



Review article

pH-responsive scaffolds for tissue regeneration: *In vivo* performance

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ABSTRACT

A myriad of pH-sensitive scaffolds has been reported in recent decades. Information on their behaviour *in vitro* under conditions that mimic the pH changes that occur during tissue regeneration is abundant. Differently, the *in vivo* demonstration of the advantages of pH-responsive systems in comparison with non-responders is more limited. The *in vivo* scenario is very complex and the intricate relationship between the host response, the overall pathological conditions of the patient, and the risk of colonization by microorganisms is very difficult to imitate in *in vitro* tests. This review aims to shed light on how the changes in pH between healthy and damaged states and also during the healing process have been exploited so far to develop polymer-based scaffolds that actively contribute *in vivo* to the healing process avoiding chronification. The main strategies so far tested to prepare pH-responsive scaffolds rely on (i) changes in ionization of natural polymers, ionizable monomers and clays, (ii) reversible cross-linkers, (iii) coatings, and (iv) production of CO₂ gas. These strategies are analysed in detail in this review with the description of relevant examples of their performance on specific animal models. The versatility of the techniques used to prepare biocompatible and environment-friendly pH-responsive scaffolds that have been implemented in the last decade may pave the way for a successful translation to the clinic.

Statement of significance

We report here on the most recent advances in pH-responsive polymer-based scaffolds that have been demonstrated *in vivo* to be suitable for wound and bone healing. pH is a critical variable in the tissue regeneration process, and small changes can speed up or completely stop the process. Although there is still a paucity of information on the performance in the complex *in vivo* environment, recently reported achievements using scaffolds endowed with pH-responsiveness through ionic natural polymers, ionizable monomers and clays, reversible cross-linkers, coatings, or formation of CO₂ ensure a promising future towards clinical translation.

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1. Introduction

pH-responsive scaffolds are a subclass of stimuli-responsive systems that are sensitive to dynamic pH environment. These scaffolds are gaining increasing attention in biomedical applications and tissue regeneration, since they are able to respond to a range of pH changes within the body arising from either physiological or pathological conditions [1,2]. Moreover, the pH-responsive scaffolds

may finely regulate the release of therapeutic substances as a function of the tiny changes in pH that occur during tissue regeneration, allowing very localized and on-demand drug delivery [3,4]. A successful design of pH-responsive scaffolds requires first a deep understanding of the influence of pH during acute and chronic wound healing as well as other complex tissue regeneration processes.

Healthy skin has an acidic surface with pH values ranging between 4 and 6 in the stratum corneum, while neutral pH is maintained in the underlying layers of the epidermis and the dermis tissues [5]. The acidic surface layer, known as the acid mantle, is important for both the function and the integrity of the skin as a natural barrier. In wounds, pH values are dynamic as they dif-

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fer depending on the time-course and the wound healing stage. In acute wounds the pH decreases, as a result of temporary acidosis [6]. This natural occurring acidic milieu is critical to impair microbial growth in the wound bed and improve oxygen delivery as described by the Bohr-effect. Once the wound is repaired the pH increases to physiological pH 7.4 and then decreases back to maintain the acidic pH of intact skin surface [7].

Chronic wounds are characterized by increased pH values for most of the healing process. The nonhealing wound bed remains shifted towards alkaline pH, although differences are observed between early and late stages depending on several factors [6,8]. In chronic wounds the balance between tissue degradation and tissue reassembly is disrupted due to activated catabolic processes, which prolong the inflammatory stage [9]. As long as the wound is in the inflammation phase the pH remains alkaline [10]. However, the presence of pus and necrotic tissues on the wound bed may result in acidic pH. This explains why chronic wounds show increased pH milieu immediately after a surgical debridement [11].

The persistent alkaline milieu of the chronic wound-bed displays an ideal environment for the growth of pathogenic bacteria, and subsequently increases the risk of biofilm formation [12]. Biofilms support the growth and survival of various pathogens disregarding the host conditions [8,13]. In turn, bacterial colonization contributes to shifting the pH towards more alkaline conditions, prolonging the inflammatory state of the wound and, thus, worsening the progression of the healing process [14]. Indeed, the increase in pH decreases tissue oxygen tension. Access of oxygen to the damaged skin depends on blood perfusion and also on oxygen diffusion, and it has been shown that a shift of 0.9 pH units may cause a 5-fold change in oxygen released to the skin [15]. Moreover, pH values that differ from those typical of the healing skin environment have an impact on the activity of many therapeutic agents, including antimicrobials [6]. Such a key role of pH in wound healing has prompted the development of wound dressings intended to maintain pH homeostasis [16,17], and a variety of products have already reached the market [18]. In parallel, intense research on the design of wound dressings able to monitor the pH [19–22] or to deliver the adequate drug at the right healing step in response to wound biomarkers has been carried out in the last years [23–25].

The information about the variations of pH during regeneration of other tissues, such as bone, is more limited because of the difficulties of *in situ* measurements. Recent developments on more versatile dyes and tools may allow in the next few years a continuous monitoring of pH changes during tissue healing *in vivo* [26,27], which is required for the successful design of the responsive scaffolds. So far, it is clear that the extracellular pH plays a critical role in regulating the bone remodelling process by directly affecting the viability and activity of osteoblasts [28]. Osteoblasts, which are the main cells responsible for bone construction and formation, are reported to have increased activity at weakly alkaline pH ranging between 7.0 and 7.6 [29,30]. Alkaline pH enhances osteoblasts proliferation. Moreover, the optimum activity of the alkaline phosphate (ALP) of osteoblasts was observed to be at pH 8 [28,31]. Indeed, osteoblasts are affected by acidosis which compromises the normal matrix mineralization [32]. Thus, high local pH during bone formation is considered essential as it promotes mineralisation, while acidosis promotes bone resorption [29,30,33]. The activity of osteoclasts is higher at low pH enhancing the resorption, while it becomes impaired above pH 7.4 [34]. On the contrary, alkalosis induces osteoblastic collagen synthesis. Shifting pH towards acidic environment can be a result of both physiological and pathological conditions. Bone fractures develop acidic milieu in the fracture site because of the disruption of local blood supply, which ends up in lactic acid accumulation. This local acidification induces cell death of osteoblasts [35].

A bone healing *in vivo* model was used to directly measure the pH in bone fracture in the first days of bone recovery. It was found that bone fractures show a decrease from the physiological pH 7.4 to more acidic pH 6.8 in the inflammatory stage, mainly in the initial 2 days of healing. The study reported until day seven of the healing process, and there was a tendency for the pH to increase towards the alkaline condition [36], which is optimal for osteoblasts activity. A decrement of pH in the first days and the subsequent increment of pH in the following week was also confirmed, although indirectly, in other studies using long bone repair models [37].

Bone infection, i.e., osteomyelitis, may also significantly alter the microenvironmental pH. Acute bone infection developed within 2 weeks of disease onset results in decreased pH [38]. Meanwhile, with the continued increase in the bacterial load accompanied with disturbances in the regeneration-resorption balance as well as the catabolic reactions involved, the environment of chronic infections tends to get higher pH values [39].

The aim of this review was to elucidate how the changes in pH between healthy and damaged states and also during the healing process are being exploited so far to develop scaffolds that can actively contribute *in vivo* to the healing process avoiding chronification. The number of reports on pH-responsive scaffolds that have been tested *in vitro* or in cell cultures is very high. Differently, *in vivo* demonstration of the validity of the approach is still limited. Thus, to carry out the review the literature search was collected using Web of Science as the main database. The search was based on the following keywords: “pH-sensitive AND scaffold”, “pH-sensitive AND dressing”, “pH-sensitive AND wound healing”, “pH-responsive AND scaffold”, “pH-responsive AND dressing”, “pH-responsive AND wound healing”. The resulting articles were refined using the term “*in vivo*”. Secondary searches were carried out using the keywords “acid-responsive AND scaffold”. The findings were focused on experimental papers on *in vivo* tissue regeneration applications using animal models. Reports that describe pH-dependent phase transitions solely used for *in situ* gelling of scaffolds were discarded, and the reader is referred elsewhere [40,41].

In the next sections, pH-responsive scaffolds that have been tested *in vivo* are analyzed in detail. The information has been categorized in terms of the main strategies and components used to prepare pH-responsive scaffolds; namely, (i) changes in ionization of natural polymers, ionizable monomers and clays; (ii) reversible cross-linkers; (iii) coatings; and (iv) production of CO₂ gas, as summarized in Fig. 1.

2. Changes in ionization of natural polymers, ionizable monomers and clays

Both cationic and anionic polymers and clays are being widely tested as components of pH-responsive scaffolds. Ionic polysaccharides and polypeptides are particularly suitable because of their natural renewable sources, biocompatibility and versatility for further functionalization [42]. Moreover, some of them show intrinsic capability to mimic the extracellular matrix and to promote cell attraction [43]. The ionic groups in a polymer are responsible for accepting or releasing protons from/to the surrounding media in response to the pH changes. The pKa value sets the pH point at which a change in the polymer conformation or solubility occurs. Such a structural change is transmitted to the scaffold as a whole, experiencing swelling or shrinking [42]. Furthermore, pKa and pH determine the feasibility of reversible interactions of polymers and clays with therapeutic agents and wound components through ionic interactions, which, in turn, provides the opportunity to develop pH-triggered site-specific release of drugs for precise on-demand support of the healing process.

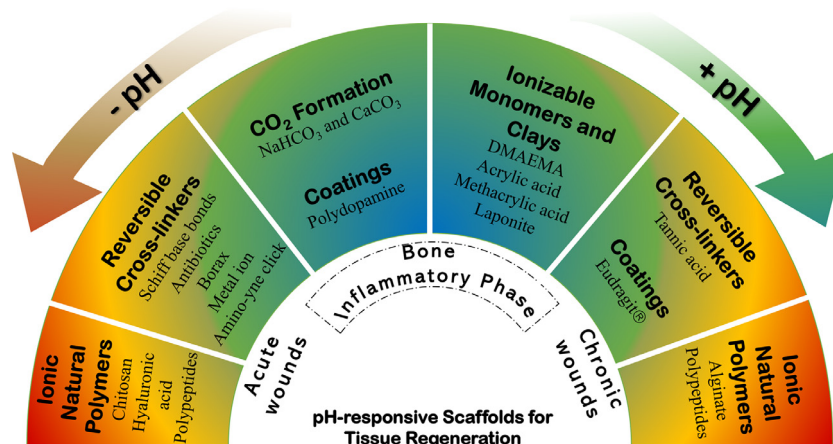


Fig. 1. Main strategies and components investigated to endow scaffolds with responsiveness to the different pH changes that may occur during tissue healing.

2.1. Chitosan

The polycationic chitosan is an outstanding example of component of pH-responsive hydrogel wound dressings [44]. At acidic pH, the amine groups in chitosan become protonated leading to an increase in the swelling of the hydrogel. The increase in the network mesh size facilitates that previously loaded therapeutic substance can diffuse out and become accessible to the surrounding tissue [45].

Chitosan films prepared by casting of chitosan solution with tannic acid have been tested for pH-responsive release of neomycin (Table 1, entry 1) [46]. The antibiotic drug was added to the films during preparation. *In vitro* release experiments evidenced an intense burst (>80%) in the first hours at pH 4.0 due to chitosan dissolution. The burst was less intense (60%) at pH 5.5, while sustained release occurred at pH 7.5. Prolonged release at pH 7.5 was demonstrated in microbiological tests useful for eradicating *Staphylococcus aureus*. Both non-loaded and neomycin-loaded films were tested in full-thickness incisions made on 10–12 weeks old Long Evans male rats. Wound closure was monitored for seven days, and the results revealed better performance of the films compared to the non-covered wound [46]. No significant differences in the skin recovery process were observed between blank and drug-loaded films probably because the test was carried out in a non-infected wound model.

Chitosan was also used to create pH-responsive hydrogels after crosslinking with genipin (Table 1, entry 2) [47]. *In vitro* swelling studies evidenced pH dependence with 230% (w/w) of water uptake at pH 7.4 and 160% at pH 8.2. The hydrogels had capability to neutralize the pH of the surrounding medium; namely, after some days of immersion the pH of the medium initially at 8.2 decreased 0.8 units, while the medium at pH 7.4 decreased 0.3 units. The antibacterial activity of these hydrogel films was also studied against *Escherichia coli*, and the crosslinked hydrogels were able to reduce the bacterial growth by ~70% in comparison with the 90% of reduction from the control films of chitosan. *In vivo* performance studies were carried out in 4-week-old female C57BL/6 wild type mice with induced stage 1 pressure ulcers. Left and right sides of the thoracic area were treated with gauze (control) and sterilized chitosan-genipin hydrogel, made of low molecular weight chitosan and 2 % of genipin. Control and hydrogel treated wounds were sealed with Tegaderm™ adhesive dressing. No significant differences were found in wound closure and re-epithelialization after 14 days. However, wounds treated with hydrogels exhibited thicker stratum spinosum and higher granulation tissue at shorter time points, which means a stronger healing response compared to gauzes [47]. Further studies are needed using chronic wound mod-

els or infected models to evidence the pH responsive character of the hydrogels.

Chitosan together with poly(lactic-co-glycolic acid) (PLGA) were used to coat halloysite nanotubes [48]. The obtained composite films showed pH-responsive *in vitro* release of the antibiotic minocycline; being faster at pH 9.0 compared to pH 5.5. The films were then tested on burn wounds of male rats for 12 days. The drug-loaded composite films exhibited faster size reduction and no infection compared with the control group (Table 1, entry 3). However, the effect of the antibiotic was not significantly studied since the wounds tested *in vivo* were not infected.

