [12], due to its poor solubility in aqueous solution, chitin is not as readily applicable as CS. The partial deacetylation of chitin leads to CS, in which the presence of amino groups significantly enhances the solubility of the polysaccharide in aqueous solution [10]. The degree of chitin deacetylation is correlated with the amino acid density of CS, and consequently to the degree of charge density. CS is a weak base (pKa = 6.5), and is soluble in acid solution due to the protonation of its amino groups. In contrast, at basic or neutral pH, CS is water insoluble [4]. CS is available with different degrees of deacetylation and at different molecular weights; the aqueous solubility of the biopolymer is driven by intermolecular hydrogen forces and ionic and hydrophobic interactions [10]. The chemical structure of CS is composed of N-acetylglucosamine and glucosamine. In biological systems, glucose generates glucosamine, which in turn produces glycosaminoglycans (GAGs), an essential component of cartilage and extracellular matrix tissues [13].

As a polycation at acidic pH, CS is able to interact with polyanions via electrostatic interactions, leading to the formation of polyelectrolyte complexes and nanoparticles [4]. Due to their properties, CS based materials are highly attractive in clinical applications in the form of nanoparticles, nanogels, nanofibers, sponges, scaffolds, beads, films and membranes [5]. CS is a versatile matrix that positively impacts the wound healing process, antimicrobial cell defense, and anti-inflammatory cell responses, as well as possessing permeation enhancing properties [14].

In cutaneous wound healing, topical application of CS-base materials has been reported to promote and accelerate the tissue repair process [4,10]. The N-acetylglucosamine residues in CS chain bind to specific receptors in vivo, activating macrophages and leading to further important events in the healing process, such as the release of cytokines and biological mediators [10]. Interleukin 8 (IL-8) and other cytokines are released at the wound bed upon fibroblast stimulation by CS. CS also stimulates hemostasis at the wound bed by activating platelets and accelerating blood clotting, an important event in the early phase of wound repair (inflammatory phase) [15]. Wound healing is a complex and dynamic tissue repair process, in which several biological molecules play important and orchestrated functions [2–4]. CS aids wound healing by stimulating fibroblast proliferation, macrophage activation, cytokine production, angiogenesis, collagen and hyaluronic acid formation, and deposition at the wound site [15]. The care of skin lesions is a critical healthcare problem, and recently there has been a tremendous amount of research into the development of CS-based wound dressings due to the hemostatic action and beneficial effects of CS in wound repair [4,10,16]. Many of these wound dressings contain immobilized nanoparticles and CS-based material [4].

In addition, CS has inherent antimicrobial properties due to its cationic nature, which allows CS to bind to anionic groups of bacteria, algae, and fungi [4]. CS also reduces inflammatory pain in a dose-dependent manner by impairing the expression of cyclooxygenase-2 protein and prostaglandin E2, and decreasing the levels of pro-inflammatory cytokines [4]. This property is especially

beneficial in topical applications of CS based materials to the wound bed of painful wounds, such as skin abrasions, ulcers, burns and skin grafted areas [4]. CS based nanomaterials have been successfully employed as drug delivery systems in vivo. CS has permeation enhancing properties owing to its cationic character, allowing the interaction of the polymeric chain with cell membrane constituents, leading to a reorganization of the structure of tight junction-associated proteins [11]. Indeed, the cationic character of CS allows it to interact with negatively charged keratinocytes in the skin, improving permeation and effecting the passive targeting of drugs loaded into CS-based nanomaterials to the deep layers of skin [17]. The presence of positive charge in the CS chain is responsible for its mucoadhesive characteristics via electrostatic interactions [10]. The biodegradability of CS in human body is due to the actions of lysoenzyme, colon bacteria and some gastrointestinal enzymes [18,19]. Low molecular weight chains of CS can be excreted by the kidney, whereas high molecular weight CS can be degraded into smaller chains before renal clearance [11]. Therefore, CS is a promising renewable material for several biomedical applications. In particular, CS-based nanomaterials have been used as promising materials in several biomedical applications due to the inherent advantages of CS and nanotechnology. In this context, the following sections discuss the recent state of the art in the design of CS-based nanomaterials for biomedical applications.

3. Why Use CS-Based Nanomaterials for Skin Tissue Regeneration?

CS based nanomaterials have made a significant contribution to the development of new materials suitable for use in a variety of biomedical applications such as in drug delivery, biosensors, diagnostics, and tissue engineering [15]. The most common forms of CS based nanomaterials are nanoparticles, nanofibrous scaffolds, and nanogels. Nanomaterials offer important advantages related to their nanometer size, such as their very large surface area compared with their volume and the ability to enter into cytoplasmic space (high cellular uptake) [6]. The incorporation of therapeutic agents into CS nanoparticles might improve their chemical stability, bioavailability and biocompatibility, while allowing the pharmacokinetics and pharmacodynamics of the therapeutic agent to be modified, enhancing the efficacy of the active agents and reducing their toxicity [6,11].

In tissue engineering, the topical administration of nanomaterials has been reported to significantly improve skin tissue repair [20]. This is due to the fact that many biomolecules and extracellular matrix (ECM) components interact at the nanoscale [21]. Collagen is the major biological component found in the ECM, and it has a fibrous structure with fibers in the range from 50 to 500 nm. CS-nanobased materials, in particular, CS-nanofibers releasing active drugs, are considered as suitable scaffolds for skin tissue engineering [22]. For successful restoration of the original tissue architecture, the presence of a scaffold is necessary, since biological cells are unable to re-establish their original structure without the presence of an extracellular guide (scaffold material) [23]. Interestingly, cells growth on nanofibrous scaffolds have a similar phenotype to cells grown in the natural site. This effect can be understood by considering the similar nanostructure of

natural ECM components (collagen) with nanofibrous materials. Therefore, topical administration of nanofibrous materials can be used for skin tissue regeneration due to their ability to guide human native cells along their surface, thus repairing the wounded area [23]. CS-based fibrous nanomaterials thus have great potential to promote skin repair by mimicking the natural environment for human cells and tissues [15,21]. In addition, CS-based nanofibrous materials have been employed as vehicles for the sustained release of therapeutic drugs, and biological molecules such as DNA and proteins [15].

There are several techniques for the synthesis of three-dimensional nanofibers, such as phase separation, self-assembly, template synthesis, drawing and electrospinning [15]. Among them, electrospinning is the most commonly employed technique due to its efficacy and simplicity. It can be used for the preparation of nanofibers with diameters in the range of 5 to 500 nm [24]. The technique is based on the addition of a polymer solution into a syringe followed by ejection of the polymeric solution from a metal capillary with the application of a high voltage power source [24]. Nanofibrous materials cannot be formed from aqueous solutions of pure CS using the electrospinning technique [16]. Therefore, CS is modified or blended with other natural and/or synthetic polymers for the purpose of tissue engineering, wound healing and drug delivery [16,25,26]. Recently, Zhao et al. reviewed the synthesis of nanofibers with renewable polymers, including CS [20]. In their review article, the authors highlighted the harmful aspects of preparing CS nanofibers in organic solvents for biomedical applications [20]. Therefore, the majority of CS-based nanofibrous materials are based on the combination of CS with other polymers in aqueous solution, to enhance the mechanical properties of the nanofibers [20]. Depending on the chemical composition of the CS-based nanofibrous materials, these nanofibers can be successfully used for skin regeneration, tissue engineering, wound healing and hemostasis due to their biocompatibility and biodegradability, their antimicrobial, hemostatic, and mechanical properties and their ability to permit cell adhesion and proliferation on the nanofiber surface [5,15,20,23].

Besides CS-based nanofibrous scaffolds, CS-based nanoparticles (CS NPs) have also been employed in wound healing, for the sustained release of encapsulated or entrapped therapeutic agents (such as growth factors or antibiotics) in skin tissue regeneration [16,27]. As stated above, CS itself has beneficial effects on wound healing. This effect might be enhanced by the combination of CS with therapeutic agents, in the form of CS-based NPs. The methods used to synthesize CS NPs are based on reverse micellization [28], reverse emulsion [29], emulsion-droplet coalescence [30], desolvation [31], emulsion solvent diffusion [32], and electrostatic complexation [33–35]. Among them, electrostatic complexation is the simplest method, and is performed at room temperature and atmosphere in aqueous medium, yielding nanoparticles with reproducible size distributions [36]. Protonated amino groups of CS in acid medium have the ability to form complexes with anionic macromolecules (hyaluronic acid, alginate, DNA), or with molecules such as (poly) phosphate, sulfate, and citrate, leading to the formation of CS NPs [36]. CS NPs have properties that are inherently suitable for biomedical applications (non-toxicity, hemocompatibility, mucoadhesion and

antimicrobial activity), in addition to their ability to efficiently incorporate therapeutic agents [36]. Hydrophilic, hydrophobic and macromolecules can be incorporated into CS NPs, leading to sustained release of the encapsulated drugs. This strategy has been used for various biomedical applications, including skin tissue regeneration.

Another important form of CS nanomaterials is nanogels. Nanogels are hydrogels with sizes in the nanodimensional range [17]. The stimulus-responsive nature of some nanogels can be triggered by changes in pH, temperature and ionic content and this is of particular importance for drug delivery in topical applications [17]. Nanogels are nanoscale cross-linked networks of the same or different types of polymers, with an elevated capacity for water absorption [10]. Some nanogels (hydrogels) have the ability to swell (expansion) upon water absorption due to the presence of hydrophilic groups of polymer chains [10]. Nanogels able to absorb water have structures similar to human tissues, making these materials suitable for tissue engineering. Nanogels can host therapeutic agents, biomacromolecules (growth factors, proteins), and NPs within their crosslinked network [37]. The polymeric structure of the nanogels protects the entrapped molecules from rapid dissolution, allowing a sustained and localized release of the therapeutic agent directly to the target site [10]. Nanogels can be classified as reversible or permanent depending on the type of bonds joining different macromolecular chains. When secondary forces, such as H-bonding, and ionic or hydrophobic forces have the main role in forming the nanogel network, the nanogel is called reversible. When the network is formed by covalent bonds through the crosslinking of polymers, it is called permanent [38]. Therefore, several methods of nanogel preparation can be used to design new materials with advantageous properties for skin regeneration. Nanogels have been extensively used in regenerative medicine as scaffolds for cell adhesion, allowing cellular remodeling that mimics the natural cellular environment [10]. In this direction, nanogels have been employed in surgical coatings, artificial skin, cartilage and corneas. Nanogels can be administered via the oral, nasal, ocular, topical, vaginal and subcutaneous routes [10]. Therefore, nanostructured CS can be prepared in various different forms (nanoparticles, nanofibers, nanogels) suitable for skin regeneration (Figure 1). The next sections highlight selected publications that report different forms of nanostructured CS materials used for skin tissue regeneration. There are a large number of papers in this field. We have selected publications that demonstrate and discuss the preparation of the nanomaterials, characterize them by different techniques, and perform *in vitro* and/or *in vivo* studies of the CS-based nanomaterials. The most relevant results are also shown in Tables. Due to manuscript length restrictions, we are not able to present and discuss all of the literature in this area.

