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Controlled local drug delivery strategies from chitosan hydrogels for wound healing

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## ABSTRACT

**Introduction:** The main target of tissue engineering is the preparation and application of adequate materials for the design and production of scaffolds, that possess properties promoting cell adhesion, proliferation and differentiation. The use of natural polysaccharides, such as chitosan, to

prepare hydrogels for wound healing and controlled drug delivery is a research topic of wide and increasing interest.

**Areas covered:** This review presents the latest results and challenges in the preparation of chitosan and chitosan-based scaffold/hydrogel for wound healing applications. A detailed overview of their behavior in terms of controlled drug delivery, divided by drug categories, and efficacy was provided and critically discussed.

**Expert opinion:** The need to establish and exploit the advantages of natural biomaterials in combination with active compounds is playing a pivotal role in the regenerative medicine fields. The challenges posed by the many variables affecting tissue repair and regeneration need to be standardized and adhere to recognized guidelines to improve the quality of evidence in the wound healing process. Currently, different methodologies are followed to prepare innovative scaffold formulations and structures. Innovative technologies such as 3D printing or bio-electrospray are promising to create chitosan-based scaffolds with finely controlled structures with customizable shape porosity and thickness. Chitosan scaffolds could be designed in combination with a variety of polysaccharides or active compounds with selected and reproducible spacial distribution, providing active wound dressing with highly tunable controlled drug delivery.

Keywords: Chitosan, hydrogels, controlled drug delivery, wound healing, tissue regeneration

Article highlights box

- In the highly complex skin wound healing process, chitosan-based hydrogel presents unique flexible properties: tunable water content, mechanical strenght, stability.
- Chitosan can be easly associated with natural polymers of plant or animal origins to improve cell adhesion and proliferation.

- Several drugs can be incorporated in the chitosan-based hydrogels: antimicrobial, antiinflammatory drugs, growth factors, antioxidants, nutrients, etc. Drug delivery kinetic can be tuned during the hydrogel preparation phase as a function of the final application.
- Pivotal challenges are posed by the many variables affecting skin repair and regeneration (i.e. wound environment, temperature, humidity, stage, etc.) and still not faced in a standardized manner. Recognized experimental guidelines are needed.
- Expert opinion, limits, challenges and future trends for innovative technologies and the design of chitosan scaffolds are discussed.

## 1. Introduction

Tissue repair and regeneration is an essential body ability to feact against infection and preserve integrity and function. This extraordinary capacity involves the reaction of the immune system and the activation of cell proliferation and differentiation process allowing the repair of the physical damages and the restoring of the original functional capabilities. A common example of tissue repair experienced by all of us at least once in the life is rapresented by the wound healing process. Wound healing is a highly complex skin process involving defined steps from vascular response and blood coagulation, to formation of fibrin network, re-epithelialisation, collagen maturation and remodelling of connective tissue [1]. In such a complex system, the definition and classification of the wounds types is still under scientific discussion and improvement and a plethora of definitions have been proposed. Among wounds, attention can be focused on the acute and chronic types (Table 1) depending on the healing time. Acute wounds derive mainly from mechanical injuries or surgical interventions and are usually managed efficiently with good chances of success in a time frame of some weeks. On the other hand, wound healing is not always a granted process and dysfunctional skin responses can result in tissue repair failure. Chronic wounds are traditionally divided etiologically, since, apart from burn wounds, they are more and more associated to age-

related pathologies, such as diabetes (i.e. diabetic foot ulcers), vascular insufficiency (i.e. venous leg ulcers), prolonged decubitus or cancer. The underlying impairment deriving form these pathologies negatively contributes to the resolution of those sores, which fail to proceed through an orderly and timely reparative process to restore the functional and aesthetic status of the tissue over a period of 3 months [2].

Another classification is to evaluate if the wound is cleaned or infected. In association with the wound stage, type of treatment, skin ageing, nutrition and glycemic level, it is well known that impaired healing of chronic wounds strongly depends by bacterial burden [3]. Pooling microorganisms (microbiomes) can colonize and grow in wounds bringing to severe infectionrelated complications, such as amputation (i.e. diabetic foot ulcer) or sepsis and death. Staphylococcus, Pseudomonas, Corynebacterium and Anaerococcus are examples of the ten most abundant bacterium genera found in chronic wounds. On the other hand, microbiome can be exploit as diagnostic tool in wound treatment to modulate therapies, as easly accesibile and highly-reactive information site. Recent studies demonstrated that wound depth and duration are associated with different microbial variety and loading. For these reasons, it is of pivotal importance to characterize the microbiome to distinguish between benign and problematic bioburden, and selectively localize bacterial colonies, together with the presence of pathogens. Currently, clinical practices are mainly based on culture approaches and a general use of antibiotic coktails to control infections. This standard approach often leads to biased results and an increase of antibiotic-resistant environments. In such a context, the use of molecular genomic tools to identify and quantify bacteria and the use of localyzed drug delivery systems may rapresent a valid therapy that move toward a more effective targeted application.

Other important wound complications are the inflammation (wounds are painful and hard to move), scarring and loss of function (i.e. a blood vessel or a nerve is damaged).