In another study chitosan nanocomposite films were prepared by incorporating carbon dots (CD) that exhibited antimicrobial properties and pH-sensitive fluorescence (increasing fluorescence signal as pH increased from 4 to 9) (Table 1, entry 4) [49]. Thus, the films allowed monitoring pH oscillation within the wound milieu throughout the healing process. Chitosan and chitosan-CD films (2.25 cm² surface area) were used to treat 1 cm² wound (rat model). Chitosan-CD films showed better wound closure than the control (non-treated group) and evidenced relevant changes in pH during the *in vivo* healing process (Fig. 2a). The intensity of the fluorescence in fresh wounds was low indicating acidic pH, then the intensity increased and, thus, the pH at day 9. The fluorescence emission decreased again at day 18 indicating healing completion (pH 5–6). Overall, CDs were beneficial acting not only as pH monitoring sensors in the dressing but also as enhancers of oxygen permeability, hence improving tissue growth. It should be noted that the wound was not infected, thus the antibacterial property is still to be confirmed *in vivo*.

2.2. Alginate

Sodium alginate (SA) is one of the most widely used polysaccharides for skin wound healing [50]. The carboxylic groups abundantly present in SA polymer backbone have pKa values of 3.4 and 4.4, thus at alkaline pH the carboxyl groups in alginate are converted into negatively-charged carboxylate ions, which endows wound dressings with high water sorption capability [51]. The pH-responsive swelling can also be exploited for pH-sensitive release; wound alkaline pH accelerates drug release. The responsiveness can be enhanced through combination with other components such as clays that reinforce ionic interactions with the drug or using polymers that also undergo shrinking-swelling cycles when pH oscillates from acid to alkaline. Composite hydrogels prepared with SA and Laponite clay nanoparticles have shown superior mechanical properties and site-specific drug release for the recovery of Achilles tendon injury (rat model) (Table 1, entry 5) [52].

Table 1*In vivo* testing of scaffolds made of ionic natural pH-responsive polysaccharides.

No.	pH-responsive component	Scaffold features	<i>In vivo</i> model	Main outcomes	Ref.
1	Chitosan	Chitosan-tannic acid (CT) nanocomposite films had smooth surface and closed porous structure with thin interconnected texture, higher in the case of neomycin-loaded CT films.	Adult Long Evans male rats. The wounds created on the left dorsal flank were 0.5–1 cm long and ~15 mm full-thickness incisions.	Both tested films (CT and neomycin-loaded CT) demonstrated significant wound recovery.	46
2	Chitosan	Porous crosslinked hydrogels cross-linked with genipin in contrast with non-porous chitosan films. Water uptake of 230% at pH 7.4 while 160% at 8.2. Reduction of 70% of bacterial growth.	Stage 1 pressure ulcers were induced in the back of 4 week-old female C57BL/6 wild-type mice. One side of the back was used as a control treated with gauze and the other side treated with hydrated hydrogels.	Wounds treated with chitosan-genipin hydrogels exhibited on day 3 a thicker stratum spinosum, correlated to keratinocyte migration, and increased granulation tissue. Similar granulation tissue was observed for wound control on day 7.	47
3	Chitosan	The films were wafer-like cut into 2 cm pieces with a smooth surface. The drug-loaded halloysite nanotube were modified via a layer-by-layer polymer coating method.	Two full-thick burn wounds (1.5 cm x 1.5 cm) were made on male rats using an electric heater.	The wounds treated with PLGA/chitosan with the halloysite nanotube with or without drug loading showed wound size reduction and enhanced healing compared with the control group.	48
4	Chitosan with carbon dots (CD)	Nanocomposite films were prepared by mixing 3% chitosan and various CD contents from 0 to 2%, and subsequent freeze-drying.	Rat model with 1 cm ² wounds that were treated with chitosan or chitosan-CD films (2.25 cm ²).	The chitosan/CD nanocomposites showed superior wound healing ability over the control group, and capability to monitor pH of the wound through fluorescence emission of CDs in the film. Synergistic antibacterial and oxygen permeability effects of chitosan and CD.	49
5	Sodium alginate (SA) and clay	Freeze-dried hydrogels prepared by mixing SA with Laponite and PEGylated IGF1.	The hydrogels were tested on rat Achilles tendon injury model. A hydrogel disc (diameter: 5 mm; height: 0.6 mm) was placed on top of the Achilles tendon anastomosis, and the skin was sutured.	The scaffold confined the release to the tendon injury, minimizing drug access to the blood stream. Minor inflammatory response and good tissue integration.	52
6	Sodium alginate (SA) and acrylic acid	SA was condensed with ethylene oxide and then added to acrylic acid monomers for free radical polymerization. The hydrogels swelled at pH values greater than 7.4.	The hydrogels were tested on Wister albino rats for 9 days. Each full-thickness wound was 6 cm long paravertebral.	Wounds treated with the hydrogels showed accelerated wound healing, better epithelisation, and regular hair growth.	53
7	Sodium alginate (SA)	SF-SA NPs were prepared by coacervation. The NPs were dispersed in PNIPAM monomers before free-radical polymerization. The hybrid hydrogel (PNIPAM & SF-SA NPs) was a cylindrical scaffold with 6 mm diameter. Highly porous with interconnected pores of about 50 µm size each.	The hydrogel was tested on Male Wistar albino rats for 21 days. Circular full-thickness wounds with 20 mm diameter were created on the paravertebral area of the rat, and then infected with MRSA suspension. 4 groups: control, PNIPAM hydrogel (no drug), PNIPAM & SF-SA NPs with vancomycin, and PNIPAM & SF-SA NPs with vancomycin and EGF.	The PNIPAM hydrogels containing SF-SA NPs (with or without EGF) had better wound healing and closure results compared with both the control and PNIPAM blank hydrogels with no infection.	51
8	Sodium alginate (SA)	The hydrogel scaffolds were prepared with mixtures of SA and SF and also encapsulating SF NPs. The hydrogels had high porosity (between 63% and 77%) with pore connectivity.	Male Wistar albino rats were damaged in the medullary cavity with a dental drill. The wound was infected by injecting MRSA suspension. After three weeks, the bone wound was expanded to 4 mm and treated with: (a) nothing; (b) control SF/SA hydrogel; (c) SF/SA hydrogel with TEC; (d) SF/SA hydrogel with PM-loaded SF NPs; and (e) SF/SA hydrogel with TEC and PM-loaded SF NPs.	Bones implanted with SF/SA hydrogel with TEC and with or without PM-loaded SF NPs showed higher rate of osteoid tissue and collagen formation. TEC decreased infection and inflammation.	39
9	Oxidized sodium alginate (OSA)	The hydrogel discs were prepared with gelatin methacryloyl (GelMA) and (OSA), and containing or not gentamicin sulfate and phenamil (PM). High swelling ratio of about 550% at pH 4.5.	Subcutaneous infection model (Sprague Dawley male rats) consisted in a 1.5 cm incision to allow the implantation of hydrogels (8 mm diameter) and subsequent injection of <i>S. aureus</i> . The wounds were sutured and evaluated for 7 days. Critical-sized cranial bone defect (8 mm diameter) on Sprague Dawley (SD) male rats were treated with: (a) scaffolds directly loaded with PM, (b) scaffolds with PM-loaded mesoporous nanoparticles, (c) blank scaffolds loaded with BMP-2; and (d) scaffolds directly loaded with PM and then added BMP2. Evaluation was made 3 months after implantation.	Scaffolds loaded with gentamicin and PM cured the subcutaneous infection. Scaffolds containing both PM and BMP2 provided the highest bone volume/tissue volume ratio and bone mineral density.	54

(continued on next page)

Table 1 (continued)

No.	pH-responsive component	Scaffold features	In vivo model	Main outcomes	Ref.
10	Poly(L-lysine)-b-poly(phenylalanine) (PLL-PPA) and poly(glutamic acid)-b-poly(phenylalanine) (PGA-PPA)	PLL-PPA micelles had 195 nm diameter and PGA-PPA micelles had 170 nm diameter. Micelles were mixed and a hydrogel was obtained by cross-linking with genipin.	Male Sprague-Dawley rats with a single full-thickness excision wound (20×20 mm) on the dorsal flank. The wounds were treated with a hydrogel without drugs, a hydrogel loaded with low content in curcumin (0.5 mg) and a fix content in amphotericin B (50 µg); and a hydrogel loaded with high content in curcumin (1.5 mg) and a fix content in amphotericin B (50 µg).	In comparison with both the control and blank groups, the treated wounds resulted in clear wound closure with enhanced granulation and epithelial lining formation and no signs of inflammation or infection.	56

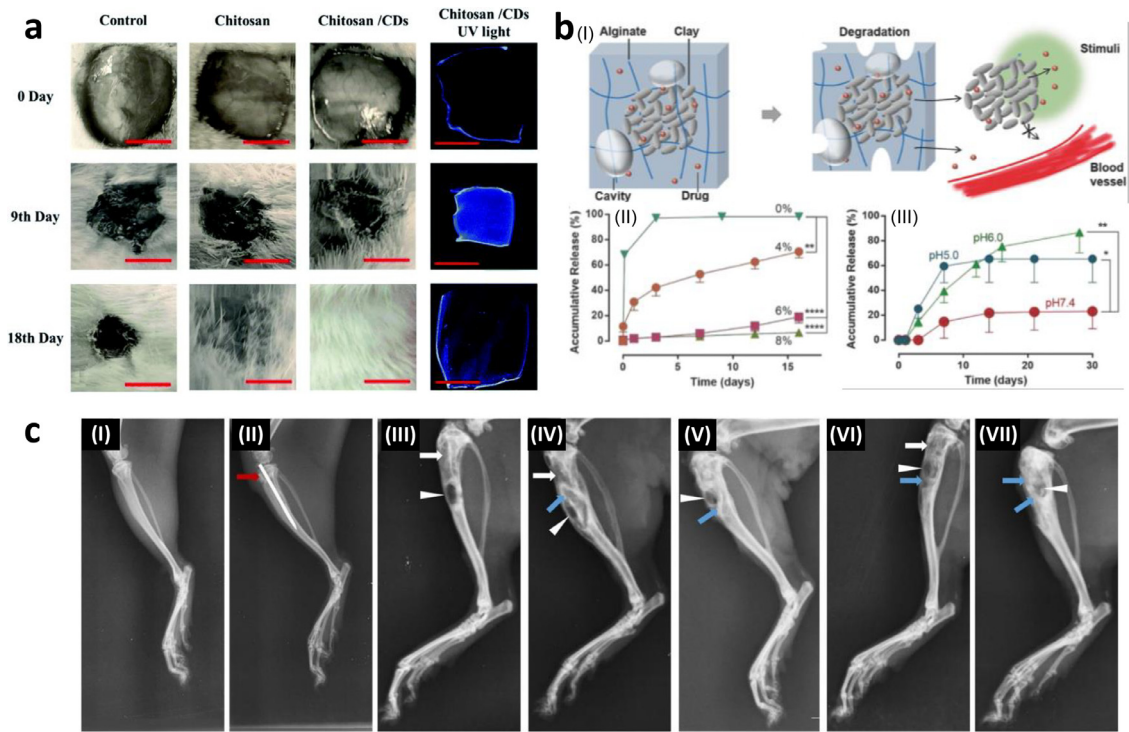


Fig. 2. (a) Images of wounds that remained untreated (control) and that were treated with chitosan or chitosan-CD nanocomposites taken at different stages (0, 9, and 18 days). The fluorescence images on the right panel show the pH sensitivity of the chitosan/CD nanocomposite, which increased the fluorescence signal when the pH in the wound became alkaline. Reproduced from Omid et al. [49] (Creative Commons Attribution 3.0 Unported Licence). (b) Scheme of an alginate hydrogel (blue lines) containing micro-sized pores (gray bubbles) and Laponite clay nanoparticle aggregates (gray discs) that adsorb drug molecules (red) through ionic interactions; (I) the drug is released in acid environment as the network degrades and the drug-clay interactions become weaker; (II) the drug weakly interacting with alginate was released fast while as the content in clay increased from 4 to 8% the release rate slowed down at pH 7.4; (III) a decrease in pH notably accelerated the release from alginate-Laponite (6%) hydrogels. Reproduced from Li et al. [52] with permission of John Wiley & Sons, Inc. (c) Radiograph images of the bone (I) before surgery, and (II) after infection with MRSA and introduction of Kirschner wire (indicated with a red arrow). Subsequent images correspond to bones after removal of the Kirschner wire and then treated for 6 weeks with (III) nothing (empty; control group), (IV) control SF/SA hydrogel; (V) SF/SA hydrogel with TEC; (VI) SF/SA hydrogel with PM-loaded SF NPs; and (VII) SF/SA hydrogel with TEC and PM-loaded SF NPs. White arrows indicate bone destruction, white arrowheads show bone window, and blue arrows points to new bone formation. Reprinted from Motasadzadeh et al. [39] with permission from Elsevier.

A positively charged PEGylated IGF1 mimetic protein was shown to interact stronger with the negatively charged clay and thus reinforced the capability of SA scaffolds to control the release under physiological conditions (Fig. 2b). A decrease in pH weakened the drug-clay and drug-SA interactions and enhanced the release rate. *In vivo* studies (rats) demonstrated that the PEGylated IGF1 was mainly delivered to the tendon, minimizing the access to blood (as compared with a site injection) and that promoted the scaffold-tendon integration as the SA scaffolds degraded [52].

Fast and marked pH-responsiveness of SA hydrogels has been observed for interpenetrating networks made with acrylic acid. The hydrogels showed the capability to inhibit *in vitro* the growth of *E. coli* and *S. aureus* bacteria and promoted *in vivo* wound healing (Wister albino rats) (Table 1, entry 6) [53]. Nevertheless, the reasons for the antimicrobial activity were not disclosed and the *in vivo* tests did not include infected wounds. More promising

performances were recorded by combining SA with silk fibroin (SF), which also bear free carboxylic acid groups in the glutamic acid and aspartic acid amino acids. Combinations of SF-SA successfully provided pH-responsive scaffolds suitable for treating severe infected wounds as well as bones, as described below [39,51].