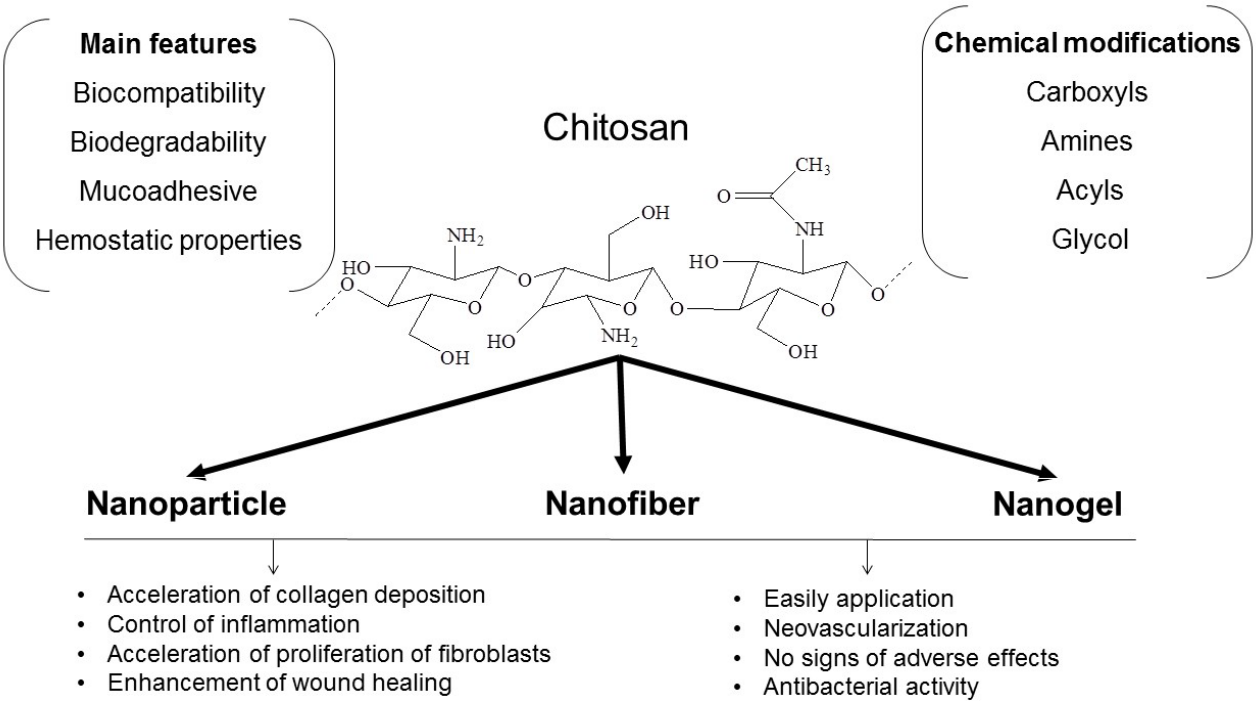

Figure 1. Schematic representation of CS and its properties, which make this biopolymer suitable for skin regeneration.

4. Selected Examples Of CS-Based Nanofibrous Materials for Skin Regeneration

A nanofibrous membrane comprised by collagen type I, CS, and polyethylene oxide (PEO) was synthesized by electrospinning and further crosslinked by glutaraldehyde vapor [24]. This CS-based nanofibrous material has a diameter of 134 ± 42 nm before crosslinking, which increased to 398 ± 76 nm, after the crosslinking. The biocompatibility of the nanofibers was assayed using the 3T3 fibroblast cell line. *In vivo* studies demonstrated that the CS-based nanofibrous material promoted skin regeneration in a superior manner compared with commercial collagen sponge wound dressings and gauzes [24].

More recently, a biomimetic nanofibrous matrix was synthesized via the electrospinning of polycaprolactone (PCL)/cellulose acetate (CA) and layer-by-layer self-assembly (LBL) of negatively charged collagen (type I) and positively charged CS for skin tissue repair [39]. *In vivo*, LBL structured $(CS/collagen)_n$ nanofibrous mats significantly promoted wound healing by enhancing cell migration, vascularization and re-epithelialization. The expression of α-tubulin, collagen IV and integrin β1 was up-regulated, as was the phosphorylation of focal adhesion kinase (FAK) at Try-397. Furthermore, histological evaluations demonstrated that CS/collagen coatings promoted neovascularization, and thus accelerated tissue repair. The ability of this CS-based nanofibrous material to promote skin regeneration was also assayed by the *in vitro* cell migration protocol. To this end, a wound healing assay was performed by culturing normal human dermal

fibroblasts (NHDF) on matrices with gaps created by an insert. This *in vitro* "wound gap" was fully healed after seven days, during which NHDF cells migrated onto the CS/collagen scaffold. In contrast, after seven days, the "wound gaps" of the control groups remained unclosed [39]. These results highlight the promise of the CS-based nanofibrous material for skin regeneration.

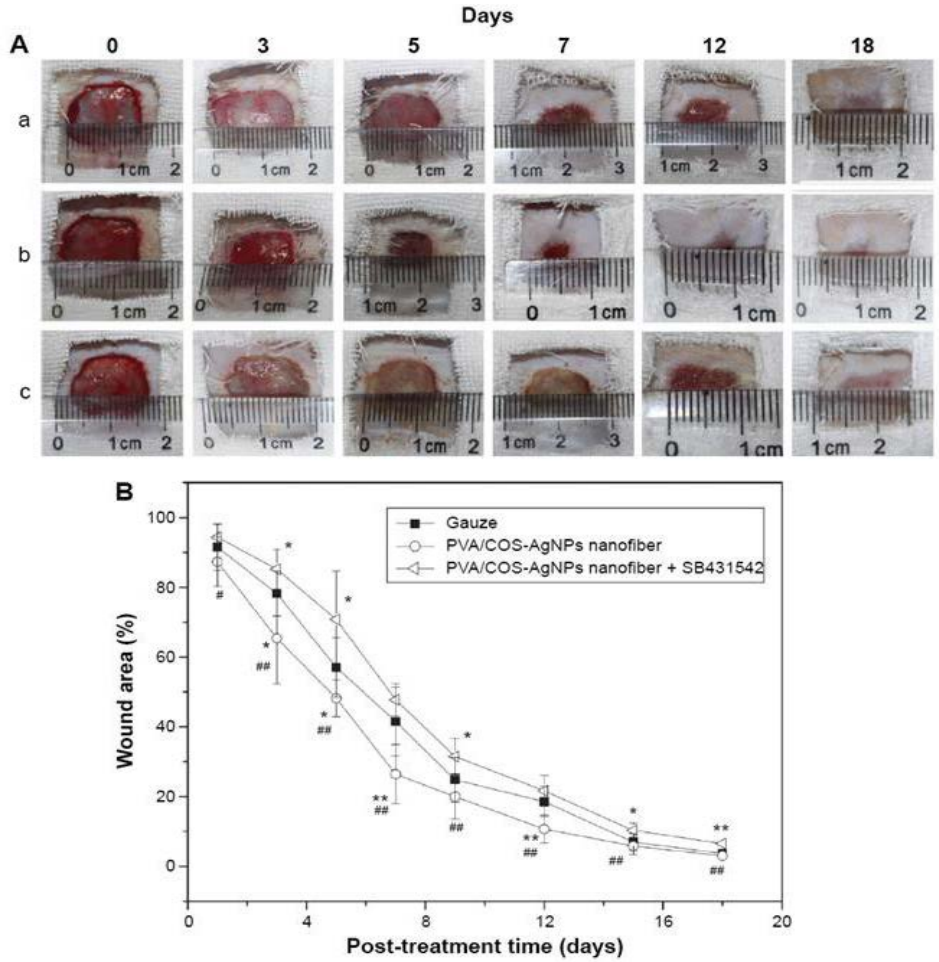

Figure 2. Topical application of PVA/CS-AgNPs nanofibers decreases the wound area of wounded rat skin, leading to a superior cosmetic appearance. A: Macroscopic evaluation of full-thickness incisional wounds treated with (a) commercial gauze, (b) PVA/CS-AgNPs nanofiber, and (c) PVA/CS-AgNPs nanofiber plus SB431542 (an inhibitor of TGFβ1 receptor kinase) at 0, 3, 5, 7, 12, and 18 days after surgery. B: Percentage wound area at different healing times, under different treatments. Each wound area measurement was compared with the wound area on day 0. Values are mean ± standard deviation. *P < 0.05 and **P < 0.01 vs. gauze group, #P < 0.05 and P < 0.01 vs. PVA/COS-AgNPs nanofiber plus SB431542 group. Abbreviations: PVA, poly(vinyl alcohol); COS, chitosan oligosaccharide; AgNPs, silver nanoparticles. Reproduced from reference [40], with permission from Dove Medical Press Limited, under the terms of the Creative Commons Attribution License.

Similarly, silver nanoparticle/CS oligosaccharide/poly(vinyl alcohol) (PVA/COS-AgNPs) nanofibers for wound healing have been synthesized via electrospinning [40]. The synthetic polymer poly (vinyl alcohol) (PVA) is biocompatible and has suitable mechanical properties and can thus be safely used for wound healing applications. This CS-based nanofibrous material was found to promote skin regeneration and upregulate the expression of cytokines related to the TGFβ1/Smad signaling pathway, involved in skin tissue repair (Figure 2). Indeed, TGFβ1 is important modulator of scar tissue formation. Inhibition of the TGFβ1 pathway by the addition of SB431542 interfered with the positive effects of the PVA/COS-AgNPs nanofibrous material on the initial stages of wound repair and cytokine expression. The CS-based nanofibrous material also accelerated wound closure in rat skin by promoting collagen fiber regeneration (Figure 2) [40].