The prevalence of acute and chronic wounds worldwide is becoming a severe medical concern, since those lesions result in considerable morbidity and a burden on healthcare costs. Impaired and

delayed healing, both of acute or chronic wounds, urges the need of therapeutic agents than can efficiently contribute to the recovery of the damaged tissue [4].

Wound treatments are different as a function of the wound type and the patient, but they usually include a cleaning step, the use of antibiotics and the selection of an appropriate dressing. An ideal wound dressing [5] should act on more levels, first of all by physically protecting wound bed preventing microbial and particulate contamination; moreover, it should promote a moist wound environment, avoid desiccation while absorbing excess exudate and allowing gas exchanges and transpiration. The dressing should be non-toxic, non-allergenic, non-adherent and should promote autolytic debridement. In case of difficult-to-heal wounds, dressings can be exploited as platforms, composed for example of biopolymers, that can promote and initiate wound healing by delivering active drugs that contribute to the control of microbial or fungal contamination and infection or to the stimulation of tissue regeneration. Dressings designed to have biological activity either on their own or through the release of bioactive constituents are defined as advanced [6]. Finally, from a healthcare point of view, the dressing should be easy to use, acceptable to the patient and cost-effective [7].

Among active dressing, hydrogels present unique properties suitable for wound care. Hydrogels are prepared starting from both natural or synthetic polymers (i.e. chitosan, hyaluronic acid, chondroitin sulfate, collagen or cellulose-derivative poloxamers) and present a liquid and solid state that generally not exceed the 10% [8]. In the solid state, the polymer intra- and inter-chain interactions determine the consistency and the mechanical properties of the gel, and, together with the physico-chemical properties of the polymer, the ability of the scaffold to absorb and release water and active ingredients. The liquid state is water or aqueous solutions to guarantee compatibility with the living tissue, mantaining moisture and skin hydratation. In addition, the high water content of hydrogels insure soft and confortable implants that complie with good bendage and applicability. A great advantage of hydrogels is presented by they flexible properties: water content, mechanical strenght,

stability, drug delivery kinetics, etc. can be tuned during the preparation phase as a function of the final application, making them excellent candidates for woud healing applications.

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#### 2. Chitosan properties

Many biopolymers satisfy the requirements cited above and also possess the virtue of being biodegradable. Among them, chitosan in particular possesses remarkable and useful properties to help wound healing. Chitosan is a polysaccharide comprising copolymers of glucosamine ( $\beta(1-4)$ linked 2-amino-2-deoxy-D-glucose) and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose). It can be derived by partial chemical or enzymatic deacetylation of chitin (from 60 up to 99% deacetylation degree), one of the most abundant naturally occurring biopolymers commonly coming from crustacean shells. This definition includes a variety of polymers with different molecular weight and degree of deacetylation: this 'tunable' aspect of chitosan allows optimization of its biological and toxicity profile [9, 10, 11]. Chitosan is biocompatible [12], biodegradable, bioresorbable [13] and has low to absent toxicity [14]. Hydrogels are prepared by solublizing the polymer in mild acid pH conditions, following reversible or irreversible gelation by inter-chain cross-linking under basic conditions. The nature of the base and its concentration, together with the presence or absence of chelating metal ions account for the nature and the strenght of the intra- and inter-chain non-covalent interactions, such as ionic interactions, hydrogen-, or hydrophobic bonds, and thus for the quality of the resulting gel. By introducing cross-linking agents or photopolymerization, chitosan performances can be modulated to form chitosan cross-linked with it self, with tunable inter-penetrating networks, or hybrid polymeric forms. It can be modulated to form different types of structure: gels, membranes, sponges, films, micro- and nano-particles, nanofibers or beads [15]. The properties of chitosan strongly depend on its production process. Both in its entirety (from 300 to 1000kDa) or in the form of its chitooligomers and monomers, obtained by enzymatic or chemical degradation, chitosan demonstrated to accelerate wound healing [16].

Chitosan appears not to cause local irritation and implantation data show either no significant dose site reactions or mild, typical foreign body reactions, rendering this generally recognized as safe (GRAS) excipient ideal for the preparation of drug delivery systems [17], for oral, and mucosal administration (especially of anionic drugs) and wound dressings. Chitosan has a lot of recognised biological activities, including enhancement of permeation and absorption [18] of drugs in formulations. Moreover it possesses additional properties that render it particularly attractive for the preparation of wound dressings [19, 20], such as intrinsic antibacterial activity [21, 22], mucoadhesion [23], in situ gelling properties [24] and ease of manufacture, haemostatic action [25], chemoattraction and activation of neutrophils and macrophages to start wound healing promotion of granulation tissue and re-epithelization [26], limitation of scar formation and retraction; increased in vitro angiogenesis, anti-inflammatory effect. The polycationic nature of chitosan also allows explaining chitosan analgesic effects. Indeed, the amino groups of the D-glucosamine residues can protonate in the presence of proton ions that are released in the inflammatory area, resulting in an analgesic effect [27].

#### 3. Chitosan-based DDSs for wound healing

As previously described, chitosan itself is able to positively contribute to wound healing [28, 29], for its biological and mechanical characteristics. Recently, chitosan films were prepared from solutions containing rose Bengal and tested for laser-activated adhesion to wounds and suture-less tissue repair [30]. The strength and tensile characteristics of those films were evaluated, revealing that medium molecular weight chitosan gave the best performances in terms of bonding and tensile strength and E-modulus.