A hybrid hydrogel system based on silk fibroin-sodium alginate nanoparticles embedded in poly(N-isopropylacrylamide) hydrogels (PNIPAM & SF-SA NPs) was developed for the controlled release of vancomycin from the SF-SA NPs and epidermal growth factors (EGF) from PNIPAM. The NPs were prepared by coacervation of SF-SA dispersions using an anti-solvent. Then, the NPs (200–240 nm) were dispersed in NIPAM monomers before free-radical polymerization in moulds. The resultant cylindrical hydrogels with 6 mm diameter were highly porous with interconnected pores of about 50 µm size each (Table 1, entry 7) [51]. The NPs retained the pH-responsive swelling and showed faster release of vancomycin at pH

8.5 compared to pH 7.4, although the embedding in the hydrogel constrained the swelling process and thus prolonged the release process for more than 20 days. Similarly, the EFG directly dispersed in the PNIPAM networks was sustainably released *in vitro* at 37 °C due to the limited swelling of the hydrogel at this temperature.

The *in vivo* study on male Wistar albino rats (MRSA infected burns) showed that after 14 days of treatment the hydrogels containing vancomycin-loaded SF-SA NPs (with or without EGF) had better wound healing and closure results with no infection compared with both the control (no treatment) and blank hydrogels. At day 21, a complete wound repair with the formation of thick epithelialization layer was recorded in the group treated with PNIPAM & EGF/SF-SA NPs hydrogels (Table 1, entry 7) [51]. This treatment also promoted uniform collagen distribution while inhibiting inflammation and the expression of TGF- β 1. This wound regeneration as well as the infection control was achieved from the prolonged and pH-dependent release of EGF and vancomycin in the alkaline milieu of severe burn wounds.

Similarly, mixtures of SF and SA were used to prepare hydrogel scaffolds containing the antimicrobial teicoplanin (TEC) and the bone regeneration phenamil (PM)-loaded SF nanoparticles (PMSFNPs) for the treatment of chronic bone infections (Table 1, entry 8) [39]. In this case, SF reinforced the mechanical properties of the SA hydrogels once SF transformed into β phase applying ultrasounds and subsequent immersion in a mixture of methanol (90%) and CaCl_2 (200 mM; 10%). *In vitro* tests evidenced that the release of both PM from PMSFNPs and TEC from SF/SA&TEC hydrogel scaffold was faster at pH 8.5 than pH 7.4. The scaffolds were challenged *in vivo* against chronic osteomyelitis induced by injecting methicillin-resistant *S. aureus* (MRSA) in the bone cavity (male Wistar albino rats) and maintaining a Kirschner wire in the infected bone for three weeks (Fig. 2c). Infected bones were then cleaned from the excess of pus, drilled to 4 mm in diameter, and the scaffolds were implanted. Bones treated with SF/SA hydrogels containing TEC and with or without PM-loaded SF NPs showed higher rate of osteoid tissue and collagen formation after 6 weeks treatment. Controls without TEC showed chronic osteomyelitis with inflammation and bone destruction. The alkaline pH-sensitive release of TEC from the scaffolds clearly resulted in decreased infection and inflammation. The *in vivo* results also showed that scaffolds combining both TEC and PM could eradicate the infection while ensuring new bone regeneration, and thus they were considered the most effective for osteomyelitis treatment.

A pH-sensitive hydrogel was fabricated using gelatin methacryloyl (GelMA) and oxidized sodium alginate (OSA) to promote bone regeneration through the dual-release of PM and gentamicin sulfate (Table 1, entry 9) [54]. Gentamicin was dispersed directly in the GelMA and OSA mixture before photopolymerization, while PM was previously loaded into mesoporous nanoparticles. The hydrogels swelled more and disintegrate at pH 4.5 compared with neutral pH because of the disentanglement of SA network and Schiff base bonds formed between GelMA and OSA. In good agreement, drug release was faster at acidic pH although sustained for several days. The hydrogels were first tested *in vivo* in a subcutaneous infection model (Sprague Dawley male rats). Dual loading of gentamicin and PM allowed complete control of the infection in one week. The hydrogels were then tested *in vivo* in a non-infected critical-sized cranial bone defect (8 mm diameter) (Table 1, entry 9). Scaffolds with gentamicin were not tested, and a GelMA-OSA scaffold containing bone morphogenetic protein 2 (BMP2) was used as positive control. Treatments with GelMA-OSA scaffold that combined PM and BMP2 resulted in the highest bone volume and bone mineral density, suggesting a synergic effect of both compounds and revealing the suitability of GelMA-OSA network to facilitate the bone healing.

Polysaccharide-based scaffolds in which the mechanism responsible for pH-responsiveness is primarily due to reversible Schiff base bonds are discussed in details in Section 3.

2.4. Polypeptides

Amino acids bearing ionizable moieties are also being explored as main components of amphiphilic polypeptides that can self-assemble into pH-responsive micelles. For example, poly(L-lysine)-b-poly(phenylalanine) (PLL-PPA) is suitable for form micelles that are protonated at acidic pH showing positive surface charges, while poly(glutamic acid)-b-poly(phenylalanine) (PGA-PPA) forms negatively charged micelles at neutral-alkaline pH [55]. Combination of both types of micelles was explored to form hydrogels (after cross-linking with genipin) that can rapidly release amphotericin B (antimicrobial) from PGA-PPA and slowly release curcumin (healing promoter) from PLL-PPA at the acidic environment of the wound (Table 1, entry 10). The designed micelles were spherical in shape with 195 nm diameter in PLL-PPA and 170 nm diameter in PGA-PPA, and after swelling their diameter increased by 10–15 folds. At physiological pH they maintained their spherical shape, while at pH 4.5 the PGA chains became un-charged, changing the conformation of the micelle, therefore releasing the drug from the core [56]. In a full-thickness wound model (Sprague-Dawley rats) the dually-loaded hydrogels resulted in clear wound closure in comparison with untreated control and blank hydrogel. The wounds showed enhanced granulation and epithelial lining formation as well as no signs of inflammation or infection. However, it should be noted that the wounds were not initially infected and thus the antimicrobial performance was not verified *in vivo*.

2.5. Ionizable monomers

Another approach to endow scaffolds with pH-responsiveness is the copolymerization with synthetic monomers bearing ionizable groups. Tuning the ratio of ionizable to non-ionizable monomers allows a fine control of the pH-responsiveness of the final network. Examples of scaffolds made of ionizable monomers that have been tested for *in vivo* regeneration are summarized in Table 2.

2.5.1. Dimethylaminoethyl methacrylate (DMAEMA)

The amino groups of the dimethylaminoethyl methacrylate (DMAEMA) can be protonated at acidic pH, remaining positively charged. Copolymer hydrogels made of DMAEMA undergo swelling at pH below the pKa of 7.5 and, relevantly, they can regulate the activity of bacteria and also enzymes in the wound bed [57].

Copolymerization of DMAEMA and 2-hydroxyethyl methacrylate (HEMA) in different ratios was investigated to form hydrogels that can increase oxygen transport and cell proliferation in the wounds (Table 2, entry 1) [58]. DMAEMA/HEMA 30/70 mol/mol scaffolds showed pH-dependent pore size with a two-fold increase when the pH dropped to 6.5. Since hypoxia is characteristic of chronic wounds impeding wound healing and collagen synthesis, the capability of the hydrogels to increase oxygen permeability was verified (Fig. 3). The highest pO_2 recorded for DMAEMA/HEMA 30/70 mol/mol scaffolds had a positive effect on cell proliferation and viability on day 3 inside these scaffolds compared with other ratios and the HEMA control. When subcutaneously implanted in Wistar rats, the pH-responsive DMAEMA/HEMA scaffolds increased the granulation tissue and upregulated factors that promote tissue regeneration.

DMAEMA was also combined with hyaluronic acid (HA) in a ratio of 1000:1 for wound dressings that can sustainably release herbal extracts. The obtained highly swellable hydrogel was able to significantly accelerate by itself the wound closure rate of full-thickness skin incisions (2.5 cm; rat model) in seven days (56%

Table 2*In vivo* testing of scaffolds made of ionizable synthetic monomers.

No.	pH-responsive component	Scaffold features	<i>In vivo</i> model	Main outcomes	Ref.
1	Dimethylaminoethyl methacrylate (DMAEMA)	DMAEMA/HEMA solutions (10/90, 20/80, and 30/70, mol/mol) were polymerized and cross-linked in the presence of sacrificial PMMA microspheres to render highly porous hydrogels with uniform interconnected pores.	Scaffold (8 mm x 2 mm) implanted subcutaneously in 8–12 weeks old Wistar rats. The incisions were sutured and then covered with triple antibiotic ointment.	DMAEMA/HEMA 30/70 scaffolds promoted the highest level of cell infiltration, showing mitogenic, angiogenic, and remodeling capabilities. Treated wounds showed significant increase in granulation tissue while inducing cytokine inflammatory profile and tissue remodelling.	58
2	Dimethylaminoethyl methacrylate (DMAEMA)	DMAEMA:HA 1000:1 hydrogels were prepared by free radical polymerization	Diabetic female Albino Wistar rats full-thickness excision wound model (2.5 cm).	DMAEMA/HA scaffolds before and after being loaded with a herbal extract of <i>Didymocarpus pedicellatus</i> promoted re-epithelization, granulation and growth of blood vessels.	59
3	Acrylic acid (AAc)	NIPAM (0.42 g) and AAc (0.08 g) were polymerized and cross-linked to obtain pH and temperature-responsive hydrogels with pore size between 8.24 and 8.36 μm . Water uptake was 190% of the dried weight at pH 4 and 800% at pH 10. Silver nanoparticles (AgNPs) were loaded by soaking.	BALB/c mice with a full-thickness excisional wound on the back. The wound was inoculated with <i>S. aureus</i> . The mice were randomly divided in three groups to be treated daily with the hydrogel without AgNPs, the hydrogel with AgNPs, and a silver sulfadiazine cream, for 9 days.	The hydrogel with AgNPs consistently reduced the number of bacteria in the wounds and promoted faster wound closure, higher rate of wound reepithelialization and increased collagen deposition.	63
4	Methacrylic acid (MAC)	Composites of MAC, carboxymethyl cellulose (CMC) and dopamine and reinforced with cellulose nanofibrils obtained by free radical polymerization.	Deep second-degree burn wound model. The groups of animals were treated with the blank hydrogels, CNF loaded hydrogels and CNF/levofloxacin loaded hydrogels or non-treated as a negative control.	Efficient recovery when treated with levofloxacin/CNF@H-2 hydrogel.	64

compared to 26% control group). Impregnation with the herbal extract of *Didymocarpus pedicellatus* favored even more the healing (72% wound closure) (Table 2, entry 2) [59].

2.5.2. Acrylic acid (AAc)

Acrylic acid (AAc) is widely used as a comonomer in a variety of medical devices owing to the capability of its polymers to swell at pH above its pK_a , which is close to 4.25 [60]. Hydrogels made of AAc moieties rapidly release neutral and anionic drugs. Differently, cationic drugs that interact ionically with the AAc moieties may notably alter the swelling profile and also cause unexpected drug release profiles [61]. The combination of pH- and temperature-sensitive monomers allows obtaining hydrogels that show pH-triggered drug release but that avoid burst delivery, providing sustained release kinetics. These dually sensitive hydrogels can be prepared by combining AAc with temperature-responsive monomers, such as NIPAM, at adequate monomer ratios to still obtain the phase transitions at the right temperature interval [62].

Relevantly for wound healing, cross-linked hydrogels prepared with NIPAM (0.42 g) and AAc (0.08 g) showed excellent capability to load silver nanoparticles (AgNPs) (Table 2, entry 3) [63]. The hydrogels shrank at pH below 5.5 and strongly swelled at pH above 7.4. Similarly, the release of AgNPs was minimal at acidic pH and almost complete in 24 h in a medium of pH 7.4 that mimicked the scenario of an infected wound. The hydrogels were challenged against a murine wound infection model in which *S. aureus* was inoculate one day before starting the treatment. Compared to blank hydrogels and a common silver sulfadiazine cream, hydrogels loaded with AgNPs notably reduced the number of bacteria since day 2 and the reduction continued during posterior days. This treatment facilitated epidermal tissue migration, with faster wound closure and denser collagen I deposition at day 9.

2.5.3. Methacrylic acid (MAC)

Methacrylic acid (MAC) performs similarly to AAc and has been used as a comonomer to confer pH-sensitiveness to a variety of scaffolds. Composites made of MAC, carboxymethyl cellulose (CMC) and dopamine and reinforced with cellulose nanofibrils were tested for wound dressing (Table 2, entry 4). The amounts of MAC and cellulose nanofibrils were varied to tune the swelling and the mechanical properties. Levofloxacin was also incorporated into the hydrogels. *In vivo* evaluation was carried out in a deep second-degree burn wound model [64]. The groups of animals were treated with the blank hydrogels, CNF-hydrogels and levofloxacin-loaded CNF-hydrogels or left untreated. Wound closure ratios were higher after 7 days treatment with the hydrogels containing CNF and levofloxacin, compared to the hydrogels without the drug. Also, inflammatory markers (IL-1 β) were lower in CNF-hydrogels and levofloxacin-loaded CNF-hydrogels. The wounds were not intended to be infected and therefore the reason behind the favored healing by levofloxacin presence was not unveiled.