A CS composite sponge in halloysite (a type of clay) nanotubes (HNTs) has been synthesized for use in skin tissue regeneration [41]. The flexible 3D porous CS sponge on the surface of HNTs has suitable properties for wound healing applications due to its biocompatibility, hemostatic character, and high mechanical strength. HNTs ($Al_2Si_2O_5(OH)_4.NH_2O$) are natural inorganic nanomaterials of length of 0.2–15 μm. The inner and outer diameters of HNTs were found to be in the ranges of 10–40 nm and 40–70 nm, respectively. The blood clotting ability and platelet activation of this CS-based nanomaterial was evaluated in a whole-blood clotting experiment. The authors observed that CS-HNT nanocomposite sponges promoted superior blood clotting in comparison with bare HNTs or pure CS. When whole blood was dripped onto the surface of the sponges, the blood hardly infiltrated the nanomaterial. The CS-HNT sponge material showed an 89% increase in clotting capability in comparison with that of pure CS. Overall, the CS-HNT sponge increased the rates of blood clotting and platelet activation ability. Topical applications of CS-HNT sponges on excisional wounds in Sprague-Dawley rats revealed that these nanomaterials accelerated wound closure, by enhancing collagen deposition and promoting re-epithelialization of the wounded area [41].

Mahdavi et al. [42] reported the preparation of CS-based biopolymers containing medical grade nanodiamonds (MND) (3 nm, up to 3 wt%) and bacterial cellulose (33 wt%) by electrospinning. Scanning electron microscopy revealed the formation of uniform fibers with diameters in the range of 80–170 nm. This nanofibrous material has suitable physical, chemical, and mechanical properties and appropriate cytocompatibility for skin tissue applications [42].

A very interesting nanofibrous material composed of a poly(lactic acid)/collagen nanofibrous scaffold coated with CS was prepared by electrospinning in order to sustain the release of an aloe vera gel for skin tissue regeneration [22]. Poly(lactic acid) is FDA approved, and collagen is a natural component of extracellular matrix, therefore, a collagen nanofibrous scaffold is a suitable material for skin regeneration. Aloe vera is a well-known active agent for burn healing. The CS-based nanofibrous material promoted the sustained release of aloe vera due to the addition of a CS-coating layer on the nanofibrous material. When the aloe vera-releasing CS-based scaffold was tested on mouse fibroblasts (L929), it supported cell attachment, allowing fibroblast proliferation. Indeed, L929 proliferation was enhanced from 48 to 72 h after seeding, indicating

the capacity of the CS-based nanofibrous scaffold for skin regeneration. It is well known that fibroblast proliferation is an important event during the proliferative phase of skin regeneration. The addition of a CS layer on the surface of collagen nanofibers significantly enhanced the mechanical properties of the nanofibers [22].

Zhou et al. (2017) developed a nitric oxide releasing-wound dressing composed of poly(ε-caprolactone) (PCL) and CS (PCL/CS-NO). Nitric oxide (NO) is known to play key roles in the wound healing process [43]. Scanning electron microscopy (SEM) images revealed the formation of a fibrous structure in the PCS/CS dressing with an average diameter of 0.74 ± 0.25 μm, and an average pore size of 3.57 ± 0.85 μm [44]. This porous nature allied with the high surface-to-volume ratio of the material is considered an advantage for a wound dressing. The efficacy of the NO-releasing wound dressing was evaluated in a mouse wound model. The results showed that topical administration of PCL/CS-NO accelerated wound closure compared with the control groups, and at day 14 after wounding, the wound was 95.31% closed. In addition, the PL/CS-NO treatment led to superior granulation at the wound site and abundant collagen formation. Therefore, this system is capable of delivering therapeutic amounts of NO directly at the wound site, enhancing the wound healing effect [44].

Finally, a nanofibrous material comprising poly(3-hydroxybutyrate-co-3 hydroxyvalerate)/CS was prepared as a scaffold for skin regeneration [45]. The fiber diameter was found to be 231 ± 78 nm, and the average pore area was 5.3×10^4 nm^2. The cytocompatibility of this nanofibrous material was demonstrated using fibroblasts (L929 cell line). *In vivo* studies revealed that the nanomaterial enhanced the wound repair process in rats. Actually, after seven days of treatment, the wound areas in animals treated with the nanomaterial were found to be significantly smaller (26% ± 11%), in comparison with control group (59% ± 17%). Moreover, the CS-based nanofibrous material decreased inflammation at the wound bed, advancing the beginning of the proliferative phase of the wound healing process. Morphological characterization demonstrated intense adhesion and proliferation of L929 fibroblasts on the surface of the nanofibrous material after 12h of seeding, leading to intensive cell proliferation, which is desirable for skin regeneration [45].

Table 1 summarizes these representative examples of CS-based nanofibrous materials for skin regeneration, and the major in vitro and in vivo achievements in this area.

Table 1. CS-based nanofibrous materials for skin regeneration.

Nanofibrous material	Major *in vitro* results	Major *in vivo* results	Referenc
Collagen type I, CS, polyethylene oxide	Biocompatible to 3T3 fibroblast cell line	Skin regeneration ability superior than commercial Dressings	[24]
Polycaprolactone/cellulose acetate and layer-by-layer self-assembly of collagen (type I) and CS	Ability for skin regeneration as assayed by in vitro cell migration protocol using normal human dermal fibroblasts	Accelerated wound healing by enhancing cell migration, vascularization and reepithelialization.	[39]

Silver nanoparticle/CS /poly(vinyl alcohol)/ AgNPs nanofiber	-	Upregulation the expression of cytokines involved in skin tissue repair	[40]
CS composite sponge in halloysite nanotubes	Blood clotting and platelet activation ability	Accelerated collagen deposition and reepithealization of the wounded area	[41]
Uniform fibers of CS/medical grade/nanodiamonds/bacteria	Cytocompatibility to mouse skin fibroblast cells (L929)	-	[42]
Poly(latic acid)/collagen nanofibrous scaffold coated with CS-containing aloe vera	Seeding and proliferation of L929 on the nanofibrous scaffold surface	-	[22]
Nanofibrous poly(3-hydroxybutyrate-co-3 hydroxyvalerate)/CS	Cytocompatibility to L929	Decreased inflammation and accelerated proliferation of fibroblasts at wound site	[45]

5. Selected Examples of CS-based Nanoparticle (NP) Composites for Skin Regeneration

This section presents and discusses examples of CS-based NP composites. It should be noted that in some cases CS is employed in the matrix, while the NPs are produced from other materials. In other examples, the NPs themselves contain CS in conjunction with other materials, as explained below for each example.

A nanodressing composed of titanium dioxide (TiO_2) nanoparticles loaded with pectin/CS has been prepared for skin regeneration [46]. The photoactive TiO_2 has antibacterial effects, while pectin is a natural prophylactic agent present in many healing ointments due to its curative and styptic actions and its defense against poisoning by toxic cations. The nanocomposite is formed by the dispersion of polyelectrolyte nanorod TiO_2 in a pectin/CS network. The polymeric network is formed due to the electrostatic attractions between ionized carboxyl groups (COO^-) of pectin and ionized amino groups of CS (NH_3^+). The authors demonstrated the antibacterial effect of the nanodressing by using the agar disc diffusion method; however, the minimum inhibitory concentration (MIC) values were not evaluated. The hemocompatibility of the CS-based nanodressing was demonstrated, since only 1.14% of erythrocytes underwent hemolysis upon whole blood contact with the CS-TiO_2-pectin dressing over 60 min. It should be noted that a hemolysis rate lower than 5% is permissible for biomaterials [46]. In addition, the cytocompatibility of the material was demonstrated towards mouse embryonic fibroblast (NIH3T3) and mouse fibroblast (L929) cell lines. In vivo studies revealed the acceleration of wound closure upon wound treatment with the CS-TiO_2-pectin nanodressing, compared with control groups. One important advantage of the nanodressing is that it did not dissolve, and efficiently adhered to the wound bed during the treatment, and it was thus easily removed without damaging the wound site and adjacent skin. Finally, histological evaluations of the wound site

demonstrated that after 16 days of treatment, the wound area was completely covered with new epithelium, and a well-developed epidermis and dermis was observed in the regenerated skin [46]. These results demonstrated the potential of the CS-based nanodressing in skin regeneration, with antibacterial activity, biocompatibility, hemocompatibility and the ability to accelerate wound closure.

In a similar strategy, a CS-based scaffold material was prepared and characterized for skin regeneration [47]. To this end, fish collagen/CS/chondroitin sulfate scaffolds were synthesized by freeze-drying and entrapped with basic fibroblast growth factor (bFGF)-containing poly(lactic-*co*-glycolic acid) (PLGA) microspheres. PLGA is a biocompatible and biodegradable polyester copolymer approved by the Food and Drug Administration (FDA), and bFGF is known to play an important role in wound healing process. *In vitro* studies demonstrated the superior proliferation of fibroblasts treated with the bFGF-composite, compared with the control groups. *In vivo* experiments were performed using a full-thickness rat skin wound model and they revealed that the CS-based scaffolds effectively supported, promoted and accelerated fibroblast infiltration from adjacent tissue. Hence, the CS-based scaffold is biocompatible and has the ability to promote fibroblast proliferation and skin tissue regeneration [47].

In another study, a CS-based nanocomposite composed of CS, poly(N-isopropylacrylamide)-co-(2-dimethylaminoethymethacrylate), P(NIPAAM-co-DMAEMA) and PLGA-modified gold nanoparticles (AuNPs), with a core-shell structure and a size in the range of 10–120 nm, was synthesized [48]. CS was employed as a surface modifying agent, and PNIPAAM is a thermo-responsible hydrophilic biocompatible polymer. The presence of AuNPs in the nanocomposite is responsible for its antibacterial activity based on near infrared (NIR) photothermal therapy. NIR laser (810 nm) therapy is a simple and efficient tool for the destruction of pathogenic bacteria, and has been approved by the FDA for clinical purposes. The authors demonstrated that incubation of Pseudomonas aeruginosa and Acinetobacter baumanhii strains with the nanocomposite, followed by different doses of low level NIR laser radiation, has antibacterial effects [48].