The intrinsic characteristics of chitosan can be further improved by its chemical association with other polymers [31, 32] and drugs or by its chemical modification into a wide variety of derivatives [33-35].

Chitosan is very frequently associated with natural polymers of plant or animal origins. Harkins et al. [36] have prepared lyophilized composites of chitosan and cellulose: the resulting dressings

showed comparable levels of blood absorption with respect to commonly used commercial dressings, killed both Gram positive (*S. aureus* and *VRE*) and Gram negative (*E. coli* and *P. aeruginosa*) bacteria over a period of 24 h, allowed the adhesion and proliferation of fibroblasts without inflammatory effects. Similar results were obtained by Lin et al. [37] who prepared membranes from chitosan and bacterial cellulose. Those membranes showed interesting tensile characteristics and good antimicrobial properties; moreover, histological examinations revealed that wounds treated with those membranes epithelialized and regenerated faster than those treated with bacterial cellulose alone or Tegaderm.

Another polysaccharide that is very frequently associated with chitosan is alginate [38, 39]. Dantas et al. [38] demonstrated for the first time that sodium alginate/chitosan-based films combined with a laser therapy promote excellent biological activities in burn healing compared to the well known cellulose dressing film. The combination of laser therapy and sodium alginate/chitosan-based dressing improves burn healing, apparently by modulating the epithelisation, blood vessels formation and collagenization processes.

Innovative chitosan-based formulations were also proposed, such as a fibrin–chitosan–sodium alginate composite (F–C–SA) in sheet form, by studying the optimum quantities of fibrin, chitosan and sodium alginate to get improved mechanical properties when compared with its chitosan counterpart [40]. The biomaterial with required porous size, surface morphology and high strength was thus tested for wound dressing applications in dogs.

Hydrogels in which chitosan is associated with alginate are commonly used for the delivery of drugs, as will be discussed later.

A bioadhesive wound-dressing material based on the combination of gelatin or collagen and chitosan with a proper ratio was developed and successfully applied in biomedical fields [41-46]. Parvez et al. [47] prepared films by increasing chitosan concentration in a fixed amount of gelatin. They demonstrated that the 10:3 (gelatin:chitosan) composite films showed the best physico-mechanical, thermal, and antimicrobial properties among the other ratios blend films, suggesting its

promising use as carrier for controlled release drug. In vivo tests carried out on rat model to test wound-healing activity showed excellent rapid healing of the wound surface than those dressed with eco-plaster and gauze with an 80 % efficacy.

The combination of chitosan and hyaluronic acid or silk fibroin was also exploited as potential wound dressing materials [48-51].

An interesting application was proposed by Lu et al. [52], based on an in-situ photopolymerized hydrogel dressings offer advantages over the use of preformed dressings such as conformability in any wound bed, convenience of application, and improved patient compliance and comfort. Hydrogel membrane was created in-situ through ultraviolet cross-linking of a photocross-linkable azidobenzoic hydroxypropyl chitosan aqueous solution. The hydrogel membrane demonstrated to be stable, flexible, and transparent with excellent tunable fluid uptake ability, water vapor transmission rate, water retention, and bioadhesion. In vitro experiments using two major skin cell types (dermal fibroblast and epidermal keratinocyte) revealed the hydrogel membrane was impermeable to bacteria but permeable to oxygen and have neither cytotoxicity nor an effect on cell proliferation.

For example, membranes composed of chitosan-sulfonamide derivatives showed increased biodegradation and swelling, as well as improved antimicrobial activity and are very promising for the treatment of burn wounds [53].

This review will focus on the most recent chitosan-based drug delivery systems (DDSs) for the treatment of wounds (Table 2) [54, 55] that, given the interesting characteristics of this material, deserve an in-depth discussion. In detail, the delivery of four drug categories were considered:

- Antimicrobial drugs
- Anti-inflammatory drugs
- Growth factors
- Other supplements

#### 3.1 Delivery of antimicrobial drugs

Traumatic and chronic wounds are very prone to painful infections that could last weeks. Dressing can have a twofold role to control infection, acting both as physical barrier against invasive exogenous bacteria and antimicrobial delivery system to treat and/or prevent bioburden [56]. As described in the Introduction, the wound microbiome is a complex mix of bacteria protected from antimicrobial drugs by their biofilms. The administration of antimicrobial drugs with the dressing could be a usefull and critical element in the control of microbial proliferation and the resolution of the sore. The development of antibacterial-containing dressings could present several advantages in terms of localized prolonged antibacterial release, reduced toxicity, adverse effects and reduced drug somministration (reducing antibiotic resistance).

Chitosan-based dressings have been developed in an array of types, including scaffolds, films/membranes, sponges and beads. Here, we report some of the most recent examples covering the different types of chitosan hydrogels used for antimicrobial drug delivery.