2.6. Clays

Many natural clays have been claimed to have antimicrobial properties and, indeed, some of them have shown outstanding capabilities to kill *in vitro* a broad spectrum of microorganisms commonly present in wound infections [65]. Clays contain a wide variety of cationic ions (mainly Al³⁺ and Fe²⁺), but the overall surface charge is usually negative. Nevertheless, they can efficiently adsorb bacteria (also with a negatively charged surface). The repulsion/attraction balance depends on the pH of the medium and the production of siderophores and other chelators by bacteria. In aqueous medium, some ions are released and became oxidized, generating reactive oxygen species (ROS). In parallel, Al³⁺ can bind to the membrane phospholipids, causing poration, which facilitates the penetration and accumulation of Fe²⁺ into the bacteria. There-

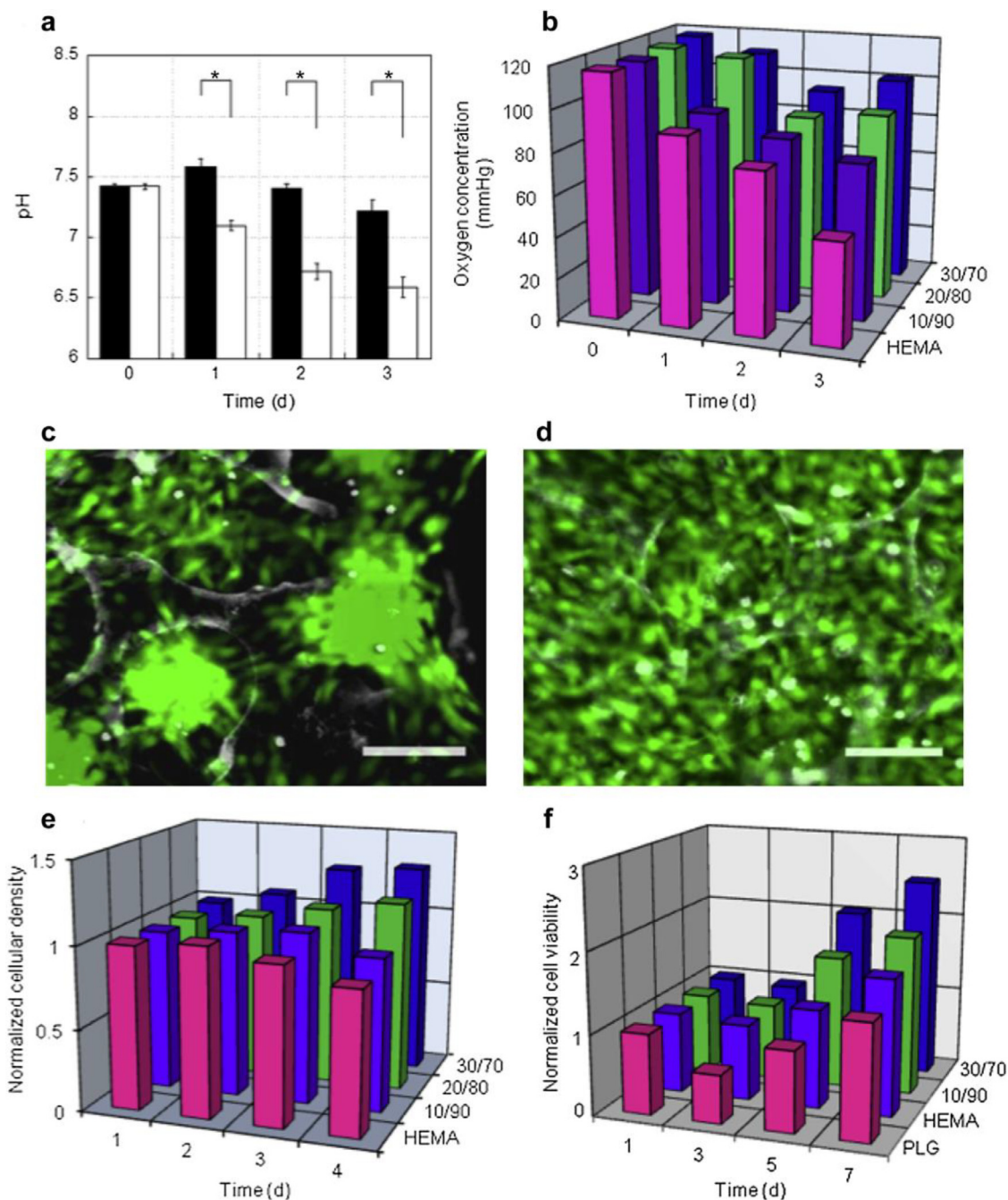


Fig. 3. (a) Changes in pH of the growth medium when DMAEMA/HEMA 30/70 mol/mol scaffolds were seeded with NIH/3T3 cells (black bars: 10^6 cells/scaffold; white bars: 10^7 cells/scaffold); (b) oxygen concentration inside cell-cultured HEMA and DMAEMA/HEMA 10/90, 20/80 and 30/70 mol/mol scaffolds; (c) Live/Dead assay of NIH/3T3 cells seeded on HEMA for 72 h; (d) Live/Dead assay of NIH/3T3 cells seeded on DMAEMA/HEMA 30/70 mol/mol scaffold for 72 h; (e) normalized cell density as a function of time; and (f) normalized cell viability under hypoxic conditions for scaffolds seeded with 10^7 NIH/3T3 cells. Reprinted from You et al. [58] with permission from Elsevier.

fore, ROS level inside the bacteria increases causing DNA damage [66,67]. Despite the excellent antibacterial and antibiofilm activity *in vitro*, most natural clays fail to efficiently kill microorganisms *in vivo* when tested as suspensions on infected wounds [68]. Among other possible reasons, insufficient release of ions and partial inactivation by the wound compounds may explain this failure. Maintenance of a wet environment around the clays and replacement of interlayer cations by more cationically charged compounds may enhance their capability to kill microorganisms. In addition to the antimicrobial properties, several other features of clays may facilitate wound healing. The released ions may promote angiogenesis as recently reported for electrospun scaffolds of

poly-ε-caprolactone (PCL) and vermiculite tested *in vivo* in diabetic wounds [69].

Regarding pH-responsive scaffolds, clays can efficiently modulate the release profiles of active substances that adsorb on their surface. Polyurethane foams prepared with alginate:bentonite hydrogels (10:0, 7:3, 5:5, and 3/7 w/w) have shown faster release of a model protein at pH 8.2 compared to 4.2 because of disentanglement of alginate chains, but the presence of bentonite attenuated the burst release observed in the first 10 h [70]. Relevantly, some clays, such as hectorite, can form stable hydrogels by themselves, which can serve as wound dressings without the need of adjuvant polymers. Hectorite hydrogels containing halloysite nan-

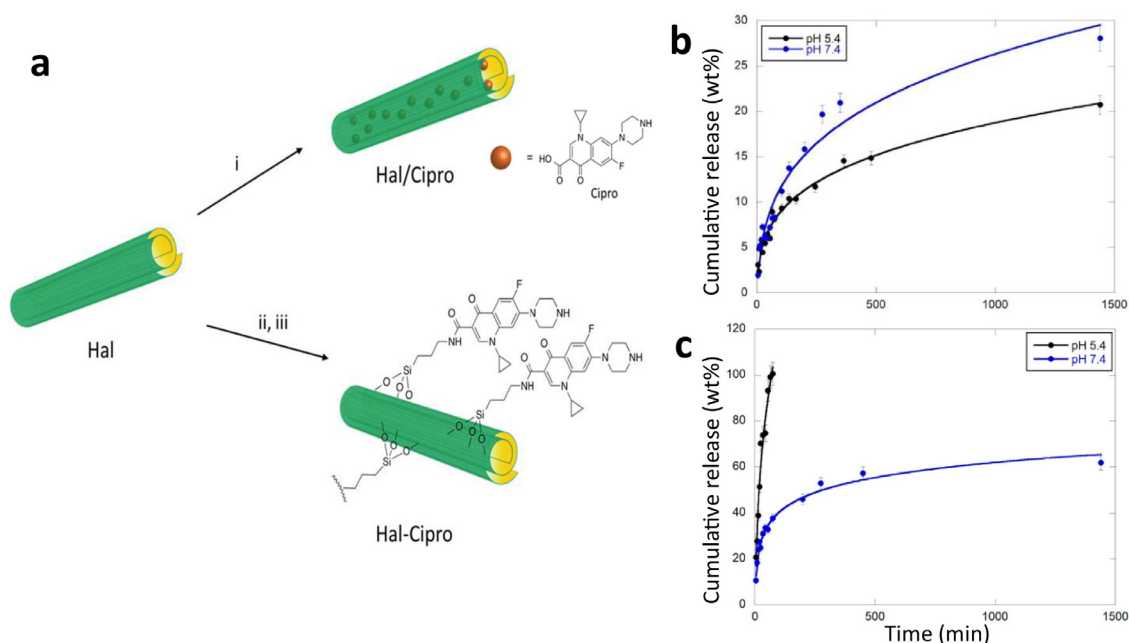


Fig. 4. (a) Halloysite nanotubes were loaded with ciprofloxacin by (i) soaking in a drug solution in methanol; or (ii, iii) covalent immobilization through amide bonds. (b) Clay hydrogels prepared through route (i) released ciprofloxacin faster at pH 7.4 due to a weakening of hydrogen bonds and ionic interactions. (c) Clay hydrogels prepared through route (ii-iii) showed burst release at pH 5.4 as amide bonds were rapidly broken. Adapted from Massaro et al. [71] with permission from Elsevier.

otubes loaded with ciprofloxacin showed strongly pH-dependent release profiles (Fig. 4). Ciprofloxacin was readily adsorbed on the inner (mainly) surface of the clay nanotubes through hydrogen bonding and ionic interactions (Fig. 4ai); such interactions were stronger at acid pH, which explained faster ciprofloxacin release at pH 7.4 compared to pH 5.4 (Fig. 4b). As an alternative, ciprofloxacin was also loaded onto the clays by means of pH-sensitive covalent bonds, while some non-covalently bound ciprofloxacin molecules contributed to generate supramolecular assemblies with the covalently attached drug molecules (Fig. 4 aii, iii). In this case, ciprofloxacin release occurred faster at pH 5.4 as the protonated drug rapidly disassembled while the pH-sensitive amide bonds also broke (Fig. 4c). Hectorite hydrogels with the ciprofloxacin-adsorbed halloysite nanotubes were more cytocompatible than the free drug when tested on human fibroblast cell lines [71]. Although the relevance of the pH-responsiveness of clays has yet to be demonstrated *in vivo*, the results already obtained allow envision promising performances for regenerative medicine.

3. Reversible cross-linkers

A variety of reversible cross-linking strategies have been explored to endow scaffolds with pH-responsiveness suitable for *in vivo* tissue healing (Table 3). The pH-sensitiveness results from a reversible linkage which is designed to be stable at specific pH values and disrupts when the pH of the environment becomes acidic or alkaline. In addition to the Schiff base bonds, many active ingredients have even been demonstrated potentially useful for this purpose acting as “green” reagents.

3.1. Schiff base bonds

Chitosan quaternary ammonium salt (QCS) was used to strengthen the antimicrobial capability of chitosan and to design a dual-crosslinked mussel-inspired hydrogel (Table 3, entry 1) [72]. Mixing QCS solution with oxidized dextran and dopamine resulted in double Schiff base bonds (Fig. 5a). The hydrogels had a homogeneously porous network structure with pore size ranging from 20

μm to 50 μm . The swelling ratio at pH 7.4 was around 500%, but the hydrogel was destroyed at pH 5.0 due to the dissociation of the bonds. The Schiff base bond made the hydrogels able to release silver nanoparticles (AgNPs) and deferoxamine (DFO), which were loaded during preparation, in a sustained pH-responsive behaviour. This was confirmed *in vitro* with a burst release (83.9% of AgNPs and 90% DFO) at pH 5.0. The *in vitro* synergistic bactericidal effect of hydrogel@AgNP&DFO was evidenced against *S. aureus* and *E. coli*. The *in vivo* studies on diabetic male Sprague Dawley rats for 14 days confirmed the effectiveness of hydrogel@AgNP&DFO with faster closure (79% on day 7) and enhanced angiogenesis compared with the hydrogel solely. This composite hydrogel also resulted in almost complete re-epithelialization with accelerated collagen deposition reaching 60% on day 14, while only 25% in the control hydrogel group. The *S. aureus*-infected rat model confirmed the *in vivo* antibacterial performance showing the least number of bacterial colonies in the wounds treated with the composite hydrogel [72].

QCS was also used to prepare syringeable reversible gels through Schiff base bonds with benzaldehyde-modified Pluronic F127 (PF) micelles encapsulating curcumin (Table 3 entry 2) [73]. Various QCS/PF hydrogels were tested using different polymer ratios and total content in PF to regulate the hydrogel pore size (20–55 μm) and to obtain mechanical properties comparable to the human skin. The *in vitro* pH-responsive release behavior of the encapsulated curcumin (CUR) was investigated likewise the *in vitro* antibacterial efficacy and cell biocompatibility. The drug release was found higher at the most acidic pH 6.0 with a CUR release of 78% at day 12, while at pH 6.8 and pH 7.4 the percentage released was 61% and 28%, respectively. The antibacterial activity of unloaded hydrogels exhibited a great killing ratio of 90% for *S. aureus* and *E. coli* when in contact for 2 h at 37 °C. The hydrogel with a QCS/PF ratio of 2/18 (%) exhibited good biocompatibility, displaying a number of cells comparable with the control. *In vivo* wound healing was tested in a full-thickness skin defect model on Female-Kunming mice (5–6 weeks old) treated with Tegaderm™ (control), QCS-PF hydrogel and QCS-PF-CUR hydrogel. Wound closure was higher for the different hydrogel groups than the con-

Table 3*In vivo* testing of pH-responsive scaffolds made of reversible cross-linkers.