Jung et al. [49] encapsulated ceramide into CS-coated PLGA NPs for the treatment of atopic dermatitis, which is a complex skin disease. Ceramide is a typical agent used in the treatment of atopic dermatitis due to its ability to regenerate the stratum corneum of the skin, relieving the disease symptoms. Due to its hydrophilic character and low capacity for dispersion, the direct use of ceramide in skin is of limited value. To overcome this limitation, ceramide was entrapped into CS-coated PLGA NPs. The addition of CS on the surface of ceramide-containing PLGA NPs enhances the initial NP adherence to the skin surface, allowing a sustained and localized release of ceramide from the nanomaterial to the affected skin. As demonstrated in Figure 3A, the CS coating facilitated the initial NP adherence to the affected skin, avoiding an initial burst of ceramide release from the NP. CS then underwent sustained degradation caused by the weakly acid environment of the skin, leading to controlled and localized release of therapeutic amounts of ceramide. CS coating of nanomaterials is suitable for the control drug release at different pH values, as CS dissolves at pH 5, increasing

the rate of drug release. Therefore, the CS coating allows temporal control of ceramide release from PLGA NPs, suggesting the ability to modulate the release profile of the drug (ceramide) according to changes in the pH of the medium. Indeed, changes in pH, temperature and irradiation conditions are promising tools for triggering drug release from nanomaterials. Figure 3B-D shows representative images of uncoated and CS-coated PLGA NPs stored at different temperatures, revealing the spherical shapes of the nanoscale particles, and the presence of the CS layer at the NP surface. CS-coated PLGA NPs containing ceramide were found to have an average diameter of 211.4 ± 35.2 nm, and a positive zeta potential of 49.31 ± 2.4 mV, due to the presence of protonated amino groups of CS on the NP surface. CS-coated PLGA NPs containing ceramide were not toxic to dermal fibroblasts, and the NPs were demonstrated to be effective in a rat atopic dermatitis model by promoting *stratum corneum* regeneration [49]. Thus, CS-PLGA/ceramide has potential in the treatment of atopic dermatitis.

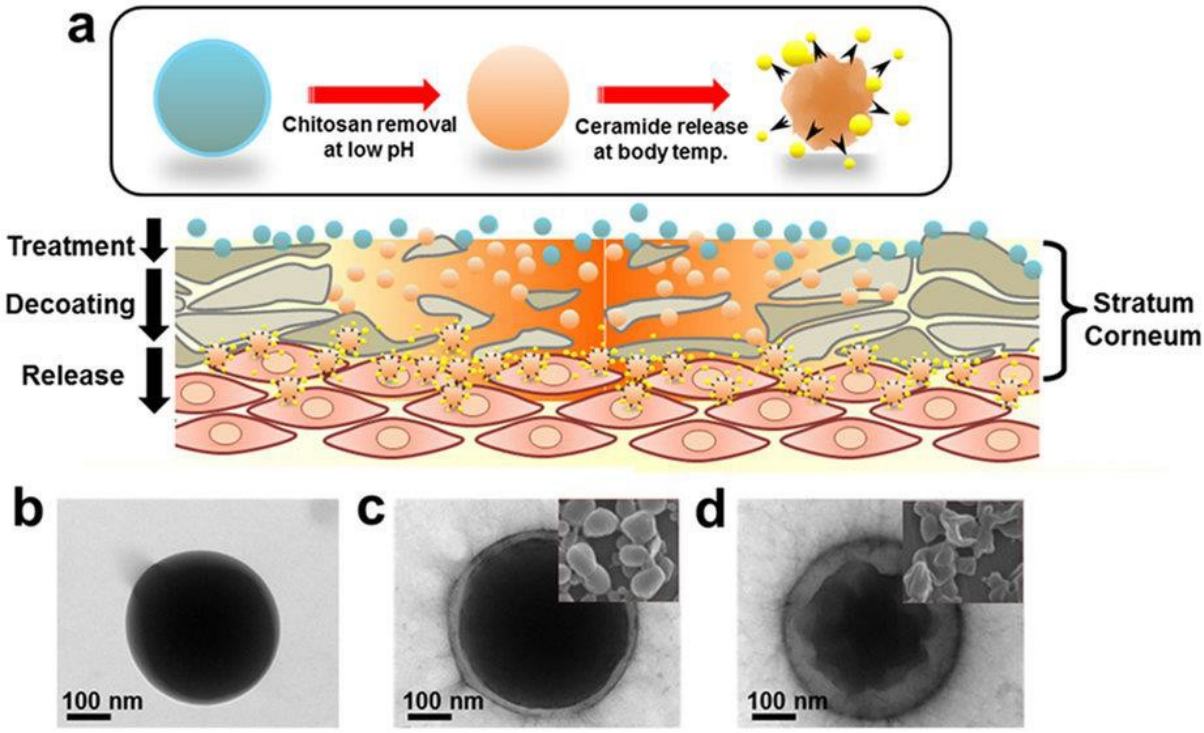

Figure 3. Schematic representation of ceramide-containing CS/PLGA NPs on the treatment of rat atopic dermatitis lesions (a), and confirmation of the shape of PLGA NPs, chitosan coating, and shrinkage of PLGA by transmission electron microscopy (TEM) and scanning electron microscopy (b, c, d). Representative TEM image of uncoated PLGA NP (b). Representative image of CS-coated PLGA NP and representative SEM image of PLGA NPs stored at 4°C (c). Representative TEM image of CS-coated PLGA NP and a SEM image of uncoated PLGA NPs stored at 36.5°C (d). Reproduced from reference [49] under a Creative Commons Attribution 4.0 International License.

The free radical NO is an important endogenously occurring molecule, involved in the control of several biological processes, including antimicrobial activities [33,50]. Due to its intrinsic instability NO donors and NO prodrugs have been incorporated into nanomaterials for biomedical applications, including skin regeneration [35,51,52]. To this end, a NO-generating nanocomposite composed of tetramethylorthosilicate (TMOS), CS, polyethylene glycol (PEG), glucose, and sodium nitrite ($NaNO_2$) was prepared in sodium phosphate buffer [53]. Redox reactions between the chemicals in the nanocomposite are initiated from electrons in glucose molecules, leading to the reduction of nitrite (NO_2^-) to NO. The nanocomposite was freeze-dried after the redox reactions, yielding a fine powder of NO-containing NPs.

Once the powder nanocomposite is exposed to an aqueous medium, the water channels inside the nanomaterial are opened, allowing the release of the entrapped NO in a sustained manner. Hence, water present in wound exudates can be used to trigger NO release from this nanomaterial. The NO-releasing-CS-based nanocomposite exerted antibacterial activity against methicillin-resistant SA (MRSA) *in vitro* and in *in vivo* (abscesses in mice). In addition, the nanocomposite impaired angiogenesis in MRSA abscesses, avoiding bacterial dissemination from infected abscesses. This observed antibacterial effect was attributed to the presence of NO and CS in the nanocomposite. The nanocomposite not only demonstrated antibacterial effects but also preserved the skin architecture by preventing collagen degradation by MRSA, as well as accelerating skin tissue repair [53].

CS based NPs represent a promising approach for the encapsulation of therapeutic agents in tissue engineering. Curcumin (CUR) is a well-known antioxidant and anti-inflammatory agent that might have beneficial applications in skin regeneration [54]. However, the administration of CUR faces some limitations such as the instability and low bioavailability of the drug. In order to overcome these limitations, CUR was loaded into CS based NPs followed by impregnation into a collagen-alginate scaffold, yielding a nanohybrid scaffold for the treatment of diabetic wounds [55]. CS was employed to promote sustained CUR release and because of its intrinsic wound healing properties. The encapsulation of CUR into CS NPs allowed sustained drug release directly at the site of the damaged tissue, avoiding rapid clearance of the drug from the inflammation site. Collagen was employed as an established skin regeneration scaffold. The authors demonstrated that the nanohybrid CS-based scaffold is biocompatible, promotes sustained drug release and has the capacity for water uptake in chronic wound sites. CUR-containing CS NPs were synthesized by the ionic gelation method with tripolyphosphate and have an average particle size of 196 nm, a positive zeta potential of + 30 mV (due to the protonated amino groups of CS chains), and a spherical shape. Kinetic studies revealed CUR release of 94.66 ± 5.23% from the nanohybrid scaffold over 14 days, ensuring sustained drug release suitable for limiting wound inflammation. In vivo studies were performed by the topical application of CUR-releasing-CS NPs/collagen scaffold to streptozotocin (STZ) diabetic wounds. STZ causes the selective destruction of pancreatic β-cells, compromising the synthesis and release of insulin and causing diabetes [55]. Diabetic wounds are chronic wounds that are difficult to heal and the treatment of diabetic wounds remains a challenge [56]. The authors observed that

topical application of the nanomaterial significantly accelerated the wound contraction of diabetic animals, compared with the control groups [55]. Indeed, the nanomaterial reduced inflammation and increased collagen deposition, leading to the formation of a well-structured thick granulation tissue. The presence of CS in the nanomaterial was responsible not only for the sustained CUR release, but also for fibroblast proliferation due to the depolymerization and release of N-acetylglucosamine units [55].

Recently, CUR was entrapped into CS/poly-glutamic acid/Pluronic NPs in a CS dressing for wound healing [57]. CS NPs were synthesized by a simple ionic gelation technique, and have an average hydrodynamic size of 193.1 ± 8.9 nm, a polydispersity index of 0.29 ± 0.02, and a zeta potential of + 20.6 ± 2.4 mV. Although the loading efficiency of CUR was found to be only 52.8 ± 4.7%, the encapsulation of CUR into CS NPs promoted sustained drug release for up to 48h. Pluronic was employed to improve the solubility of CUR/CS/poly-glutamic acid NPs in the CS dressing. In vitro analysis demonstrated that the NPs have no cytotoxicity towards human skin fibroblasts or RAW 264.7 cells. In vivo studies demonstrated that the CS/-PGA/Pluronic/curcumin NPs enhanced wound closure, and histological evaluation showed a reduced inflammation response at wound sites treated with the nanocomposite wound dressing [57].

In a similar strategy, gelatin/CS/epigallocatechin gallate NPs were incorporated into a poly (γ-glutamic acid)/gelatin hydrogel, composed of carbon fibers and gentamicin, leading to a sandwiched scaffold for skin regeneration [58]. This dressing scaffold material was designed to avoid wound inflammation, to reduce the bacterial burden, and to enhance re-epithelialization. Epigallocatechin gallate has been used to treat dermal wounds due to its immunomodulatory and anti-inflammatory properties. An in vitro wound healing study demonstrated that the sandwich dressing significantly enhanced wound regeneration by improving epithelial covering. In vivo studies revealed that the sandwich dressing can be easily applied and removed from the wound bed, enhancing tissue repair by neocapillary formation and decreasing the degree of inflammation [58].