Noel and colleagues [57] worked on chitosan films and sponges for the local delivery of antibiotics. Amikacin and daptomycin were predissolved in the acidic solvent with chitosan, then the solution was cast and dried. Resulting films showed a rapid release of drugs (86% and 32% for amikacin and daptomycin, respectively, after 1h) with an almost complete recovery of loaded antibiotics within 72 hours (96% and 88% for amikacin and daptomycin, respectively) and a promising inhibition of *S. aureus* growth (between 85 and 99% inhibition percentage). Analogous results were obtained by loading amikacin or vancomycin into pre-prepared dry chitosan sponges that were immersed in antibiotic solution: turbidity tests showed inhibition of the growth of *P. aeruginosa* and *S. aureus* respectively for 48 or 72 hours [58].

Porous membranes were prepared by freeze-drying chitosan gelified with dibasic sodium phosphate or (3-glycidoxypropyl)methyldiethoxysilane incorporating gentamycin sulfate and silver nanoparticles as antimicrobial agents: membranes showed an enhanced stiffness, and in vitro drug release evaluation showed an initial burst release of about 70% of loaded gentamycin sulfate at 24 h, followed by a moderate release over subsequent days [59]. Tested against *S. aureus, E. coli, E. faecalis, P. aeruginosa and P. mirabilis* highly efficient results were observed. A derivative of chitosan, grafted with 2-hydroxyethylacrylate (CS-g-PHEA), was used to prepare sponges loaded with levofloxacin by cryogenic induced phase separation and freeze-drying [60]. The resulting sponges released levofloxacin, sparingly soluble in water, within 24h by a combination of erosion and diffusion mechanisms, whose cumulative amount was inversely proportional to the percentage of loaded drug; the release was strongly accelerated if final sponges were neutralized with NaOH before release tests.

Chitosan is often associated with other polymers in the preparation of antibiotic-loaded wound dressings: Sung et al. [61] prepared polyvinyl alchol (PVA)/chitosan physical hydrogels loaded with minocycline by repeated freeze-thawing cycles: the antibiotic was released from gels with a Fickian diffusion mechanism, driven by the increased swelling ability given by the presence of chitosan. The combination of PVA and chitosan allowed to improve the flexibility, elasticity and swellability of the wound dressing. Tested on rat models, these hydrogels showed greater healing effects than did the hydrogel without the minocycline or conventional products (Figure 1). In another paper by Zhang et al. [62], PVA was modified to prepare a series of novel crosslinked hydrogels with chitosan, loaded with gentamicin sulphate as model drug. The sustained release of the drug within 50 hours almost doubled with the increase of the PVA/chitosan ratio in crosslinked gels, in comparison with the physical blend of the polymers or chitosan alone. Tested *in-vitro* against *S.aureus* and *E.coli* efficient bacterial suppression was observed.

Ciprofloxacin was loaded into composite hydrogels into which chitosan was associated with poly ethilene glycol (PEG) by blending polymer solutions that were subsequently neutralized and lyophilized [63]. The addition of PEG lead to a consistent increase of in vitro drug release (up to 35%) in comparison with chitosan scaffolds (20%) which was further confirmed by zone of inhibition and turbidity measurements as well as by *in-vivo* evaluation of scaffolds: PEG/chitosan scaffold adhered better to wounds and reduced the healing time. The same antibiotic was loaded into thermoresponsive chitosan hydrogel prepared with thiolated chitosan with poly (N-isopropyl acrylamide) [64]. Films prepared from this solution showed a good mucoadhesivity and were able to load about 3.5 mg of ciprofloxacin per cm<sup>2</sup>. About 77% of the drug was released, after a 35% burst, in 48 hours. Another quinolone drug, norfloxacin, was included in collagen/chitosan scaffolds prepared by freeze-drying [65]. Scaffolds with different polymer ratio and concentration showed high release efficiency-values that ranged from 74 to 87%. Scaffolds with total polymers of 4% released the drug faster than analogous scaffolds with higher polymer concentration but same collagen/chitosan ratio. Such scaffolds were able to enhance the rate of wound healing of rat full thickness skin defects without side effects.

In these studies chitosan and chitosan-composite scaffolds were prepared by finely tuning processing parameters and additives and the results proposed by the different Authors clearly show a suitable anti-bacterial activity for wound healing applications. As for the *in-vivo* trials few, not-standardized data were available.

## 3.2 Delivery of anti-inflammatory drugs

Recent advances in the use of wound dressings as drug delivery systems suggested that wound healing involves different molecular events requiring communication between cells and various physiological processes, such as haemostasis and inflammatory phase. The inflammatory phase starts within a few minutes of injury up to 24 h and lasts for about 3 days. This therefore necessitates effective analgesic delivery within this inflammatory period.

Pawar et al. [66] prepared streptomycin (STP) and diclofenac (DLF) loaded film dressings by blending Polyox® with four hydrophilic polymers [hydroxypropylmethylcellulose, carrageenan, sodium alginate or chitosan] using glycerol as plasticiser. The films were characterised by scanning electron microscopy, differential scanning calorimetry, X-ray diffraction, and Fourier transform

infrared spectroscopy, texture analysis (tensile and swelling characteristics) and in vitro dissolution profiles using Franz diffusion cell. Drug dissolution profiles showed the difference between the swelling capacity of the unplasticised and plasticised films and the release form the plasticised film was significantly higher ( $60.03 \pm 5.56\%$  STP,  $63.39 \pm 1.92\%$  DLF). The film dressings formulated in this study the continuous delivery of the anti-inflammatory drug for three consecutive days was showed, making them suitable candidate for wound healing treatments.