No.	pH-responsive component	Scaffold features	<i>In vivo</i> model	Main outcomes	Ref.
1	Schiff base bonds (chitosan quaternary ammonium salt)	Reversible Schiff base bonds between chitosan and oxidized dextran conjugated with dopamine. Pore size ranging from 20 μ m to 50 μ m. High swelling rate at pH 7.4, and disintegration at pH 5.0.	Diabetic male Sprague Dawley. The 1 cm diameter wounds were made using a skin needle biopsy punch and were infected with <i>S. aureus</i> .	The wounds treated with Hydrogel@AgNP&DFO resulted in faster wound closure, complete re-epithelialization and enhanced collagen deposition compared to control hydrogels. Fast and effective antibacterial activity against <i>S. aureus</i> .	72
2	Schiff base bonds (chitosan quaternary ammonium salt)	Dynamic Schiff bond between chitosan and Pluronic F127 micelles loaded with curcumin to create syringeable reversible gels.	Full-thickness skin defect model (1 cm diameter) Female Kunming mice (30–40 g, 5–6 weeks old). Three groups, control with Tegaderm™, QCS-PF and QCS-PF-CUR	Higher wound closure rates for the hydrogels, which showed less inflammatory cells and more fibroblasts. The CUR-loaded hydrogels exhibited more blood vessels, hair follicles and thickened epidermis.	73
3	Schiff base bonds (chitosan quaternary ammonium salt)	The hydrogels were prepared with reversible Schiff base bonds between chitosan functionalized with catechols (CQCS) and the aldehyde groups in dibenzaldehyde-PEG (DBPEG).	Hemostatic ability was tested in Sprague-Dawley rats (male) and wound closure of infected full-thickness skin defect model (8 mm in diameter) in ICR mice (female). The wounds were infected with MRSA, control group was treated with Tegaderm™ and the experimental group with CQCS-DBPEG gel	Wounds treated with the gel showed no bacterial signal after 6 days, while the control group was still infected, and hair follicles at the wound site (none in the control). The gel-treated wounds also exhibited less inflammatory infiltration and higher vascularization, and the skin regenerated was better.	74
4	Schiff base bonds (N-carboxyethyl chitosan (CEC) and benzaldehyde-terminated Pluronic F127/CNT)	Reversible hydrogels obtained by Schiff base bonds and physical cross-linking of the micelles. CNTs communicated photothermal antimicrobial activity.	The hydrogels were tested on a mouse liver trauma model regarding haemostatic capability and on full-thickness skin wound-infected Kunming female mice. Wounds infected with <i>S. aureus</i> were treated with dressings with and without CNT and exposed or not to NIR (808 nm; 10 min).	The wounds covered with Mox-CEC/PF/CNT2 and CEC/PF/CNT2 hydrogels groups showed complete closure after 14 days with higher collagen deposition compared with the ones covered with Tegaderm™ and CEC/PF/CNT0 hydrogel. NIR photothermal treatment allowed eradication of the infection.	75
5	Schiff base bonds (Oxidized hyaluronic acid, OHA)	Syringeable hydrogels were prepared with OHA, poly- ϵ -L-lysine and Pluronic F127, and loaded with adipose mesenchymal stem cells (AMSCs)-derived exosomes.	Full-thickness diabetic wound model (male ICR mice). 8-mm wounds were treated with saline (control), the hydrogel without exosomes, the free exosomes, and the hydrogel containing exosomes for 21 days.	The wounds treated with hydrogels loaded with exosomes showed superior healing ability and wound closure compared with the control group. The treated wounds had more granulation tissue and collagen fibers due to the sustained release of exosomes.	77
6	Schiff base bonds (Oxidized hyaluronic acid, OHA)	Reversible Schiff base formation between OHA and antimicrobial peptide.	Full-thickness wound model in BALB/c female mice (7 mm) were infected with <i>S. aureus</i> and then untreated (control) or treated with the antimicrobial peptide as solution or as hydrogel.	The wounds treated with the hydrogels resulted in the best closure rate while shortening the inflammation period. They also exhibited neovascularization in the wound site and collagen formation while regenerating new hair follicles.	78
7	Covalent organic frameworks (COFs) with Schiff base bonds	COFs made of dimethoxyterephthaldehyde (DMTA) and 1,3,5-tris(4-aminophenyl) benzene (TAPB) were loaded with curcumin through Schiff base bonds and then electrospun with poly(ϵ -caprolactone).	Male Sprague-Dawley rats with a full-thickness wound defect model. Rats were divided in 3 groups: Tegaderm™ (control), PCL (blank) and PCL with the CUR-COF.	PCL with the CUR-COF displayed the largest wound contraction area (51%) compared with the control (33%) or the PCL (45%) at day 7. The CUR group showed less inflammatory cells, and more proliferation of fibroblasts cells and growth of hair follicles and sebaceous glands.	80
8	Tannic acid	Highly porous cylindrical hydrogels (6 mm diameter) were prepared via hydrogen bonding between gelatin (10 mL; 10% w/v) and tannic acid (1.2 mL; 0.3–0.6 g/mL). The hydrogels were freeze-dried. Some hydrogels were previously loaded with allantoin.	Hemostatic activity was evaluated in rat tail-amputation model. Full thickness skin wound model in male Sprague-Dawley rats with two full-thickness on either side of the dorsal region with a diameter of 2.0 cm. Wounds were treated with the hydrogels with and without allantoin for 14 days. Non-treated rats were used as control.	Both hydrogels showed excellent wound healing results, with less granulation tissues and less inflammation at the wound site than the control. Combination of tannic acid with allantoin provided synergistic effects on stimulation of skin regrowth while reducing pain and itchiness.	82

(continued on next page)

Table 3 (continued)

No.	pH-responsive component	Scaffold features	<i>In vivo</i> model	Main outcomes	Ref.
9	Tobramycin	Hydrogels formed by imine bonds between dialdehyde-modified carboxymethyl cellulose (DCMC) and tobramycin (7:3 mass ratio). Uniform pore size between 1 and 10 μm ; swelling ratio of 1910%. Some hydrogels also contained borneol forming inclusion complexes with a cationic β -cyclodextrin derivative.	The hydrogels with and without borneol were tested on Kunming mice for 7 days. Non-treated wounds and wounds treated with the borneol inclusion complex were used as controls.	The hydrogels with and without borneol reduced faster the wound area and provided effective anti-inflammatory properties.	83
10	Gentamicin	Chemically cross-linked hydrogels of HEMA and AAC with dynamic ionic cross-linking of gentamicin.	Male BALB/c mice with <i>S. aureus</i> -infected full-thickness wounds created on the back (1 $\times$ 1 cm dimensions).	The hydrogels resulted in better wound healing with decreased infection compared with the commercial dressing and the control group.	84
11	Vancomycin	Vancomycin was covalently and ionically bound to specific demineralized bone extracellular matrix (SDECM).	Male Sprague Dawley (SD) rats for 6 weeks. The bone defect was created by a midline incision exposing the parietal bone. The defects were infected with MRSA suspension, and then filled with the SDECM with or without vancomycin, covered with collagen plugs infected with MRSA, and sutured. Controls were treated with collagen plug without SDECM.	Bone implanted with vancomycin-loaded SDECM showed pH-responsive sustained release of vancomycin while promoting osteogenesis and immunoregulation effects.	85
12	Borax	Cellulose nanofibrils (CNF) were coated with tannic acid (TA) and loaded with AgNPs (Ag-TA@CNF) and then dispersed in SA and PVA medium. Borax was used as cross-linker. Hydrogels exhibited high and uniform porous structure with interconnectivity, and bioadhesive properties. <i>In vitro</i> drug release evidenced the pH-dependence.	Female Kunming mice with a bacterial infected wound (6 mm in diameter) in the back. Wounds were treated with hydrogels and fixed with Tegaderm TM . The control group was only covered with Tegaderm TM and remained untreated.	<i>In vivo</i> experiments evidenced accelerated wound healing in comparison with the control, in terms of denser collagen deposition, more activated fibroblast cells and fewer inflammatory cells after the 14 days treatment.	86
13	Borax	Cylindrical hydrogels made by cross-linking of PVA with borax and including resveratrol-polyethylene glycol-cellulose nanofibrils.	Female Kunming mice with partial thickness wounds (6 mm diameter) on the dorsum. The wounds were covered with <i>S. aureus</i> -containing PVA-borax hydrogel. Then, hydrogels with and without resveratrol-polyethylene glycol-cellulose nanofibrils were applied on top and secured with Tegaderm TM . The dressings were replaced every 3 days.	The hydrogels with resveratrol accelerated the closure and the healing of the wounds with no visible open cuts and new epidermal tissue appeared after 12 days treatment.	87
14	3-Acrylamidophenylboronic acid (AMB)	Scaffolds prepared with chitosan, AA, hydroxyethyl methacrylate, disulfide based dimethacrylate (DSDMA), 3-acrylamidophenylboronic acid (AMB), and nano-hydroxyapatite. AMB formed boronate bonds with DFO.	Rats with anterior cruciate ligament injury reconstruction were treated with hydrogels w/o boronate esters and w/o DFO. The scaffold (thin strips) was wrapped in the tendon graft.	Treatment with hydrogels combining boronate esters with DFO notably enhanced the expression of HIF-1 α and VEGF at the interface between tendon grafts and bone and favored bone healing and fusion of tendon grafts with the bone interface.	88
15	3-Aminophenylboronic acid	HA grafted with phenylboronic acid and mixed with polyvinyl alcohol (PVA) and containing polydopamine and bFGF-loaded microparticles.	Rabbits with chronic and acute tendon injuries. Treatments were: suture of the incision (control group); wrapping/filling with hydrogel without bFGF; and wrapping/filling with hydrogel with bFGF.	After 42 days of healing, the group treated with bFGF-loaded hydrogels showed higher tendon tensile strength, lower content in inflammatory cells, and higher collagen deposition than the other groups.	89
16	Catechol-metal ion (catechol-Fe)	The hydrogels (QCS-PA@Fe) had porous network structure with pore diameter between 179 μm and 69 μm . The swelling ratio also varied from 267% in the hydrogels containing the highest PA@Fe to 174% in the hydrogels with the lowest PA@Fe.	MRAS was subcutaneously injected (Kunming mice) in four different places and 24 h later the hydrogels were injected into the abscess. A 808 nm laser was applied for 10 min on the injection area of some abscess. Non-treated abscesses were used as controls. The hydrogels were also tested on 2 cm full-thickness skin incisions on the back of the rats and in 7 mm full-thickness wounds infected with <i>S. aureus</i> . Some treatments included NIR irradiation for 10 min.	Plate count of abscesses after treatment revealed a remarkable decrease in cfu only in the group treated with the hydrogel and subsequent NIR irradiation. The hydrogels favored wound closure with denser collagen deposition. MRSA-infected wounds treated with the hydrogels combined with NIR application showed less inflammatory infiltration and accelerated healing.	90

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Table 3 (continued)

No.	pH-responsive component	Scaffold features	In vivo model	Main outcomes	Ref.
17	Amino-yne click reaction	Reversible hydrogels were made with adipic dihydrazide-modified hyaluronic acid (HA) and dipropiolate ester of polyethylene glycol through enamine bonds. Some hydrogels were also loaded with tannic acid, deferoxamine and AgNPs.	<i>S. aureus</i> -infected wounds (1.5 cm) on SD rats. Treatments involved the hydrogel, the hydrogel with AgNPs, the hydrogel with deferoxamine, and the hydrogel with both components.	pH-sensitive hydrogels loaded with AgNPs and PHMD showed the best wound healing performance: highest closure rate, enhanced IL-10 and VEGF production, and decreased TNF- α .	91

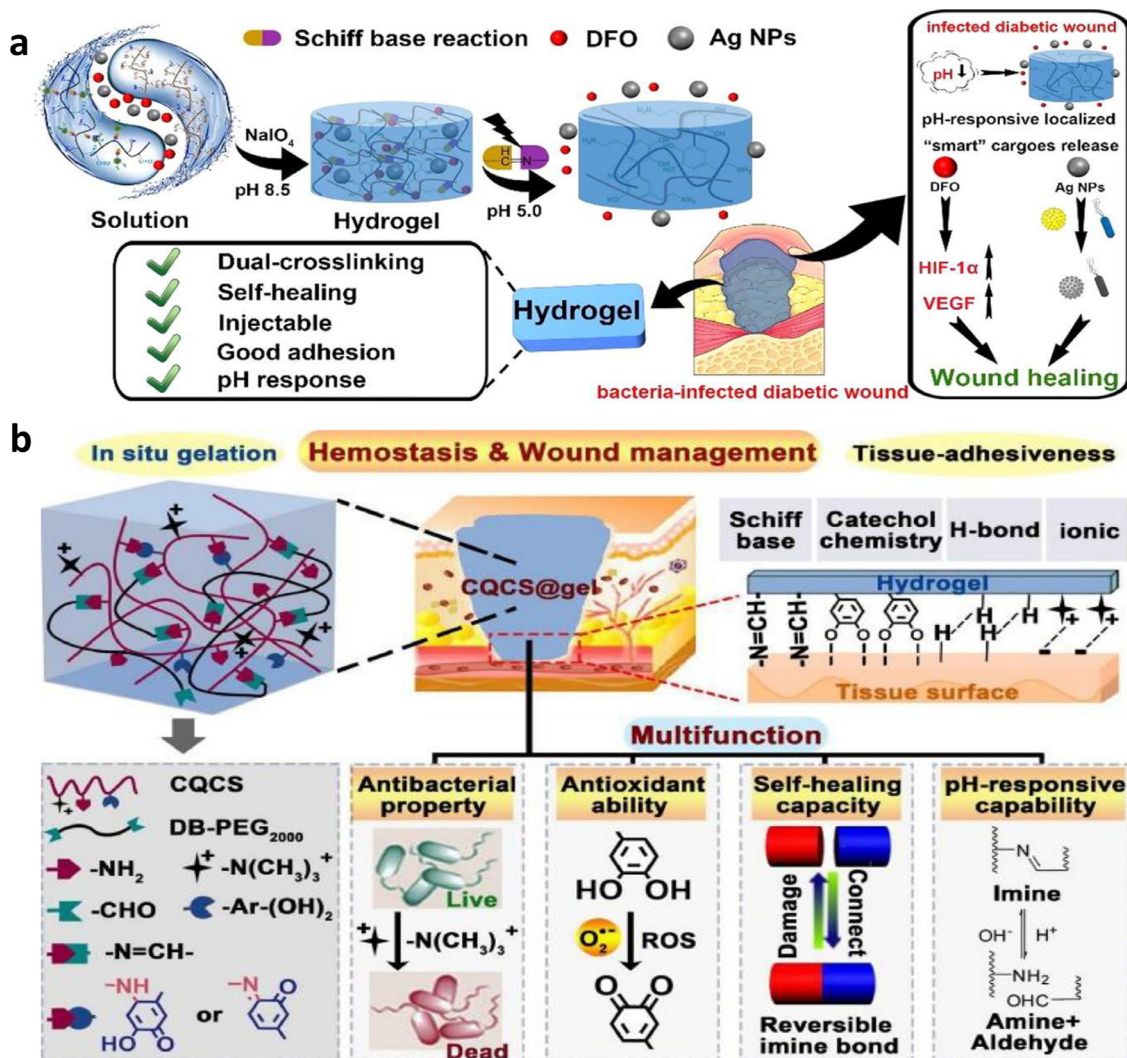


Fig. 5. (a) Double cross-linked hydrogels were prepared by Schiff base reactions between dopamine and oxidized dextran and subsequent interaction with quaternized chitosan, with additional formation of catechol-catechol adducts through dopamine. The hydrogels released pro-angiogenic drug deferoxamine (DFO) and AgNPs when pH decreased to pH acid favoring the healing of bacteria-infected diabetic wounds. Reprinted from Hu et al. [72] with permission from Elsevier. (b) Reversible hydrogels prepared with quaternized chitosan functionalized with catechols (CQCS) and dibenzaldehyde-PEG (DBPEG) (Schiff base reaction) showed strong adherence to the wound and developed antibacterial and antioxidant performances in infected wounds. Reprinted from Fang et al. [74] with permission from Elsevier.

tol. Mice wounds treated with QCS-PF-CUR had more blood vessels and hair follicles, thickened epidermis, and enhanced proliferation of fibroblasts. The curcumin-loaded hydrogels also exhibited the least expression of TNF- α (inflammation) with the highest expression of VEGF (angiogenesis).