Recently, Nawaz et al. [59] reported the preparation of 5-fluouracil-containing CS NPs by nanospray-drying for transdermal drug delivery applications. The transdermal drug delivery was performed with microwave-treated skin (2450 MHZ, 5 min, 5 + 5 min, 3985 MHZ 5 min) mediated via NPs through the skin layers. The transdermal drug delivery was facilitated by the combination of CS and microwave energy that fluidized lipid/protein residues in the epidermis and dermis, and induced changes in lipid conformation from trans to gauche. The ability of CS NPs to permeate human skin has not been demonstrated. Therefore, microwave assistance was employed to allow CS NPs transdermal permeation across the skin layers [59]. More studies are necessary to better understand the interactions of CS NPs with human skin layers.

The antimicrobial cationic peptide temporin B (TB) was encapsulated into CS NPs for the treatment of infected wounds [60]. TB is a small alpha-helical cationic peptide isolated from amphibian skin secretions that is highly cytocompatible and has antimicrobial properties. CS NPs were synthesized by ionic gelation with an average hydrodynamic size of 185 ± 10 nm, a PDI of

0.063 and a positive zeta potential of + 8.8 ± 0.1 mV. The encapsulation efficiency of TB into CS NPs was found to be 74.7% ± 2.3%. The cytocompatibility of TB-containing CS NPs was demonstrated using the mouse embryo fibroblast BALB/3T3 clone A31 cell line. The antibacterial activity of TB-containing CS NPs was evaluated against clinical isolates of Staphylococcus epidermidis. The authors elegantly demonstrated that TB-CS NPs are able to deliver TB directly to the bacterial surface, due to the adsorption of the NPs onto bacterial surface, thus allowing the release of high concentrations of TB directly at the bacterial membrane and causing cell death [60].

Ramasamy et al. reported the preparation of a hybrid nanomaterial containing EGF-epidermal growth factor for skin regeneration [61]. The nanomaterial comprises solid lipid based core nanoparticles coated with a bi-layered structure produced by the layer-by-layer self-assembly of hyaluronic acid and CS. The core-shell CS based nanomaterial was found to have a spherical shape, and was physically stable, with a hydrodynamic size of 280 nm, a monodisperse size distribution and appropriate cytocompatibility for potential uses in skin regeneration [61].

Recently, in situ melatonin-containing CS/Pluronic F127 microspheres were prepared as an antimicrobial wound dressing [62]. A hydrogel is formed upon contact with wound exudates. Melatonin is a methoxyindole secreted by the pineal gland that has anti-oxidant and anti-inflammatory properties due to its free radical scavenging ability. Melatonin-containing microspheres were shown to be biocompatible with skin fibroblasts and keratinocytes at concentrations that have toxic effects on planktonic bacteria. The melatonin release profile from the microspheres suggested non-Fickian diffusion or anomalous transport, as described by the Higuchi model, making this material suitable for combating pathogenic bacteria at early phases of wound contamination. The antimicrobial activity of the microspheres was demonstrated against planktonic Staphylococcus aureus ATCC 29213, with a minimum inhibitory concentration of 0.125 mg/mL. The authors stated that the combination of melatonin and Pluronic F127 optimized the antimicrobial activity of CS [62]. This material might find important applications in the treatment of bacterially contaminated skin tissue.

Table 2 summarizes representative examples of CS-based nanoparticle (NP) composites used for skin regeneration and the major in vitro and in vivo achievements in the field. The table indicates the versatility of nanobased CS containing materials that can be used in wound healing.

6. CS-based nanogels: Methods for Their Preparation and Selected Examples Used in Skin Regeneration

There are several methods for the preparation of CS-based nanogels including crosslinking [63–69], dialysis [70], and radiation [71]. The majority of these methods involve mild conditions, which increases the commercial viability of these materials. The crosslinking method is most commonly used for the preparation of CS-based nanogels. The crosslinking can be performed with various molecules, such as the disodium salt of glycerol phosphate [19], poly (vinyl alcohol) [63], sodium

tripolyphosphate [64], pectin [65], sodium bicarbonate [66], genipin [67], metal ions [68] and glutaraldehyde [69]. CS-based nanogels offers advantages for skin tissue regeneration including biocompatibility, suitable scaffold morphology and porosity, cell survival efficiency, and soft flexible stability, which allows them to fill site defects and make connections with new tissue [72,73]. Therefore, CS-based nanogels are a promising matrix for regenerative skin medicine.

Table 2. CS-based nanoparticle (NP) composites for skin regeneration.

CS-based NP composite	Major *in vitro* results	Major *in vivo* results	References
Titanium dioxide NPs loaded pectin/CS nanodressing	Antibacterial effect against *E. coli*, *S. aereus*, *P. aeruginosa*, *B. Subtilis* and *A. niger*. Cytocompatibility to L929 and NIH3T3 mouse fibroblast cells	Faster wound closure with new epithelium and a well-developed epidermis and dermis in the regenerated skin	[46]
Collagen/CS/chondroitin sulfate scaffolds/ fibroblast growth factor containing poly(lactic-co-glycolic acid) microspheres	Enhanced human lung fibroblast cell line MRC-5 proliferation	The scaffold supported, promoted and accelerated fibroblast infiltration from the adjacent tissue to the wound bed	[47]
Core-shell NPs of CS, poly(*N*-isopropylacrylamide)-*co*-(2-dimethylaminoethymethacrylate) and PLGA modified gold nanoparticles	Antibacterial effects towards P. *aeruginosa* and A. *baumanhii* strains upon near infrared irradiation	-	[48]
Ceramide containing CS-coated PLGA NPs	Cytocompabibility to dermal fibroblasts isolated from skin rats	Effective in a rat atopic dermatitis model by promoting *stratum corneum* regeneration	[49]
Nitric oxide-generating-tetramethylorthosilicate/CS/PEG/glucose/sodium nitrite	Antibacterial activity Against methicillin-resistant SA (MRSA)	Inhibition of bacterial dissemination from infected MRSA abscesses in mice	[53]
Curcumin-containing CS NPs impregnated into collagen-alginate scaffold	-	Reduction of inflammation and enhanced collagen deposition in streptozotocin-induced diabetic wounds	[55]

Curcumin-containing CS/poly-γ-glutamic acid/Pluronic NPs in CS dressing	Cytocompatibility to human skin fibroblastas and RAW 264.7 cells	Enhanced wound healing with less inflammation at wound site	[57]
Gelatin/CS/epigallocatechin gallate NP incorporated into poly (γ-glutamic acid)/gelatin hydrogel	Compatibility to normal human skin fibroblast and antimicrobial effects to *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*	Easily application at the wound bed with leading to neovascularization and decrease of inflammation	[58]
5-fluouracil-containing CS NPs	Rat skin permeation study in Franz diffusion cell indicated that the transdermal drug delivery was facilitated by the combination of CS and microwave fluidizing lipid/protein residues in epidermis and dermis	-	[59]
CS NPs containing antimicrobial cationic peptide temporin B	Cytocompatibility to mouse embryo fibroblast BALB/3T3 clone A31 cell line. Antibacterial effects against clinical isolates of *Staphylococcus epidermidis*		[60]
Solid lipid based core NPs coated with a bi-layered self-assembly of hyaluronic acid and CS	Cytocompatibility to NIH3T3 fibroblast cells		[61]
Melatonin-containing CS/Pluronic F127 microspheres	Cytocompatibility to skin fibroblasts and keratinocytes at toxic concentrations to planktonic bacteria		[62]

The CS backbone contains primary hydroxyl and amine groups. These functional groups are responsible for the reactivity of the polymer [74] and for some of the intrinsic properties of CS, such as its antibacterial and hemolytic effects [72]. These functional groups also provide sites for further chemical modification. Therefore, several studies have reported chemical modifications to the CS backbone by the addition of function groups (e.g. carboxyl, amine, acyl, and glycol groups) [74].

These modifications can yield new and enhanced properties for CS, for example, water solubility over a wider pH range, which is a desirable trait for regenerative medicine [73,75,76]. There are an enormous variety of chemically modified CS materials, for example, carboxymethyl CS, which forms a pH-induced thermosensitive nanogel with enhanced mechanical properties and antibacterial action [68,77], methacrylamide CS [73], thiolated CS [78,79], hydroxybutyl CS [80], and poly(aminoethyl) CS [81]. These modified CS materials form nanogels with promising properties for skin regeneration.

Zhang et al. [81] synthesized a chemically modified CS nanogel by increasing the number of amino groups on the CS backbone, designated as poly(aminoethyl) modified CS (PAEMCS) [81]. The number of cationic amino groups is related to the antibacterial activity of CS [81–83] and thus, a multi-amino group CS-based nanogel might have increased antibacterial effects and therefore great potential as a wound dressing material. The inhibition rate of the multi-amino group CS-based nanogel was significantly higher compared with pure CS at the same concentration when tested against four of the most common bacteria that are found in wounds, after 12 h of treatment. The minimum inhibition rates of the PAEMCS-based nanogel were 53.87%, 62.97%, 33.5%, and 67.76% against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*, respectively. Interestingly, the inhibition rates were higher against gram-positive bacteria than against gram-negative bacteria. All of the results indicated that multi-amino group CS could greatly improve the antibacterial activity of CS nanogels [81].

In addition to chemical modifications of the CS backbone, other molecules can be incorporated into CS nanogels to increase their healing potential, for example, collagen [84,85] and hyaluronic acid [73] and its derivatives, which are used for skin and soft tissue regeneration [86]. Polymers can be incorporated into CS nanogel to modify the mechanical properties of the material, such as Pluronic, poloxamer [87,88] or agarose [89]. In addition, NPs can be incorporated into CS nanogels, forming composites with superior antibacterial effects, for example graphene oxide [64], silver [90,91], or zinc oxide NPs [92]. Therefore, chemically modified CS nanogels represent a promising approach for the preparation of new materials with properties suitable for skin tissue regeneration.

There are several application strategies in skin tissue regeneration, among them, the injection of nanogels. The ideal injected nanogel has solution like behavior at room temperature and semi-solid behavior under physiological conditions [93]. Injected nanogels are designed to be injected as a liquid and undergo a gelification or reticulation in situ due to an external stimulus, such as, body temperature, pH, or light [94]. This type of application has numerous advantages. For example, injected nanogels can adhere to the surrounding tissue, filling defective sites, due to their mechanical properties. Nanogels are porous structures that allow the migration and proliferation of cells and free diffusion of wastes and nutrients; after implantation the nanogels are degradable, avoiding the need for surgical procedures to remove the material [19,93].