In a work presented by Amin and Abdel-Raheem [67] different preparations of polyvinyl alcohol, chitosan hydrogel matrix-based wound dressing containing bee venom were developed using freeze-thawing method. The mechanical properties such as gel fraction, swelling ratio, tensile strength, percentage of elongation and surface pH were determined. Tested for inhibition of *S. aureus, P. aeruginosa* and *E.coli*, the results showed that no microbial contamination was observed. The bee venom (4%)-loaded wound dressing exhibited anti-inflammatory effect that was comparable to that of diclofenac gel, the standard anti-inflammatory drug. The combination of chitosan and bee venom demonstrated to significantly accelerate wound healing activity in rats under diabetic state (Figure 2). In addition the anti-inflammatory and antioxidant properties resulted to be beneficial for the skin wounds.

In a study presented by Hashemikia et al. [68] mesoporous silica particles with a hexagonal structure were synthesized and modified with (3-aminopropyl) triethoxysilane, and used as a carrier for anti-inflammatory drug, betamethasone sodium phosphate (BSP). These drug-loaded silica particles were grafted on the cotton fabric surface using chitosan and polysiloxane reactive softener as a soft and safe fixing agent to develop an antibacterial cotton fabric with drug delivery properties. The treated cotton fabrics were tested and evaluated using scanning electron microscope images, bending length, air permeability, washing durability and anti-bacterial properties. It was found that the chitosan-/softener-treated fabrics compounded with drug-loaded silica particles have a good drug delivery performance. The results obtained from release profile of BSP from various

samples in phosphate buffer saline solution at pH 7.2–7.4 demonstrated that the application of chitosan lead to a drug lower release rate from mesoporous silica for 48h, following the conventional Higuchi model. The treatment of cotton fabric with chitosan and drug-loaded mesoporous silica particles leads to develop a cotton fabric with anti-inflammatory drug delivery properties that exhibited a powerful antibacterial activity against both *E. coli* and *S. aureus* even after five washing cycles.

Abioye et al. [69] developed novel *ternary* chitosan–ibuprofen–gellan nanogel as controlled transdermal delivery tool for ibuprofen. An interesting processing approach based on a combination of electrostatic nanoassembly and ionic gelation techniques was proposed. The interactions between the poorly soluble ibuprofen and chitosan produced spherical eutectic nanoconjugates (from 4580 to 14.15 nm) presenting thermally stable amorphous characteristics, elasticity and pseudoplastic characteristics. Chitosan enhanced the skin penetration, permeability and the rate of transdermal release of ibuprofen by a factor of 4, explained by the ibuprofen–chitosan ionic interaction and its concentration.

These studies evidence that chitosan-based formulations have significant potential application in controlling the delivery of anti-inflammatory drugs over a time period compatible with the wound healing steps.

## 3.3 Delivery of growth factors

During wound healing, the mechanisms of cell growth, proliferation, migation and differentiation are activated by the temporal and spatial regulation of peptides acting as growth factors (GF). In chronic wounds the growth factor levels have been proven to decrease; thus, an interesting strategy to promote healing is their external administration. However, the direct local administration of GFs can present some limitations in terms of short half-time caused by the proteolytic enzyme degradation occurring in the wound environment and/or inactivation of the GFs by the extracellular matrix. Thus, the development of dressing containg GFs suitale for an improved controlled and prolonged release is a demand in the wound care.

Wang et al. [70] reported an interesting combination of collagen/chitosan with two functions referred to mediating rapid angiogenesis based on recombinant human vascular endothelial growth factor (rhVEGF) and antibacterial from gentamicin. The two active compounds were encapsulated in PLGA microspheres and combined with collagen/chitosan mixtures in low (lower layer) and high (upper layer) concentrations, and molded to generate the two-compartment and bi-functional scaffolds. Morphological analysis revealed a distinct double layered porous and connective structure with PLGA microspheres encapsulated. The release behavior of gentamicin from PLGA microspheres was described by a classic three-period model including burst release, linear release and slow release. The burst release happened at the first 2 days, and the drug released 31.99% of the total amount, whereas the linear release period covers from the 3rd day to the 15th day gentamicin released 72.97%). From the 16th day, the rest of the drug is released over 28 days. On the other side, the release behavior of rhVEGF from PLGA microspheres presented another trend, like a linear release relationship over 49 days except for the burst phenomenon at the first day. The Authors ascribed the release behavior and long release period to the special microcapsule structure which comprises a dense surface and encapsulated small microspheres, leading to more encapsulation of growth factors. The two different release profiles from the microsphere of the upper and lower layers is a key point. Gentamicin loaded PLGA microsphere has a release period of around a month, denoting that it can play an antibacterial function during the wound inflammation period. The rhVEGF loaded PLGA microsphere, demonstratedi a two month long time release helpfull for vascular regeneration and skin repair during the skin repairing and remodeling periods. In vitro culture of mouse fibroblasts showed that the scaffold can facilitate cell adhesion and proliferation and inhibited proliferation of Staphylococcus aureus and Serratia marcescens, exhibiting antibacterial effect.