The use of quaternary chitosan to create pH-sensitive hydrogels was also investigated through a Schiff base reaction between QCS functionalized with catechols (CQCS) and the aldehyde groups in dibenzaldehyde-PEG (DBPEG) (Table 3, entry 3) [74]. As expected, the hydrogels released faster the loaded drug (methylene blue) at acidic pH. The hydrogels showed *in vitro* cytocompatibility,

adhesive properties and antibacterial activity, and *in vivo* hemostatic ability (rats) (Fig. 5b). ICR female mice with a full-thickness skin wound (8 mm diameter) on their back were used for wound closure experiments. The wounds were infected with methicillin-resistant *S. aureus* (MRSA) and after 24 h covered with TegadermTM (control) or treated with the CQCS-DBPEG gel. The wounds treated with the gel evidenced higher contraction, much less bacterial signal at day 6, less inflammatory infiltration and more regenerated hair follicles compared to the control. The skin regeneration performance was also assessed through Masson's trichrome staining, showing more fibroblast migration and denser and better colla-

gen deposition for the CQCS-DBPEG gel group than the control. Immunohistochemical staining also evidenced higher expression of CD31 for the gels, meaning better pro-vascularization ability.

Another nanocomposite hydrogel wound dressing was fabricated using N-carboxyethyl chitosan (CEC) and benzaldehyde-terminated Pluronic F127/carbon nanotubes (PF127/CNT) (Table 3, entry 4) [75]. Both the Schiff base bonds and the physical cross-linking of the micelles contributed to the obtaining of reversible hydrogels. The resultant CEC/PF/CNT hydrogels demonstrated adequate mechanical properties, ability to absorb large amounts of water, and suitability for photothermal therapy due to the CNT. The hydrogels were loaded with moxifloxacin (Mox) at acidic pH to facilitate the diffusion into the hydrogel, and then showed *in vitro* sustained pH-sensitive release. The sustained release resulted in zone of inhibitions lasting 8 days and 9 days for *S. aureus* and *E. coli*, respectively. The hydrogels were tested on full-thickness wound-infected female Kunming mice. After 14 days the wounds covered with Mox-CEC/PF/CNT2 and CEC/PF/CNT2 hydrogels showed complete closure with higher collagen deposition compared with the ones covered with the commercial dressing Tegaderm™ or CEC/PF/CNT0 hydrogel (without CNT) as a control. Additionally, wounds treated with CEC/PF/CNT2 hydrogels that were irradiated with NIR light (808 nm; 10 min) for photothermal therapy showed faster healing rate. After 14 days, both Mox-CEC/PF/CNT2 and NIR-irradiated CEC/PF/CNT2 hydrogels showed significantly lower bacterial survival ratio compared with the other tested groups [75].

Hyaluronic acid is another example of widely used anionic pH-responsive natural polysaccharide that plays an essential role in tissue regeneration and wound healing [76]. The reversible Schiff base bond formed between oxidized hyaluronic acid (OHA) and poly- ϵ -L-lysine (antimicrobial) while combined with Pluronic F127 (temperature-responsive) resulted in multifunctional injectable hydrogels (Table 3, entry 5) [77]. The hydrogels exhibited self-recovery ability while maintaining 3D porous morphology when freeze-dried. The hydrogels sustainedly released adipose mesenchymal stem cells (AMSCs)-derived exosomes in weak acidic pH upon the breakage of the Schiff bond. Prolonged pH-responsive release behaviour resulted in the acceleration of angiogenesis responses as confirmed both *in vitro* and *in vivo*. In full-thickness diabetic wounds, hydrogels loaded with exosomes showed synergic healing effects promoting faster re-epithelialization, formation of granulation tissues and deposition of collagen fibres in the wound gap.

The suitability of reversible hydrogels made of antimicrobial peptides to heal infected wounds was further explored in a more recent study. OHA reacted with the antimicrobial peptide (KK(SLKL)₃KK) through the formation of Schiff base bonds to form a compact network structure with homogeneously distributed pores (Table 3, entry 6) [78]. The pH-triggered cleavage of the Schiff base bond made the hydrogels to release faster the antimicrobial peptide under the pathologically acidic conditions of the infected wounds. The hydrogels exhibited effective antibacterial activity against both *E. coli* and *S. aureus* at pH 5.5 in *in vitro* tests. Similarly, the *in vivo* studies performed on the infected full-thickness wounds of a female mice model confirmed enhanced wound healing with significantly better wound closure and shortened inflammatory period in the wounds treated with the hydrogels compared with controls. They also resulted in accelerated tissue re-epithelialization, collagen formation and new hair follicles in the wound site.

Schiff base bonds have also been explored to endow pH-responsiveness to covalent organic framework (COF) scaffolds. COFs are 2D or 3D structures made of organic compounds covalently linked together to form a new class of highly porous polymeric materials [79]. Although their use in regenerative medicine is still

incipient, an example of pH-dependent release for wound healing has been reported recently. COFs made of dimethoxyterephthalaldehyde (DMTA) and 1,3,5-tris(4-aminophenyl) benzene (TAPB) were shown to load curcumin by means of Schiff base bonds [80]. The complex CUR-COF was later incorporated into poly(ϵ -caprolactone) (PCL) nanofibers to create an electrospun wound dressing (Table 3, entry 7). Blank PCL fibers with free curcumin were also prepared. The fibers increased their size along the incorporation of the COF-CUR complex from 117 to 267 nm. Curcumin release was strongly dependent on the pH and ranked 21.3, 58.9 and 74.6 % released at pH 7.4, 6.5 and 5.0, respectively, after 100 h of experiment. After confirming the antimicrobial and antioxidant ability of the nanofibers, the dressings were evaluated *in vivo* for wound healing in full-thickness defects (male Sprague-Dawley rats). The animals were divided in three groups and treated each with Tegaderm™ (control), PCL mats (blank) and PCL mats containing 10% of CUR-COF. Wound contraction rates at 7 days were, respectively, 33%, 45% and 51%. Inflammatory response was found to be less for the CUR-COF-loaded dressing at day 3. This treatment also enhanced the proliferation of fibroblasts, hair follicles and sebaceous glands, confirming the better tissue regeneration.

3.2. Tannic acid

Tannic acid is a polyhydric phenol able to function as a physical or chemical crosslinker (Fig. 6a). At supramolecular level, tannic acid is able to form hydrogen bonds [81]. An example is gelatine-tannic acid hydrogels where the interaction between the carboxyl and amine groups of the gelatine backbone and the hydroxyl groups of tannic acid results in multiple hydrogen bonds that are sensitive to changes in the pH (Table 3, entry 8) [82]. The hydrogen bonds are formed in acidic conditions (pH 5.5), when the amine groups in the gelatine backbone are protonated. However, in neutral or slightly alkaline conditions (pH 7.4) the deprotonation of gelatine and the ionization of hydroxyl groups in tannic acid weaken the bonds, and thus the surrounding medium may penetrate inside the hydrogel leading to the release of tannic acid and any drug encapsulated in the hydrogel. Gelatine-tannic acid scaffolds were prepared by freeze-drying; some were previously loaded with allantoin to enhance skin regrowth. *In vitro* tests revealed that the release of tannic acid was less of 3.9% after several days at pH 5.5, while more than 15% was released in 48 h in pH 7.4 medium followed by sustained release in the days after. Preferential release under the alkaline pH typical of chronic wound environment was seen suitable for efficient inhibition of *S. aureus* and *E. coli* growth, and also to shorten the haemostasis time. The *in vivo* tests were carried out in non-infected full-thickness wounds (rat model). Gelatine-tannic acid with and without allantoin reduced faster the wound area (31% and 53% of the initial area at day 3) than Tegaderm™ (65%) and non-treated condition (74%). Moreover, the hydrogels loaded with allantoin enhanced the growth of new blood vessels, the thickness of the epidermis layer, and the collagen formation.

3.3. Tobramycin

Tobramycin is a broad-spectrum antibiotic drug that contains multiple amino groups (Fig. 6b), which can be exploited for reversible cross-linking. Tobramycin was shown to interact with the carbonyl groups of dialdehyde-modified carboxymethyl cellulose (DCMC) to form a hydrogel based on imine bonds (Table 3, entry 9) [83]. These imine bonds break (via a hydrolysis reaction) under weak acidic conditions leading to gradual degradation of the hydrogel, which in turn accelerates tobramycin release to the wound bed. The mass ratio of DCMC to tobramycin is critical to obtain hy-

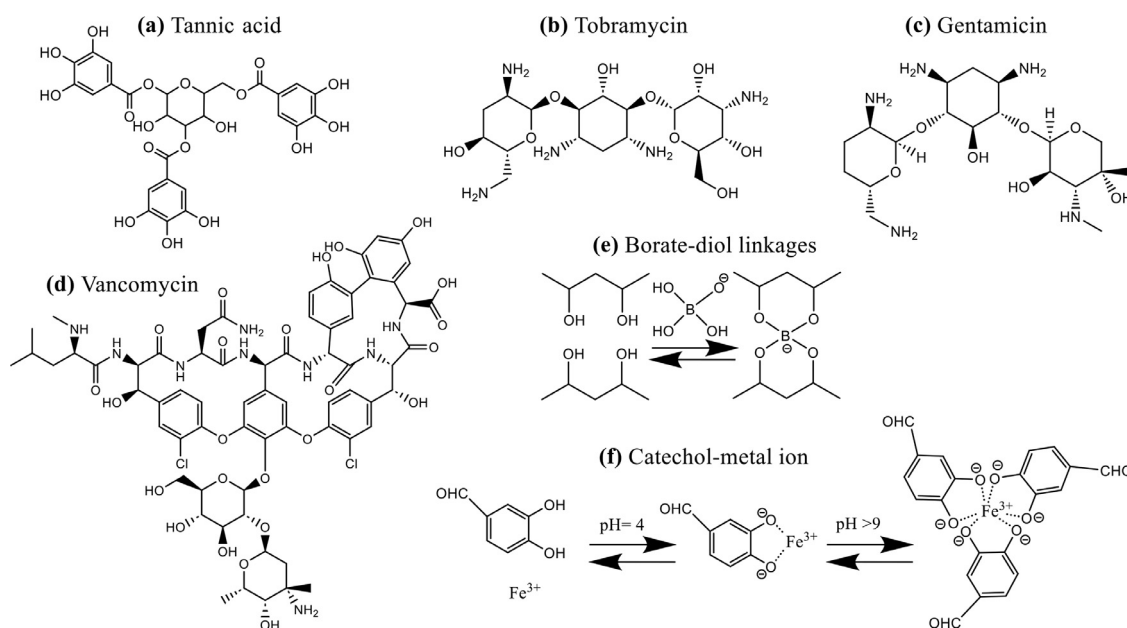


Fig. 6. (a–d) Structure of some active compounds that may perform as pH-sensitive cross-linkers, (e) mechanism of reversible linkages of borate with polyols, and (f) 3,4-dihydroxybenzaldehyde may form catechol-iron bonds and also Schiff base bonds depending on pH.

drogels with sufficient cross-linking density but still swellable and pH-responsive.

Scaffolds prepared with a DCMC:tobramycin mass ratio of 7:3 showed self-healing properties and could incorporate borneol (pain relief) forming inclusion complexes with a cationic β -cyclodextrin derivative. *In vivo* tests in skin wounds (Kunming mice) revealed that the hydrogels with and without borneol were more effective in wound closure since day 2 than the borneol complex solely. The tobramycin hydrogels acted as wound dressings with effective anti-inflammatory properties [83]. This proof-of-concept opens a window to the use of drugs bearing multiple amino groups as reversible cross-linkers that can self-regulate the release of the drug as a function of wound pH.

3.4. Gentamicin

Gentamicin containing several amino groups (Fig. 6c) was also successfully incorporated into wound dressing hydrogels as a reversible cross-linker. Hydrogels prepared by copolymerization of HEMA and AAC were loaded with gentamicin (pKa 8.2) that formed multiple point ionic interactions with the oppositely charged AAC moieties at neutral pH (Table 3, entry 10) [84]. Gentamicin-loaded hydrogels resulted in mechanically strong hydrogels that released faster the drug at pH 5.6 than at pH 7.4 (58.2% versus 12.1% in the first 48 h). The acidic environment caused the protonation of the carboxylic acid groups in AAC moieties, disrupting the interaction with gentamicin. The hydrogels were tested on a *S. aureus*-infected full-thickness wound model, after waiting for 12 h for the infection to develop. The gentamicin-loaded hydrogels showed better healing as they resulted in smaller wound area with decreased bacterial colonies on day 10, compared with the other groups (wounds treated with a commercial dressing and the non-treated wounds).