An example of this type of application was demonstrated by Moura and co-workers [19]. The authors performed a study with two CS-based nanogels, the main difference between the two nanogels

being the crosslinking step. The first CS nanogel was synthesized using the crosslinking method with the disodium salt of glycerol phosphate. The second CS nanogel was obtained by combined crosslinking with the glycerol disodium salt and genipin. SEM images showed that the CS-nanogel prepared using the combined crosslinking method exhibited much larger pores (~600 μm) in comparison with the CS nanogel crosslinked with only the glycerol disodium salt (~250 μm). The CS-based nanogels were subcutaneously implanted in rats, and after eight days of implantation, the implant was identified as a massive material in one piece. Although, there was a moderate to severe inflammatory response, no signs of adverse inflammatory or immunological effects were observed [19].

Biodegradability is an important factor that must be evaluated for the safe administration of materials to be implanted subcutaneously or in direct contact with the skin or mucous surface. CS is well known to be biodegradable, because it is decomposed by enzymes present in the skin layers, such as lysozymes and collagenases [18,19]. Cheng et al. [95] developed a CS-based nanogel crosslinked with glycerol phosphate and gelatin that was used with adipose-derived stem cells in therapeutic angiogenesis. SEM was used evaluate the biodegradability of the CS/gelatin nanogel, at day zero and after seven days in contact with a medium containing collagenase. After seven days, the nanogel showed a highly porous surface topography (~2 μm with a pore number of 44.8 ± 9.1 per power field), without disruption of the material [95].

Similarly, Herris et al. [86] studied the biodegradability of CS-based nanogels simultaneously crosslinked with glycol and glyoxal (0.005%). The CS-nanogels were submerged in a solution containing lysozyme, and 21% of the material was degraded after four weeks [86]. Figure 4 shows that degradation rates decreased with increasing concentrations of the cross-linking agent.

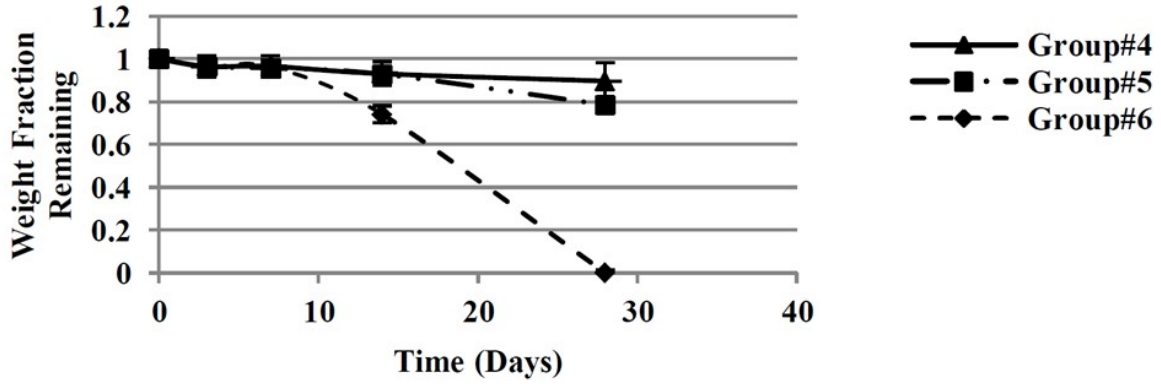

Figure 4. *In vitro* enzymatic degradation of cross-linked CS-glycol (GCs)/glyoxal (Gy) nanogels. GCs 0.0075%, 0.005%, 0.0025% for groups 4, 5, and 6, respectively. Enzyme: lysozyme. Reproduced from reference [86] from Elsevier under the terms of the creative commons attribution non-commercial non-derivatives license.

The ideal degradation time for a wound dressing material is between one and three months due to the timing of the end of granulation and the formation of inflammation tissue. These interactions are

intended to modulate wound healing and generate of scar-free tissue [86]. Biocompatibility is an important requisite for biomedical applications requiring contact with the skin layers [19]. Dang et al. [85] assessed the cytotoxicity of a CS-based nanogel containing acid-soluble collagen (ASC) (extracted from haddock skin) crosslinked with α, β-glycerophosphate. The hemolysis test showed less than 2% hemolysis, which indicates that the material is non-hemolytic. Intraperitoneal injections of CS-nanogels were performed in mice and several parameters were evaluated, after blood collection on days 7 and 14. The results showed that the injections did not cause hematopoiesis or damage to the liver or kidney functions of mice, indicating the biocompatibility of the CS-based nanogel [85].

Sapru et al. [67] evaluated the cytotoxicity and antibacterial features of a CS-based nanogel crosslinked with genipin and containing sericin. Sericins are hygroscopic and exhibit beneficial properties for skin tissue regeneration, including antibacterial, anti-oxidant, UV resistant and anti-apoptotic features [67]. The nanogels were stable (< 6 weeks), porous (57.23–75.22), and flexible [67]. The toxicity of the CS-based nanogel was evaluated by testing hemolysis and in vitro cell proliferation using human dermal fibroblasts. Similar to other reports, the hemolysis was less than 2%, indicating that the CS-based nanogel was non-hemolytic. The production of tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β) by cells treated with the nanogel was less than 55 pg·mL-1, indicating a minimal immune response and non-inflammatory behavior of the CS-nanogel [67]. Its antibacterial activity was evaluated against *Escherichia coli* and *Staphylococcus aureus* by the agar diffusion method. The highest zone inhibition reported was of 10–15 mm, indicating that the CS-nanogel indeed has antibacterial effects [67,96].

Zhou et al. [91] prepared a gelatin and carboxymethyl CS-based nanogel containing silver NPs, by irradiation-induced crosslinking. This method is considered a “green approach” and produced a homogenous distribution of silver NPs inside the nanogel network [91]. The antibacterial effect of this CS-based nanogel containing silver NPs was evaluated using the halo inhibition test against *Escherichia coli*. The nanogel containing silver NPs at 10 mM concentration exhibited an inhibition zone of 19.6 mm [91]. As silver NPs are known for their antimicrobial effect [97], the combination of CS and silver NPs demonstrated enhanced antimicrobial activity.

When these studies are considered as a whole, CS-based nanogels can be seen to have promising effects in skin tissue regeneration. The properties of CS can be enhanced by chemical modifications in the polymer backbone or by the addition of other therapeutic molecules/polymers/NPs to the CS-nanogel network. CS-based nanogels have been shown to be non-hemolytic (less than 2% hemolysis) [67,85] and have exhibited antibacterial activities that are more effective against gram positive bacteria [67,96]. The application of CS-based hydrogels can be carried out by injection or by topical wound dressing. In both cases, CS-based nanogels have great potential in the field of regenerative medicine. Table 3 summarizes representative examples of CS-based nanogel composites for skin regeneration and the major *in vitro* and *in vivo* achievements in this area.

Table 3. CS-based nanogels for skin regeneration.

CS-based nanogel	Major *in vitro* results	Major *in vivo* results	References
Poly(aminoethyl) modified CS nanogel	Antibacterial effect against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The antibacterial effect was more pronounced in gram positive bacteria	-	[81]
CS-based nanogels formed by crosslink with glycerol disodium salt and genipin	-	No signs of adverse inflammatory or immunology effects were observed after subcutaneous implants in rats	[20]
CS-based nanogel crosslinked with glicerophosphato and gelatin	Biodegradability assayed by SEM images after 7 days of contact with collagenase	-	[95]
CS-based nanogels crosslinked simultaneously with glycol and glyoxal	21% of the nanogels was degraded after 4 weeks in contact with lysozyme		[86]
CS-based nanogel crosslinked with glycerophosphate containing collagen	Less than 2% of haemolysis	Intraperitoneal injections in mice did not cause haematopoiesis or damages at liver and kidney	[85]
CS-based nanogel crosslinked with genipin containing sericin	Less than 2 % of haemolysis. Antibacterial effect against *Escherichia coli* and *Staphylococcus aureus*. More pronounced effect in gram positive bacteria	-	[67]
Carboxymethyl modified CS-based nanogel containing gelatin and silver NPs	Antibacterial effect against *Escherichia coli*. Enhanced antibacterial effect was observed by the addition of silver NPs to CS-nanogel	-	[91]

7. Final Remarks: Perspectives and challenges

This review summarizes recent progress in the design of CS-based nanomaterials for skin

regeneration, particularly focused on CS-based composite NPs, nanofibrous scaffolds and nanogels. Several scaffolds and strategies based on CS and nanomaterials have been successfully developed for wound healing and are documented in the literature reviewed here. CS has several intrinsic features that make it promising for use in skin tissue regeneration, such as: biocompatibility, biodegradability, antimicrobial activity, mucoadhesive and hemostatic character, low cost, and renewability. Moreover, CS is a versatile material that can be prepared in different forms, such as films, nano- and microparticles, membranes, nanogels, nanofibrous materials and nanocomposites. The combination of CS with biologically active molecules, other polymers and NPs highlights the multiple strategies employed in regenerative medicine using CS-based nanomaterials. CS-based NPs, nanofibers and nanogels have rheological properties suitable for topical use, acceptable skin compatibility, and offer ease of manipulation, handling and application. Moreover, the charged surface of CS allows a prolonged residence time for CS-based nanomaterials, increasing the drug concentration at the application site. CS is easily chemically modified, leading to new materials with tailored properties suitable for skin regeneration.

As stated in this review, there is a great interest in the design of smart CS-nanobased materials that respond to external stimuli such as changes in pH, ionic concentration, and temperature. These nanomaterials could allow controlled and sustained release of therapeutic amounts of entrapped drugs, directly to the desired site of application, with minimum side effects and maximum efficacy.

Although the use of CS-based nanomaterials is a promising technology for skin regeneration, the current knowledge on the safety of various nanobased materials in wound healing is still not sufficient for their clinical application [11]. Despite the several advantages of CS-based nanomaterials for skin tissue regeneration, some key challenges in this field need to be overcome. More studies aimed to further investigate long term cellular interactions with the nanobased materials are still necessary. Some issues also need to be optimized, for example, cellular differentiation, proliferation and vascularization in the engineered tissues need to be accelerated. It should be noted that the major goal of tissue engineering is to efficiently restore and improve damaged tissue by its reconstruction. In this sense, the ideal nanomaterial should be capable of mimicking the natural environment, supporting cellular adhesion, proliferation and tissue growth with adequate cell infiltration, oxygen permeation, nutrient and water supply while affording protection against infection and further mechanical damage. Although several advances have been made in this direction, further studies are necessary.