Mohandas et al. [71] developed a chitosan-hyaluronic acid composite sponge containing vascular endothelial growth factor (VEGF) loaded nanofibrin (particle size 150–180 nm) for the delivery of VEGF to diabetic wounds with poor angiogenesis. VEGF is the most potent angiogenic growth factor which stimulates multiple phases of wound healing angiogenesis and thereby accelerates, healing. The prepared sponges were characterized and evaluated the porosity, mechanical strength, swelling, degradation and hemostatic potential. VEGF release studies from chitosan-hyaluronic acid/VEGF loaded nanofibrin composite sponges (CHVFS) showed an initial burst release followed by a sustained release till 7 days which is appropriate for a wound dressing material. Cell studies with HDF cells and HUVECs revealed the cytocompatible nature of CHVFS. Moreover endothelial cells seeded on CHVFS were well proliferated and showed capillary like tube formation which is a prominent process in wound healing angiogenesis. The proposed system provides a promising way for the treatment of diabetic wounds with poor granulation tissue.

Ribeiro et al. [72] carried out an in vitro and in vivo assays to evaluate the applicability of a dextran hydrogel loaded with chitosan microparticles containing epidermal and vascular endothelial growth factors, for the improvement of the wound healing process. The macroscopic analysis showed that the period for wound healing occurs in animals treated with microparticle loaded hydrogels containing growth factors that were considerably smaller than that of control groups, and the histological analysis revealed the absence of reactive or granulomatous inflammatory reaction in skin lesions.

An other interesting study involving growth factor controlled release was propsed by Zhou et al. [73]. Recombinant human epidermal growth factor (rhEGF), known to stimulate cell proliferation and accelerate wound healing, was direct delivered at the wound site in a sustained and controllable way without loss of bioactivity would enhance its biological effects through a fibrin gel loaded with chitosan nanoparticles. rhEGF-loaded chitosan nanoparticles were prepared with the ability to protect rhEGF from proteolysis and then incorporated into a fibrin gel matrix during polymerization. The in vitro release studies showed that the fibrin gel loaded with rhEGF/chitosan

nanoparticles could achieve a more sustained release of rhEGF than either chitosan nanoparticles or an unloaded fibrin gel. When rhEGF was released from the chitosan nanoparticles, a burst release of approximately 65% of the rhEGF occurred within the first 12 h, followed by a slow release phase sustained for approximately 72 h. The burst release of rhEGF for fibrin glue was approximately 35% of the rhEGF within 12 h and 56% within 24 h. Compared with the two systems above, the nanoparticle-incorporated fibrin glue released rhEGF more gradually and more steadily. Approximately 35% of rhEGF was still not released from this system after 96 h. Moreover, the release rate could be controlled by altering the contents of fibrinogen and thrombin in this composite delivery system. rhFGF release rate decreased as the thrombin and the fibrinogen contents in the composite fibrin gels was increased. Additionally, the bioactivity of the released rhEGF was determined by assessing its ability to stimulate the proliferation of BALB/c 3T3 cells, and the results showed that rhEGF bioactivity was not affected during the preparation process and could be maintained for at least 7 days.

In these papers, the Authors demonstrated that chitosan delivery system may have great potential applications in the local administration of GF favouring the wound healing process.

## 3.4 Delivery of other supplements

Several recent papers dealing with the use of chitosan based scaffolds for wound healing applications include the use of various bioactive compounds, such as antioxidants, aminoacids and vitamins able to improve cell proliferation [74-80].

In a recent study, Kim et al. [81] investigated the use of nitric oxide (NO) as a promising therapeutic agent with antibacterial and wound-healing properties. NO-releasing films composed of chitosan (CS) and S-nitrosoglutathione (GSNO) as a NO donor were developed. Thermal analysis demonstrated molecular dispersion of GSNO in the films. In vitro release study revealed that NO release from CS/NO films followed Korsmeyer–Peppas model with Fickian diffusion kinetics. Moreover, the CS/NO film showed a stronger antibacterial activity against Pseudomonas

aeruginosa (Gram-negative) and Staphylococcus aureus (Gram-positive) than the CS film. Further, the CS/NO film accelerated wound healing and epithelialization in a rat model of full-thickness wounds as compared to the CS film. Histopathological studies revealed that CS/NO films favorably enhanced the re-epithelialization and reconstruction of wounded skin.

The chitosan/soy (cht/soy) membranes, in a rodent model under impaired healing conditions induced by corticosteroid treatment, are suitable wound dressings, allowing the progress of a normal healing path and a faster replacement of the excised epidermis in comparison to an undressed wound (negative control) [82]. The presence of granulation tissue at earlier stages of healing was indicative of an accelerated tissue reaction.

After 1 week the wounds dressed with the cht/soy membrane already presented some stratification. The smaller wound area and the thinner margins, with an almost complete repair of all the layers of the excised epidermis after 14 days of dressing, confirmed the normal progress of re-epithelialization and a better healing after dressing with the cht/soy membrane. The cht/soy membranes were easily detached from the wounds, without additional trauma and without removing the granular tissue. The overall results showed that the cht/soy-based membranes induced the formation of new tissue with good histological features in a short period of time although, in comparison to the standard controls, the wound area seems similar at the end of the experiment.