3.5. Vancomycin

Vancomycin is antimicrobial peptide also possessing multiple amino groups in its structure (Fig. 6d). In the field of bone regeneration, vancomycin was included in specific demineralized bone extracellular matrix (SDECM) scaffolds with the double aim of providing protection against osteomyelitis and also to act as an effi-

cient cross-linker that can self-regulate drug release. Powders were prepared by a combination of covalent binding of vancomycin to SDECM via carbodiimide chemistry and ionic binding of the drug with the carboxyl groups of SDECM (Table 3, entry 11) [85]. The composite exhibited faster vancomycin release at pH 5.5 ($2285.0 \pm 105.6 \mu\text{g}$) after 6 weeks compared with pH 7.4 ($686.3 \pm 43.6 \mu\text{g}$). For the *in vivo* evaluation, the powders were used to fill MRSA-infected parietal bone defects (male Sprague Dawley rats) and then covered with a MRSA-infected collagen layer before suture. The group treated with vancomycin-containing SDECM showed less bone erosion in the defected area from the first week by inhibition of osteoclast activity and excellent anti-biofilm properties compared with the groups treated with SDECM solely and the control group. After six weeks, defect bones treated with vancomycin-containing SDECM demonstrated new fully mature mineralized cancellous bone formation with 90% bone regeneration rate.

3.6. Borax

The use of borax (Fig. 6e) as a pH-reversible crosslinker has also been explored in the regenerative medicine field. The pH-sensitiveness is attributed to diol-borate (boronate) ester formation with molecules bearing alcohol groups. Boronate ester bonds are stable under pH 7.4 while a decrease in the external pH (acidic) triggers the disruption of the bond [86]. pH-sensitive hydrogels were developed preparing cellulose nanofibrils (CNF) coated with tannic acid (TA) and loaded with AgNPs (Ag-TA@CNF) for antimicrobial activity and subsequent mixing with sodium alginate and polyvinyl alcohol (PVA) (Table 3, entry 12) [86]. The hydrogel was crosslinked using borax and evidenced self-healing properties. The release of silver ions was monitored as a function of pH showing slow sustained release at pH 7.4 and a strong burst when pH drops to 4.0 because of the disruption of the borate bonds. The evaluation of the wound healing was performed using a *S. aureus*-infected wound model (female Kunming mice) (Fig. 7). The hydrogels were placed on the wound site and replaced every other day. The pH-responsive scaffold displayed superior wound healing compared to the control (non-treated) in terms of wound closure and deposition of collagen in the wound region, with more activated fibroblast cells and fewer inflammatory cells on day 14.

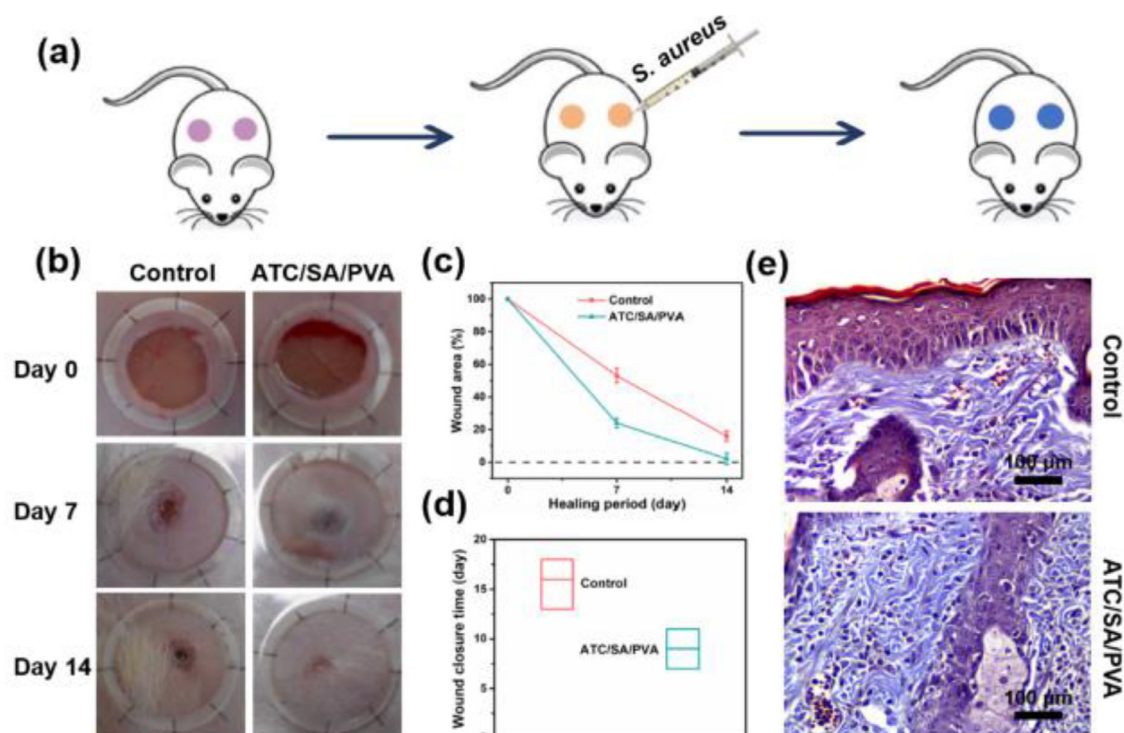


Fig. 7. *In vivo* test of borax-crosslinked Ag-TA@CNF hydrogels in a *S. aureus* infected wound model. (a) The infected wounds were treated with hydrogel and fixed with Tegaderm™ and replaced every other day; non-treated wounds (only covered with Tegaderm™) were used as controls; (b) appearance of the wounds along time; (c) changes in the wound size; (d) wound closure time; and (e) Masson's trichrome-stained tissue slices obtained on day 14. Reprinted from Liu et al. [86] with permission from Elsevier.

Borax was also used as a chemical crosslinking agent to form hydrogel networks with PVA chains reinforced with resveratrol-polyethylene glycol-cellulose nanofibrils conjugate (Table 3, entry 13) [87]. The dynamic boronate ester bonds formed allowed for faster release of resveratrol (antioxidant and antimicrobial agent) under the acidic environment of wounds compared to pH 7.4. Hydrogels containing resveratrol were demonstrated to be highly cytocompatible and effective against *S. aureus*. When tested *in vivo* on partial thickness wounds (female Kunming mice) infected with *S. aureus*, the hydrogels containing resveratrol accelerated the closure and the healing of the wounds with higher collagen deposition and hair follicles at day 12.

Tendon injuries are particularly challenging since tendon-to-bone union is a naturally complex process. Angiogenesis and osteogenesis should occur at a very well-balance rate in a hypoxic and enzyme-rich environment. To facilitate the integration of a tendon graft and bone, a 3D porous hydrogel has recently been designed by combining chitosan with an acrylic acid (AAc)-based hydrogel cross-linked with a disulfide-based dimethacrylate. Some hydrogels also contained 3-acrylamidophenylboronic acid (AMB) to reinforce the hydrogen bonds among the components (Table 3, entry 14). All hydrogels also contained nano-hydroxyapatite [88]. AAc and 3-acrylamidophenylboronic acid facilitated the loading of DFO by means of ionic interactions and boronate ester formation, which weakened under acidic conditions triggering DFO release. *In vitro* studies carried out with a model protein evidenced that the release from the hydrogels was faster at acidic pH and that the release rate increased even more in an environment rich in ROS or in DTT as both biomarkers broken the disulfide bonds of the cross-linker and caused the disentanglement of the hydrogel. In *in vivo* studies in an animal model with anterior cruciate ligament injury reconstruction (rat), treatment with hydrogels combining boronate esters with DFO and disulfide-based cross-linker notably enhanced

tendon-to-bone interface by promoting new bone formation and fusion of tendon grafts with the bone interface. Relevantly, compared to the same hydrogels without DFO or to hydrogels without 3-acrylamidophenylboronic acid, the hydrogels combining boronate esters and DFO enhanced the expression of HIF-1 α and VEGF and regulated the polarization of macrophages to M2 macrophages in the wound in the first few weeks of treatment.

Hyaluronic acid grafted with phenylboronic acid was shown to reversibly react with polyvinyl alcohol (PVA) to form scaffolds that also encapsulate polydopamine and gelatin microspheres loaded with basic fibroblast growth factor (bFGF) (Table 3, entry 15). The hydrogels were highly adhesive to the tendon providing physical support while slowly released bFGF. These hydrogels were successfully challenged against both acute and chronic tendon injuries revealing strong capability to attenuate inflammation and to enhance collagen I production [89]. Nevertheless, the pH-responsiveness was not demonstrated experimentally.

3.7. Metal Ion (Catechol-metal ion)

Divalent and trivalent cationic ions can readily form pH-responsive coordinate bonds with catechols (Fig. 6f). Combination of catechol-Fe bonds and Schiff base bonds has been explored to obtain syringeable scaffolds that form mechanically stable hydrogels at neutral pH and can rapidly disassemble under acidic conditions (Table 3, entry 16). Chitosan quaternary ammonium salt was combined with a protocatecholaldehyde that can simultaneously form coordinate bonds with Fe and Schiff base bonds with the modified chitosan. These dual-dynamic cross-linking bonds endowed the hydrogel with a remarkable pH-sensitive release behaviour. The pore diameter ranged from 179 μm to 69 μm depending on the molar ratio of amino group and aldehyde group. The hydrogels demonstrated antibacterial activity *in vitro* against

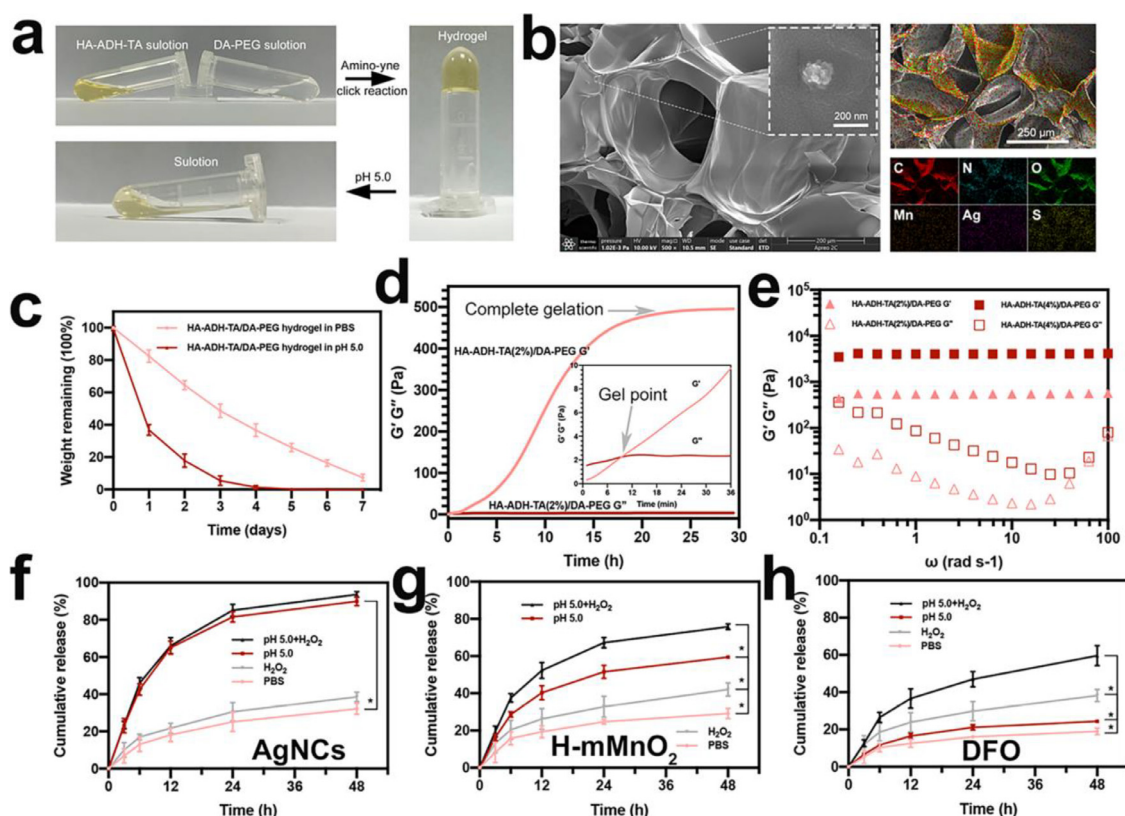


Fig. 8. (a) pH-Reversible hydrogels were prepared by mixing adipic dihydrazide-modified hyaluronic acid bearing tannic acid (HA-ADH-TA) with dipropiolate ester of polyethylene glycol at pH 7.4; the enamine bonds were reversible and dissociated in acidic environments; (b) SEM image of the hydrogel and its EDS elemental mapping, the inset view showed the encapsulated hollow mesoporous manganese dioxide (H-mMnO₂); (c) pH-dependent erosion of the hydrogels; (d) storage (G') and loss (G'') moduli during hydrogel formation, and (e) once formed at 37 °C under a strain of 1%; and (f, g, h) release profiles of silver nanoparticles (AgNCs), H-mMnO₂ and deferrioxamine (DFO) from a hydrogel loaded with all components; the legend indicates the different medium conditions. Reprinted from Deng et al. [91] with permission from Elsevier.

S. aureus and *E. coli* due to a synergic effect of chitosan quaternary ammonium groups and the catechol complex. Also, the catechol complex readily absorbed near infrared (NIR) light causing a local increase in temperature of 10 to 16 °C, which also contributed to bacteria eradication in an *in vivo* model of subcutaneous infection [90].

The wound healing capability was verified in non-infected and infected full-thickness wounds (Kunming mice). In all cases, the syringeable hydrogels showed excellent adhesion to the damaged skin. In non-infected wounds the hydrogels accelerated the healing without scar formation. In MRSA-infected wounds, the hydrogels disregarding NIR application favoured wound closure compared to non-treated wounds and wounds treated with chitosan quaternary ammonium salt. Nevertheless, the advantages of 10-min NIR irradiation were evident in terms of less inflammatory infiltration and accelerated healing due to a better control of the infection [90].