We hope that this article provides insights on the design and use of CS-based nanomaterials in combination with other polymers, active drugs and NPs that can create promising platforms for the future of tissue engineering and regenerative medicine.

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Conflict of Interest

There is no conflict of interest.

References

1. Gantwerker EA, Hom DB (2011) Skin: histology and physiology of wound healing. *Facial Plast Surg Clin N Am* 19: 441-453.
2. Sorg H, Tilkorn DJ, Hager S, et al. (2017) Skin wound healing: An update on the current knowledge and concepts. *Eur Surg Res* 58: 81-94.
3. Gonzalez ACD, Costa TF, Andrade ZD, et al. (2016) Wound healing—A literature review. *An Bras Dermatol* 91: 614-620.
4. Ahmed S, Ikram S (2016) Chitosan based scaffolds and their applications in wound healing. *Achievements Life Sci* 10: 27-37.
5. Azuma K, Izumi R, Osaki T, et al. (2015) Chitin, chitosan, and its derivatives for wound healing: Old and new materials. *J Funct Biomater* 6: 104-142.
6. Cortivo R, Vindigni V, Iacobellis L, et al. (2010) Nanoscale particle therapy for wounds and ulcers. *Nanomedicine* 5: 641-656.
7. Andrews JP, Marttala J, Macarak E, et al. (2016) Keloids: The paradigm of skin fibrosis – Pathomechanisms and treatment. *Matrix Biol* 51: 37-46.
8. Mari W, Alsabri SG, Tabal N, et al. (2015) Novel insights on understanding of keloid scar: Article review. *J Am Coll Clin Wound Spec* 7: 1-7.
9. Mordorski B, Prow T (2016) Nanomaterials for wound healing. *Curr Dermatol Rep* 5: 278-286.
10. Ahmadi F, Oveisi Z, Samani M, et al. (2015) Chitosan based hydrogels: characteristics and pharmaceutical applications. *Res Pharm Sci* 10: 1-16.
11. Elgadir MA, Uddin MS, Ferdosh S, et al. (2015) Impact of chitosan composites and chitosan nanoparticle composites on various drug delivery systems: A review. *J Food Drug Anal* 23: 619-629.
12. Patel S, Goyal A (2017) Chitin and chitinase: Role in pathogenicity, allergenicity and health. *Int J Biol Macromolec* 97: 331-338.
13. Baldrick P (2010) The safety of chitosan as a pharmaceutical excipient. *Regul Toxicol Pharmacol* 56: 290-299.
14. Dutta PK (2016) Chitin and chitosan for regenerative medicine. Springer India: 2511-2519.
15. Chaudhari AA, Vig K, Baganizi DR (2016) Future prospects for scaffolding methods and biomaterials in skin tissue engineering: A review. *Int J Mol Sci* 17: 1974.

16. Parani M, Lokhande G, Singh A, et al. (2016) Engineered nanomaterials for infection control and healing acute and chronic wounds. *ACS Appl Mater Interfaces* 8: 10049-10069.
17. Siravam AJ, Rajitha P, Maya S, et al. (2015) Nanogels for delivery, imaging and therapy. *WIREs Nanomed Nanobiotechnol* 7: 509-533.
18. Kean T, Thanou M (2010) Biodegradation biodistribution and toxicity of chitosan. *Adv Drug Deliv Rev* 62: 3-11.
19. Moura MJ, Brochado J, Gil MH, et al. (2017) *In situ* forming chitosan hydrogels: Preliminary evaluation of the *in vivo* inflammatory response. *Mater Sci Eng C* 75: 279-285.
20. Zhao Y, Qiu Y, Wang H, et al. (2016) Preparation of nanofibers with renewable polymers and their application in wound dressing. *Int J Polym Sci.* Article ID 4672839. doi: http://dx.doi.org/10.1155/2016/4672839.
21. Jayakumar R, Menon D, Manzoor K, et al. (2010) Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydr Polym* 82: 227-232.
22. Salehi M, Farzamfar S, Bastam F, et al. (2016) Fabrication and characterization of electrospun PLLA/collagen nanofibrous scaffold coated with chitosan to sustain release of aloe vera gel for skin tissue engineering. *Biomed Eng Appl Basis Commun* 28: 1650035.
23. Yildirimer L, Thanh NTK, Seifalian AM (2012) Skin regeneration scaffolds: a multimodal bottom-up approach. *Trends Biotechnol* 30: 638-648.
24. Chen JP, Chang GY, Chen JK (2008) Electrospun collagen/chitosan nanofibrous membrane as wound dressing. *Colloids Surf A* 313-314: 183-188.
25. Sun K, Li ZH (2011) Preparations, properties and applications of chitosan based nanofibers fabricated by electrospinning. *Express Polym Lett* 5: 342-361.
26. Muzzarelli RAA, Mehtedi ME, Mattioli-Belmonte M (2014) Emerging biomedical applications of nano-chitins and nano-chitosans obtained via advanced eco-Friendly technologies from marine resources. *Mar Drugs* 12: 5468-5502.
27. Oyarzun-Ampuero F, Vidal A, Concha M, et al. (2015) Nanoparticles for the treatment of wounds. *Curr Pharm Des* 221: 4329-4341.
28. Manchanda R, Surendra N (2010) Controlled size chitosan nanoparticles as an efficient. Biocompatible oligonucleotides delivery system. *J Appl Polym Sci* 118: 2071-2077.
29. Brunel F, Véron L, David L, et al. (2008) A novel synthesis of chitosan nanoparticles in reverse emulsion. *Langmuir* 24: 11370-11377.
30. Tokumitsu H, Ichikawa H, Fukumori Y, et al. (1999) Preparation of gadopentetic acid- loaded chitosan microparticles for gadoliniumneutron-capture therapy of cancer by a novel emulsion-droplet coalescence technique. *Chem Pharm Bull* 47: 838-842.
31. Agnihotri S, Aminabhavi TM (2007) Chitosan nanoparticles for prolonged delivery of timolol maleate. *Drug Dev Ind Pharm* 33: 1254-1262.
32. El-Shabouri MH (2002) Positively charged nanoparticles for improving theoral bioavailability of cyclosporin-A. *Int J Pharm* 249: 101-108.

33. Cardozo VF, Lancheros CAC, Narciso AM, et al. (2041) Evaluation of antibacterial activity of nitric oxide-releasing polymeric particles against *Staphylococcus aureus* from bovine mastitis. *Int J Pharm* 473: 20-29.
34. Pelegrino MT, Silva LC, Watashi CM, et al (2017) Nitric oxide-releasing nanoparticles: synthesis, characterization, and cytotoxicity to tumorigenic cells. *J Nanopart Res* 19: 57.
35. Pelegrino MT, Weller RB, Chen X, et al. (2017) Chitosan nanoparticles for nitric oxide delivery in human skin. *Med Chem Comm* 4: 713-719.
36. Bugnicourt L, Ladavière C (2016) Interests of chitosan nanoparticles ionically cross-linked with tripolyphosphate for biomedical applications. *Prog Polym Sci* 60: 1-17.
37. Soni KS, Desale SS, Bronich TK (2016) Nanogels: An overview of properties, biomedical applications and obstacles to clinical translation. *J Control Release* 240: 109-126.
38. Caló E, Khutoryanskiy V (2015) Biomedical applications of hydrogels: A review of patents and commercial products. *Eur Polym J* 65: 252-267.
39. Huang R, Li W, Lv X, et al. (2015) Biomimetic LBL structured nanofibrous matrices assembled by chitosan/collagen for promoting wound healing. *Biomaterials* 53: 58-75.
40. Li CW, Wang Q, Li J, et al. (2016) Silver nanoparticles/chitosan oligosaccharide/poly(vinyl alcohol) nanofiber promotes wound healing by activating TGFβ1/Smad signaling pathway. *Int J Nanomedicine* 11: 373-387.
41. Liu M, Shen Y, Ao P, et al. (2014) The improvement of hemostatic and wound healing property of chitosan by halloysite nanotubes. *RSC Adv* 4: 23540-23553.
42. Mahdavi M, Mahmoudi N, Anaran FR, et al. (2016) Electrospinning of nanodiamond-modified polysaccharide nanofibers with physico-mechanical properties close to natural skins. *Mar Drugs* 14: 128.
43. Georgii JL, Amadeu TP, Seabra AB, et al. (2011). Topical S-nitrosoglutathione-releasing hydrogel improves healing of rat ischemic wounds. *J Tissue Eng Regen Med* 5: 612-619.
44. Zhou X, Wang H, Zhang J, et al. (2017) Functional poly(ε-caprolactone)/chitosan dressings with nitric oxide-releasing property improve wound healing. *Acta Biomater* 54: 128-137.
45. Veleirinho B, Coelho DS, Dias PF, et al. (2012) Nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/chitosan scaffolds for skin regeneration. *Int J Biol Macromolec* 51: 343-350.
46. Archana D, Dutta J, Dutta PK (2013) Evaluation of chitosan nano dressing for wound healing: Characterization, *in vitro* and *in vivo* studies. *Int J Biol Macromolec* 57: 193-203.
47. Cao H, Chen MM, Liu Y, et al. (2015) Fish collagen-based scaffold containing PLGA microspheres for controlled growth factor delivery in skin tissue engineering. *Colloids Surf B Biointerfaces* 136: 1098-1106.
48. Gharatape A, Milani M, Rasta SH, et al. (2016) A novel strategy for low level laser-induced plasmonic photothermal therapy: the efficient bactericidal effect of biocompatible AuNPs@(PNIPAAM-co-PDMAEMA, PLGA and chitosan). *RSC Adv* 6: 110499-110510.