## **Expert Opinion**

Over the last decade the efforts of the scientific community for wound care improving are notably. A list of organic and inorganic compounds were tested in a variety of assembled forms to move toward appropriate and effective wound healing strategy. However, the wound healing processes need further studies to address the biological and molecular occurring events from cell proliferation and differentiation to the extra cellular matrix deposition. In this regenerative medicine scenario, the need to establish and exploit the efficacy and advantages of natural biomaterials in combination with active compounds is playing a pivotal role.

The papers proposed in this review clearly show that chitosan-based hydrogels exhibit significant capabilities in wound healing theraphy as biocompatible, bioabsorbable and cell-stimulating material. As a key point, the possibility to include drugs in the chitosan material allows to tune their localized and controlled release over different days improving tissue regeneration. In such a context, the non-covalent interactions [83, 84] between polymers and drugs play a pivotal role to understand the release mechanisms and could be optimized as a function of the applications. The wound healing process involves two distinct processes: the tissue repair and tissue regeneration, mainly correlated with the restoring of the function and the structure of tissue, respectively. The challenges posed by the many variables affecting tissue repair and rigeneration (i.e. wound environment, temperature, humidity, stage, etc.) need to be faced in an increasing standardized manner and adhere to recognized guidelines to improve the quality of evidence in the wound healing process. Even if a high number of observational data is available, the comparison of results among different biopolymer-based products in wound healing to attest the state-of-art in this field is not always easy to perform and has not yet been attempted. Consistent and reproducible approaches are needed to obtain adequate outcomes both in the preclinical and clinical studies. An effort toward the standardization of the material used and the selection of appropriate animal models and test used should be considered. As stated in the introduction, the chitosan characterization is not straightforward, but of pivotal importance to understand the properties of the biopolymer. Moreover, the animal wound model, the wound location and dimensions, the use of analgesics is only one of the parameters that should be standardized to obtain comparable data. In addition, in this standardization process, attempts to understand the biological processes involved in wound healing should increase to drive the preparation of effective hydrogel-containing active compunds for targeted therapy.

An other significant aspect that deeply need to be investigated together with the properties of the chitosan-based materials, is done by the technology. Innovative technologies could provide a further implementation about the effectiveness of the chitosan materials, i.e. electrospinning,

bioelectrospray or 3D printing [85-87]. Different ways are followed to prepare effective scaffold formulations and structures. Innovative technologies such as 3D printing or bio-electrospray are promising to create chitosan-based scaffolds with finely controlled structure with customizable shape porosity and thickness. The capability of these techniques to create micro- and nano-structures in combination with the tuning of the processing parameters allows to mimick in vivo architecture able to differently stimulate cell differentiation and proliferation.

Briefly, chitosan scaffolds could be designed in combination with a variety of polysaccharides or active compounds with selected and reproducible spacial distribution, providing active wound dressing with highly tunable controlled drug delivery. Among key factors influencing the quality of the final product, hydrogel processing parameters such as acidic dissolution, heating, sterilization and liophylization should be strictly controlled to obtain standardizable behaviour. From the quality point of view, analytical strategies allowing characterization of raw materials and the final products are a demand for the practical applicability of the chitosan-based scaffolds.

As medical frontier, living scaffolds, scaffolds containing living cells, able to recreate and stimulate tissue repair, represent a innovative therapeutic approach for wound healing. In this context, chitosan is a biopolymer facing several challenges to be used as carrier material (ie. gelling conditions, cell living, oxygen permeability etc.) and other polymer are preferably used. A scheme well describing some considerations for the design of scaffolds/dermal substitutes was provided by Hodgkinson e Bayat (Figure 3) [87].

Overall, the complexity of wound healing involves phenomena that could be not easly reproducible such as scaffold erosion and scaffold microenvironments (i.e. humidity, or exudates etc.) and the variable microbiome. Consequently, drug release profile can be altered from patient to patient in a manner independent from the initial compostion or structure of the hydrogel.

This scenario opens stimulating scientific trends toward the combination of innovative materials with new technologies for the development of highly targeted costumable products.

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## **Declaration of interest**

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This review provide an overview of new technologies used in regenerative medicine.

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**Figure 1.** Photographs of wounds treated with (a) hydrogel with 0.75% chitosan and 0.25% drug, (b) sterile gauze, (c) hydrogel with 0.75% chitosan and no drug, (d) con- ventional product at (A) 0 day, (B) 3 days, (C) 6 days, (D) 9 days, (E) 12 days and (F) 15 days of post-operation [Ref 61].



**Figure 2.** Wound healing effects of different hydrogel preparations (F1–F4) compared to control (Con) on round section full thickness excision wound in rats after 0, 7, 14 and 21 days. Values are expressed as mean  $\pm$  SE (n = 6). Data comparisons were performed using one-way ANOVA followed by Dunnett's test.

 $P \ 0.05; **P \ 0.01, \bullet ***P \ 0.001$  as compared with untreated control (Con) rats at each correspondent time point (a). Photos showing an example of the healing effect of F4 hydrogel on wound of diabetic rats compared to control (Con) (b). Ref. 67.