3.8. Amino-yne click reaction

pH sensitive hydrogels were created through amino-yne click reaction between adipic dihydrazide-modified hyaluronic acid (HA-ADH) and the alkyne groups of dipropiolate ester of polyethylene glycol (Table 3, entry 17) [91]. Some adipic dihydrazide groups in HA-ADH were further used to covalently attach tannic acid (HA-ADH-TA). The enamine bonds were reversible and dissociated in acidic environments such as the acidified wound sites (Fig. 8). The hydrogels showed good syringeability and tunable rheological properties depending on the content in the modified HA. The hydrogels were finally loaded with AgNPs (antimicrobial agent) and hollow

mesoporous manganese dioxide particles (H-mMnO₂; ROS scavenging and O₂ generator) used as a carrier of DFO (angiogenic drug). *In vitro* experiments evaluated the drug release in different environments at pH 7.4, pH 5.0, H₂O₂, and pH 5.0+H₂O₂. Consistently, the cumulative release of any of the compounds loaded was faster at pH 5.0, specially in the presence of H₂O₂ (Fig. 8f–h). While the acidic pH destroyed the cross-linking points of the polymer chains, the H₂O₂ degraded the H-mMnO₂ further accelerating the release of DFO. *In vivo* wound healing tests were performed in a diabetic rat model and the wounds were treated with the hydrogel solely, loaded with one component (AgNPs or PDO-H-mMnO₂) or all components (AgNPs plus PDO-H-mMnO₂). All hydrogels performed better than the non-treated control. The hydrogels combining all components showed the highest wound closure at day 7. AgNPs conferred antimicrobial properties while PDO-H-mMnO₂ provided antioxidant and angiogenic properties. The regeneration of the skin was also better for this hydrogel with more fibroblast, higher number of blood vessels. The collagen fibers were also thicker, denser and more regularly arranged.

4. Coatings

Material coating is another strategy discussed for pH-responsive materials. Polydopamine (pDA) is a common source of material coating for pH-responsive purposes. Polydopamine can be used to coat a variety of substrates through an oxidation process and then it can be dissolved in acidic conditions [92].

Polydopamine was used as a coating material in 3D printed polyetheretherketone (PEEK) porous scaffolds [93]. The scaffolds

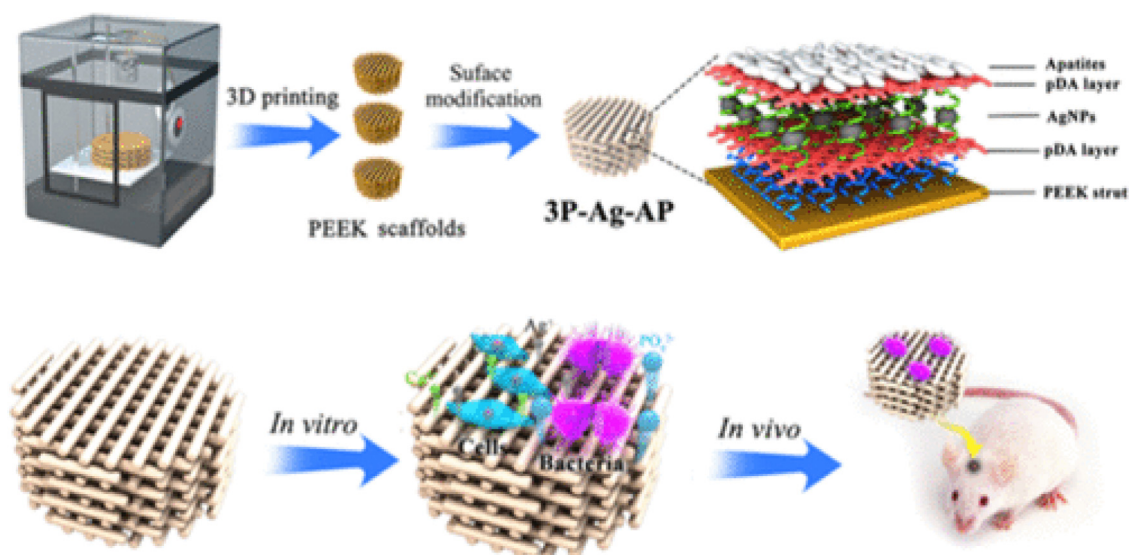


Fig. 9. Schematic illustration of the double coating of polydopamine to endow 3D printed polyetheretherketone (PEEK) scaffolds with antimicrobial (AgNPs) and calcium (apatites) sources, and the subsequent experiments carried out *in vivo* with bacteria contaminated scaffolds. Reprinted from Deng et al. [93] with permission. Copyright © 2020, American Chemical Society.

were twice coated with polydopamine where silver nanoparticles were loaded in the first layer and apatite was attached to the second layer (Fig. 9). This double coating was intended to act as a calcium supply for non-infected wounds and smartly modulate the antibacterial agent (Ag) when the pH was reduced due to the acidic bacteria environment. Indeed, the scaffolds released 1.17, 1.86 and 2.09 mg/g scaffold in pH medium of 7.4, 5.0, and 4.5, respectively. *In vivo* evaluation was carried out in critical-sized bone defects (8 mm) in the skull (Sprague Dawley rats) using scaffolds previously contaminated with *S. aureus*. The scaffolds tested were the 3D printed scaffolds as prepared (without coating), coated with AgNPs, coated with hydroxyapatite, and coated with both AgNPs and hydroxyapatite. Scaffolds containing AgNPs were the only ones that provided protection against infection development, but the dually coated scaffolds showed higher bone ingrowth evidencing the synergistic effect of AgNPs and hydroxyapatite on osseointegration and neovascularization in a highly challenging scenario.

Synthetic acrylic polymers exhibiting pH-dependent solubility, such as some Eudragit®, are also being explored for wound-regulated release of active substances. As an example, Eudragit S100, which becomes soluble at pH above 7, was used as a coating film to cap the porous layer formed by poly(lactic-co-glycolide) (PLGA) on stainless-steel microneedles (600 μm in height and 120 μm in diameter) as depicted in Fig. 10 [94]. The combination of Eudragit S100 and PLGA resulted in a porous pH-responsive polymer coating that enabled the release of the encapsulated drug when the wound medium became alkaline. The *in vitro* release demonstrated burst release of lidocaine reaching 87% in 2 h at pH 7.5, while almost no release occurred at pH 4.5. For the *in vivo* assessment, the fabricated microneedles were loaded with a near-infrared fluorescent dye, and then tested on Sprague Dawley rats with and without wounds. The wound area exhibited strong fluorescent signal, compared with no signal on the healthy skin (slightly acidic pH). Histological analysis was not carried out, and thus the wound healing effect was not significantly evidenced.

5. Production of CO₂ gas

The last route for designing pH-sensitive systems is to load in the initial material (without covalent bonding) substances, such as NaHCO₃ and CaCO₃, that react in an acid environment and produce

CO₂ gas that is released from the system allowing other loaded molecules to be released too.

Acid-responsive scaffolds for the regulation of inflammatory factors were developed by co-electrospinning using a pre-loaded solution of poly(L-lactide) (PLLA), ibuprofen and NaHCO₃ [95]. Acid responsiveness was evidenced in *in vitro* ibuprofen release experiments by the comparison of electrospun scaffolds containing NaHCO₃ (80% ibuprofen released) or not (30% ibuprofen release after 48 h). Full-thickness skin wounds (2 cm in diameter; Sprague-Dawley rats) were treated with PLLA scaffolds solely, scaffold containing ibuprofen, pH-responsive scaffold containing ibuprofen and NaHCO₃, or remained untreated (control). On the 7th day, the residual wound area was 58.5, 42.2, 25.0, and 73.1%, respectively. Immunohistochemical analysis showed that bFGF expression was higher and the inflammation was lower in the pH-responsive scaffold group.

Another acid-responsive scaffold was prepared using CaCO₃. The hydrogel system was fabricated by photopolymerization of Pluronic F127-diacrylate in the presence of nano-CaCO₃. Two types of responsive scaffolds were used. One scaffold type contained the calcium carbonate homogeneously distributed along the scaffold. The second scaffold type was partially digested in acidic medium, remaining the CaCO₃ entrapped in the middle of the scaffold forming a core-shell structure to slower the release [96]. Bone regeneration was evaluated *in vivo* in full-thickness cranial defects (New Zealand rabbits) with the insertion of the hydrogels in a skull defect. Compared to F127 solely hydrogels and F127 hydrogels with homogeneously distributed CaCO₃, the F127 shell-CaCO₃ core hydrogels promoted higher thickness of the new bone formation, more mineralization and maturity of bone tissue. The gradient distribution of Ca²⁺ of these scaffolds induced better osteoblast differentiation and migration by overexpression of Col1, BMP2 and osteopontin.

6. Conclusions and future prospects

For years it has been empirically known that the pH can condition the evolution of a wound, but the effect of this variable on the regeneration of other tissues has still barely been elucidated. It is known that a skin wound must evolve towards an acidic pH to avoid the risk of chronification, while in the case of bone an alka-

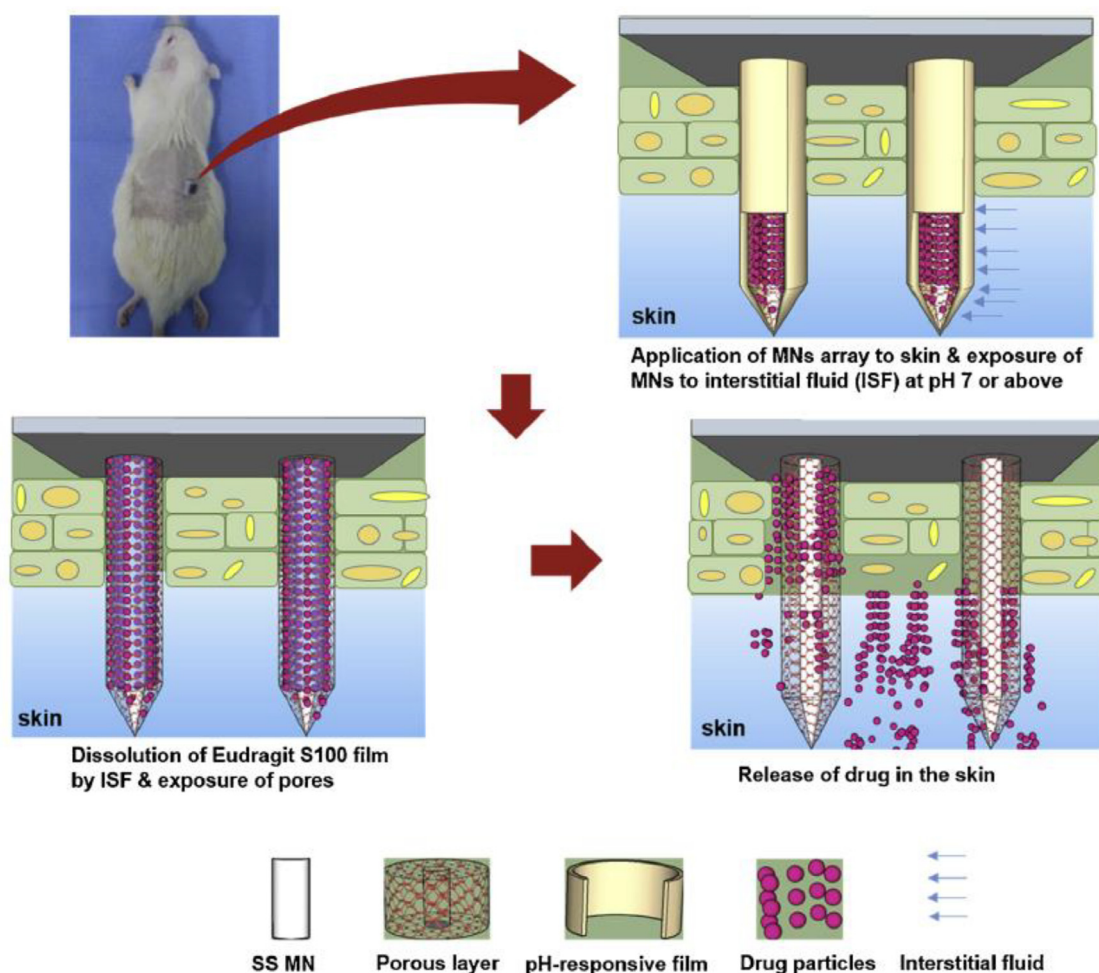


Fig. 10. Microneedles, which were 600 μm in height and 120 μm in diameter, were first coated with a porous PLGA layer and then dipped in the Eudragit S100 solution to form the pH-responsive layer covering the uniformly distributed pores where the drug was encapsulated. Once applied on healthy skin (pH acid), no release occurs. Differently in a wounded area of pH >7.0 the drug is rapidly released. Reprinted from Ullah et al. [94] with permission from Elsevier.

line pH favors its formation. Therefore, different tissues may have different optimal pHs for healing. Recent advances in pH measurement during the healing process of a tissue *in vivo* may shed light on naturally occurring pH changes that must be preserved and may also serve as the basis for the development of scaffolds that monitor the regeneration process in real-time and help in making decisions about the most appropriate treatment.

Preclinical *in vivo* evaluation of pH-responsive scaffolds has clearly demonstrated their superior healing abilities compared to non-responsive counterparts of similar design and composition. The better known the small changes in pH that occur during tissue regeneration, the more successful will be the identification of the pH-sensitive component to be used in each clinical scenario. This information is also critical to determine when to provide a certain therapeutic substance that can speed up the healing process or prevent the development of microbial biofilm. The design of pH-responsive scaffolds should also consider the raising concerns about medical device waste and its environmental impact. Interestingly, the most explored strategies and compositions of the pH-responsive scaffolds tested *in vivo* so far are based on natural polymers and clays that can be modified in versatile ways to strengthen the pH sensitiveness. Among other approaches, the grafting of ionizable synthetic polymers and the functionalization with moieties capable of participating in Schiff base bonding or that can be reversibly cross-linked with borax or multifunctional drugs have provided biocompatible and biodegradable scaffolds

with performances adjusted to the dynamic changes in the pH during tissue regeneration. Some pH-dependent solubility coatings and simple salts used to generate CO_2 also appear as feasible and scalable strategies to expand the production of pH-responsive scaffolds.

Although more information is still needed to translate pH-responsive scaffolds to the clinical field, the variety of approaches currently being explored to endow scaffolds with tunable pH sensitivity, as well as the application of green chemistry principles, may provide efficient designs that can fulfil specific therapeutic demands.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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