49. Jung SM, Yoon GH, Lee HC, et al. (2015) Thermodynamic insights and conceptual design of skin sensitive chitosan coated ceramide/PLGA nanodrug for regeneration of *Stratum corneum* on atopic dermatitis. *Sci Rep* 5: 18089.
50. Seabra AB, Kitice NA, Pelegrino MT, et al. (2015) Nitric oxide-releasing polymeric nanoparticles against *Trypanosoma cruzi*. *J Phys: Conf Series* 617: 012020.
51. Seabra AB, Duran N (2012) Nanotechnology allied to nitric oxide release materials for dermatological applications. *Curr Nanosci* 8: 520-525.
52. Seabra AB, Duran N (2017) Nanoparticulated nitric oxide donors and their biomedical applications. *Mini Rev Med Chem* 17: 216-223.
53. Han G, Martinez LR, Mihu MR, et al. (2009) Nitric oxide releasing nanoparticles are therapeutic for *Staphylococcus aureus* abscesses in a murine model of infection. *PLoS One* 4: e7804.
54. Shome S, Das TA, Choudhury MD, et al. (2016) Curcumin as potential therapeutic natural product: a nanobiotechnological perspective. *J Pharm Pharmacol* 68: 1481-1500.
55. Karri VV, Kuppusamy G, Talluri SV, et al. (2016) Curcumin loaded chitosan nanoparticles impregnated into collagen-alginate scaffolds for diabetic wound healing. *Int J Biol Macromol* 93: 1519-1529.
56. SeabraAB, Pankotai E, Feher M, et al. (2007) S-nitrosoglutathione-containing hydrogel increases dermal blood flow in streptozotocin-induced diabetic rats. *Br J Dermatol* 156: 814-818.
57. Lin YH, Lin JH, Hong YS (2017). Development of chitosan/poly-g-glutamic acid/pluronic/curcumin nanoparticles in chitosan dressings for wound regeneration. *J Biomed Mater Res Part B* 105B: 81-90.
58. Lin YH, Lin JH, Li TS, et al. (2016) Dressing with epigallocatechin gallate nanoparticles for wound regeneration. *Wound Repair Regen* 24: 287-301.
59. Nawaz A, Wong TW (2017) Microwave as skin permeation enhancer for transdermal drugdelivery of chitosan-5-fluorouracil nanoparticles. *Carbohydr Polym* 157: 906-919.
60. Piras AM, Maisetta G, SandreschiS, et al. (2015) Chitosan nanoparticles loaded with the antimicrobial peptide temporin B exert a long-term antibacterial activity *in vitro*against clinical isolates of *Staphylococcus epidermidis*. *Front Microbiol* 6: 372.
61. Ramasamy T, Kim JO, Yong CS, et al. (2015) Novel core–shell nanocapsules for the tunable delivery of bioactive rhEGF: Formulation, characterization and cytocompatibility studies. *J Biomater Tissue Eng* 5: 730-743.
62. Romić MD, Klarić MŠ, Lovrić J, et al. (2016) Melatonin-loaded chitosan/Pluronic® F127 microspheres as *in situ*forming hydrogel: An innovative antimicrobial wound dressing. *Eur J Pharm Biopharm* 107: 67-79.
63. Abureesh MA, Oladipo AA, Gazi M (2016) Facile synthesis of glucose-sensitive chitosan-poly(vinyl alcohol) hydrogel: Drug release optimization and swelling properties. *Int J Biol Macromol* 90: 75-80.

64. Zhao X, Zou X, Ye L (2016) Controlled pH-and glucose-responsive drug release behavior of cationic chitosan based nano-composite hydrogels by using graphene oxide as drug nanocarrier. *J Ind Eng Chem*: 1-10.
65. Neufeld L, Bianco-Peled H (2017) Pectin–chitosan physical hydrogels as potential drug delivery vehicles. *Int J Biol Macromol* 101: 852-861.
66. Rogina A, Ressler A, Matic I, et al. (2017) Cellular hydrogels based on pH-responsive chitosan-hydroxyapatite system. *Carbohydr Polym* 166: 173-182.
67. Sapru S, Ghosh AK, Kundu SC (2017) Non-immunogenic, porous and antibacterial chitosan and Antheraea mylitta silk sericin hydrogels as potential dermal substitute. *Carbohydr Polym* 167: 196-209.
68. Wahid F, Wang HS, Zhong C, et al. (2017) Facile fabrication of moldable antibacterial carboxymethyl chitosan supramolecular hydrogels cross-linked by metal ions complexation. *Carbohydr Polym* 165: 455-461.
69. Yu S, Zhang X, Tan G, et al. (2017) A novel pH-induced thermosensitive hydrogel composed of carboxymethyl chitosan and poloxamer cross-linked by glutaraldehyde for ophthalmic drug delivery. *Carbohydr Polym* 155: 208-217.
70. Mohan N, Mohanan PV, Sabareeswaran A (2017) Chitosan-hyaluronic acid hydrogel for cartilage repair. *Int J Biol Macromol*.
71. Mozalewska W, Czechowska-Biskup R, Olejnik AK, et al. (2017) Chitosan-containing hydrogel wound dressings prepared by radiation technique. *Radiat Phys Chem*134: 1-7.
72. Croisier F, Jérôme C (2013) Chitosan-based biomaterials for tissue engineering. *Eur Polym J* 49: 780-792.
73. Carvalho IC, Mansur HS (2017) Engineered 3D-scaffolds of photocrosslinked chitosan-gelatin hydrogel hybrids for chronic wound dressings and regeneration. *Mater Sci* 93: 1519-1529.
74. Mohan N, Mohanan PV, Sabareeswaran A (2017) Chitosan-hyaluronic acid hydrogel for cartilage repair. *Int J Biol Macromol*.
75. Mozalewska W, Czechowska-Biskup R, Olejnik AK, et al. (2017) Chitosan-containing hydrogel wound dressings prepared by radiation technique. *Radiat Phys Chem* 134: 1-7.
76. Giri TK, Thakur A, Alexander A, et al. (2012) Modified chitosan hydrogels as drug delivery and tissue engineering systems: present status and applications. *Acta Pharm Sin B* 2: 439-449.
77. Santos JCC, Mansur AAP, Mansur HS (2013) One-step biofunctionalization of quantum dots with chitosan and n-palmitoyl chitosan for potential biomedical applications. *Molecules* 18: 6550-6572.
78. Medeiros FGLB, Mansur AAP, Chagas P, et al. (2015) *O*-carboxymethyl functionalization of chitosan: Complexation and adsorption of Cd (II) and Cr (VI) as heavy metal pollutant ions. *React Funct Polym* 97: 37-47.

79. Yu P, Bao R-Y, Shi X-J, et al. (2017) Self-assembled high-strength hydroxyapatite/graphene oxide/chitosan composite hydrogel for bone tissue engineering. *Carbohydr Polym* 155: 507-515.
80. Liu X, Chen Y, Huang Q, et al. (2014) A novel thermo-sensitive hydrogel based on thiolated chitosan/ hydroxyapatite/beta-glycerophosphate. *Carbohydr Polym* 110: 62-69.
81. Song K, Li L, Yan X, et al. (2017) Characterization of human adipose tissue-derived stem cells *in vitro* culture and *in vivo* differentiation in a temperature-sensitive chitosan/B- glycerophosphate/collagen hybrid hydrogel. *Mater Sci Eng C* 70: 231-240.
82. Bao Z, Jiang C, Wang Z, et al. (2017) The influence of solvent formulations on thermosensitive hydroxybutyl chitosan hydrogel as a potential delivery matrix for cell therapy. *Carbohydr Polym* 170: 80-88.
83. Zhang Y, Dang Q, Liu C, et al. (2017) Synthesis, characterization, and evaluation of poly(aminoethyl) modified chitosan and its hydrogel used as antibacterial wound dressing. *Int J Biol Macromol* 102: 457-467.
84. Rabea EI, Badawy MET, Stevens C V, et al. (2003) Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules* 4: 1457-1465.
85. Zakrzewska A, Boorsma A, Delneri D, et al. (2007) Cellular processes and pathways that protect *Saccharomyces cerevisiae* cells against the plasma membrane-perturbing compound chitosan. *Eukaryot Cell* 6: 600-608.
86. Song Y, Zhang D, Lv Y, et al. (2016) Microfabrication of a tunable collagen/alginate-chitosan hydrogel membrane for controlling cell-cell interactions. *Carbohydr Polym* 153 :652-662.
87. Dang Q, Liu K, Zhang Z, et al. (2017) Fabrication and evaluation of thermosensitivechitosan/collagen/α, β-glycerophosphate hydrogels for tissue regeneration. *Carbohydr Polym* 167: 145-157.
88. Heris HK, Latifi N, Vali H, et al (2015) Investigation of Chitosan-glycol/glyoxal as an injectable biomaterial for vocal fold tissue engineering. *Procedia Eng* 110: 143-150.
89. Yap LS, Yang MC (2016) Evaluation of hydrogel composing of Pluronic F127 and carboxymethyl hexanoyl chitosan as injectable scaffold for tissue engineering applications. *Colloids Surf B* 146: 204-211.
90. Malli S, Bories C, Pradines B, et al. (2017) *In situ* forming Pluronic® F127/chitosan hydrogel limits metronidazole transmucosal absorption. *Eur J Pharm Biopharm* 112: 143-147.
91. Molina MM, Seabra AB, de Oliveira MG, et al. (2013) Nitric oxide donor superparamagnetic ironoxide nanoparticles. *Mater Sci Eng C Mater Biol Appl* 33: 746-751.
92. Ong SY, Wu J, Moochhala SM, et al. (2008) Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. *Biomaterials* 29: 4323-4332.
93. Zhou Y, Zhao Y, Wang L, et al. (2012) Radiation synthesis and characterization of nanosilver/gelatin/carboxymethyl chitosan hydrogel. *Radiat Phys Chem* 81: 553-560.

94. Sudheesh KPT, Lakshmanan VK, Anilkumar TV, et al. (2012) Flexible and microporous chitosan hydrogel/nano ZnO composite bandages for wound dressing: *In vitro*and *in vivo*evaluation. *ACS Appl Mater Interfaces* 4: 2618-2629.
95. Yang JA, Yeom J, Hwang BW, et al. (2014) *In situ*-forming injectable hydrogels for regenerative medicine. *Prog Polym Sci* 39: 1973-1986.
96. Hoffman AS (2012) Hydrogels for biomedical applications. *Adv Drug DelivRev* 64: 18-23.
97. Cheng NC, Lin WJ, Ling TY, et al. (2017) Sustained release of adipose-derived stem cells by thermosensitive chitosan/gelatin hydrogel for therapeutic angiogenesis. *Acta Biomater* 51: 258-267.
98. Zhang D, Zhou W, Wei B, et al. (2015) Carboxyl-modified poly(vinyl alcohol)-crosslinked chitosan hydrogel films for potential wound dressing. *Carbohydr Polym* 125: 189-199.
99. Duran N, Duran M, de Jesus MB, et al. (2016) Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity. *Nanomedicine* 12: 789-799.

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