**Figure 3.** Flow chart representing some of the design considerations for next generation dermal substitutes. The design of dermal substitutes starts with the choice of substrate material which can be from a biolog- ical source or a synthetic material. The *grey box* represents selected instructive cues and promising methods of incorporation into sub- strates. This instructive scaVold may then be inserted as an acellular substitute or cells may be introduced (*purple box*). The incorporation of viable cells into the designed substrate is also an important design consideration with several populations to select from. The 3D spatial positioning of these cells within scaVolds should improve the speed and functionality of substitute assisted healing. The eYcacy of trans- planted cells is dependent on delivery method and scaVold material design. Ref. 87.



 Table 1. A classification of wound types [Ref. http://www.woundcarecenters.org/articles/wound-types].

Wound Types	Definition	
Acute wounds	Surgical or traumatic wounds involving blood	
	vessels, nerves, muscles with variable depth	
Chronic wounds	An acute wound failing to heal in the expected time	$\langle \uparrow \rangle$
	frame.	
	Types of chronic wounds	
	Diabetic wounds	Poor or delayed healing wounds. Healing problems are
		caused by the peripheral arterial diseases and peripheral
		neuropathy that can occur with diabetes, wherein the
		small blood vessels in different parts of the body,
		especially in the extremities (hands and feet), grow
		narrower and reduce the blood circulation to those areas.
	Venous leg ulcers	Ulcers occurring in the lower limbs, resulting in a
		breakdown of the surronding cells and tissue layers.
	Pressure ulcers	Decubitus ulcers caused by prolonged, unrelieved
		pressure to an area of skin of the body.
	Ischemic wounds	Wounds resulting from arterial insufficiency: blocked
		blood flow to medium and small vascular beds in the
		body.
	Infected wounds	Wounds in which bacteria or other microorganisms have
		colonized, causing deterioration of the wound and delay
		in the wound healing.
	Biofilm-infected wounds	Wounds in which living microbes forms colonies forming
	$\langle \rangle \rangle$	a complex, three-dimensional structure resistant to
		defense mechanisms of the body.

Table 2. Overview of the select	ted drug delivery chitosa	n-based applications in wound h	ealing.		
Drug catergory	Active compound	Material	Forms	Applications	Ref.
Antimicrobial drugs	Amikacin and	Chitosan	Film and sponges	Tested for inhibition of	57
	daptomycin			S. aureus	
	Amikacin and	Chitosan	Sponges	Tested for inhibition of	58
	vancomycin			S. aureus and	
				P.aeruginosa	
	Gentamycin sulfate	Chitosan	Porous membranes	Tested for inhibition of	59
	and silver			S. aureus,	
	nanoparticles		$\geq$	P.aeruginosa, E.coli,	
				E.faecalis ans	
				P.mirabilis	
				Preliminary <i>in-vivo</i> test	
				on the shell of	
	I	Chitesen /2	<u>Currente</u>	Hermann tortoises	(0
	Levoiloxacin	Chitosan/2-	Sponges	l ested for inhibition of	60
		nydroxyetnylacrylate		S. <i>aureus</i> and	
	Minagualina	DVA (ahitagan	Undragal	P. deruginosa	61
	Contomyoin gulfata	DVA/chitoson	Hydrogel	Tested <i>In-VIVO</i> off fats	62
	Gentaniyeni sunate	P VA/chitosan	nyulogei	S gurges and $E$ coli	02
	Ciprofloyacin	PEG/chitosan	Hydrogel	Tested for inhibition of	63.64
	Cipionozacin	1 EO/emitosan	Films	$S_{aurous} P$	05,04
		Thiolated chitosan/N-	1 11115	geruginosa and E coli	
		isopropyl acrylamide films		uer uginosu and E.con	
	Norfloxacin	Collagen/chitosan	Hydrogel	Tested <i>in-vivo</i> on	65
			,	Sprague-Dawley rats	
				1	
Anti-inflammatory drugs	$(\bigcirc)$				
	Diclofenac	Polyox-HPMC-carrageenan- sodium alginate/chitosan	Film	-	66

	Bee venom	PVA/chitosan	Hydrogel	Tested for inhibition of S. aureus, P. aeruginosa and E.coli Tested on rats induced of diabetes	67
	Betamethasone	Silica/3-aminopropyl triethoxysilane/chitosan	Mesoporous particles	Tested for inhibition of <i>S. aureus</i> and <i>E.coli</i>	68
	Ibuprofen	Chitosan–gellan	Nanogel	Pig skin transdermal experiments	69
Growth factors					
u	rhVEGF	PLGA/Collagen/chitosan	Microspheres	Tested for inhibition of S. aureus and Serratia marcenses	70
	VEGF	Chitosan/hyaluronic acid	Nanofibrin composite sponges	Tested on human umbilical vein endothelial cells (HUVECs) culture	71
	VEGF	Dextran/chitosan	Microparticles	Tested on human fibroblast and Wistar rats	72
	rhEGF	Chitosan	Nanoparticles	Tested on mouse embryonic fibroblasts	73
Other supplements					
(	Nitric oxide	Chitosan/S- nitrosoglutathione	Film	Antibacterial activity against <i>P.aeruginosa</i> and <i>S. aureus</i> Tested on male Sprague-Dawley rats	81
					37

 $\bigwedge$ 

Soy proteins	Chitosan/soy	Membrane	Tested <i>in-vivo</i> on Sprague-Dawley rats	82
			Sprague-Dawley rats	
				38