Healing of Chronic-wounds – An Update of Recent Developments and Future Possibilities

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Abstract

Chronic-wounds are the result of disruptions in the body's usual process of healing. They are not only a source of significant pain and discomfort, but more importantly an unguarded port of entry for pathogens into the body. While our current understanding of this phenomenon is far from complete, findings in physiological patterns and advancements in wound-healing technologies have helped develop wound management and healing solutions to this longstanding medical challenge. This review presents an overview of known wound-healing mechanics, abnormalities that lead to chronic-wounds and a summary of established and new wound-healing technologies. Various approaches to heal wounds are discussed, from dermal replacements to advanced biomaterial based treatments, from cell, synthetic and composite based approaches to preclinical approaches which make developing such products possible. While tested breakthrough products are described, the authors focused more on recently developed innovations which are at varying stages of maturity. The review concludes with a note on future perspectives and opinions on where the field and industry are headed and where they should be.

Impact Statement

Wound-healing is an important area of research and clinical practice, and has captured the attention of tissue engineers since the nascent beginnings of the discipline. Tissue engineered skin was the first FDA-approved product, achieved in 1996. Despite this success, and the passage of time, healing wounds, particularly chronic-wounds, remains a vexing challenge. This comprehensive review paper will provide readers with a synopsis of current issues, research approaches, animal models, technologies and products that span the continuum from early

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development to clinical studies, in the hope of fueling new interests and ideas to overcome this long-standing medical challenge.

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Introduction

Dysfunctional wound-healing is a major complication of both type 1 and type 2 diabetes with a lifetime risk of developing a foot ulcer potentially as high as 25% whereas the global prevalence rate of DFU varies from 3–8% (1, 2). The incremental cost of a diabetic foot ulcer (DFU) alone is US$20,000 annually (3) in the U.S. With 1.5 million patients presenting with a new DFU each year, this costs $30 billion annually in the U.S. alone. The incidence of chronic-wounds, including DFUs, is expected to rise as the total number of people aged 20-99 with diabetes is set to increase from 425 million in 2017 to 629 million in 2030 (4).

A chronic-wound is one which either seizes to heal or does not heal in the usual, predictable stages and timeframe. Patients with chronic-wounds often present a complicated etiology. A now popular concept, moist wound-healing was first described in 1962 (5). This concept has evolved into the current practice of fluid management within a wound, which has resulted in the development of absorbent and gel-forming materials in "advanced wound-dressings." Primarily as a consequence of the regulatory pathway for wound-dressings in the US (the so-called "510(k) pathway"; discussed in a complementary paper in this issue), there has been a proliferation of these types of dressings. Simply addressing wound management may be an over-simplification of the requirements for effective chronic wound therapy.

Many of the latest technologies target the complex underlying biology of chronic-wounds with novel and elegant synergies between these aspects and the materials needed to perform functions beyond fluid management. In this review, we discuss a comprehensive spectrum of contemporary therapeutic approaches that focus specifically on chronic, non-healing wounds (Figure 1). We

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present current approaches to preclinical development, the relevance of testing in animal models, and offer opinions on research strategies for impactful clinical translation.

Methodology

A plethora of publications are available on the topic of wound healing. For the purpose of this review we limited our scope to chronic wounds only. Three levels of literature search was performed:

a) Established long-standing products: Recent reviews (last 5 years) were investigated to generate an understanding of established products and general categories of wound healing technologies. We limited our search to only products which are indicated for chronic wounds.

b) Upcoming technologies: The Web of Science database was employed using the following keywords as the "Topic" – (Chronic Wounds OR Diabetic Foot Ulcers OR Diabetic Leg Ulcers OR Venous Leg Ulcers OR Pressure Ulcers) AND (Wound treatment OR wound healing OR wound care).

c) Recently marketed products: In order to narrow down the search to only market-ready, registered products and their clinical data, we used the keywords listed in (b) AND [TM[1] OR (R)[2]].

d) Clinical trials: To search for clinical trial information, keywords used were – ("product name" AND clinical trial).

[1] TM: trademark sign e.g. ProductX™
[2] (R): registered sign e.g. ProductY®

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Features of Chronic-wounds

Healthy wound-healing has been extensively reviewed elsewhere (6-8). Briefly, an injury triggers an inflammatory response characterized by hemostasis and the recruitment of inflammatory cells by activated platelets. Neutrophils infiltrate the wound site, where they are replaced by monocytes that differentiate into macrophages. The proliferation stage involves re-epithelialization, angiogenesis, collagen synthesis, and extracellular matrix (ECM) deposition. Finally, the remodeling stage of wound-healing involves revision of collagen into a mature matrix, as well as vascular maturation and remodeling.

Chronic wounds, as mentioned earlier, do not follow the predictable stages of events. There are three major etiologies: venous ulcers (VU or VLU for legs), diabetic ulcers (DU or DFU for foot) and pressure ulcers. Venous ulcers occur primary in the leg (VLU) and are caused by dysfunctional valves. Diabetic ulcers may start as minor scratches, often going unnoticed due to impaired nerves especially in lower extremities, and can become severely infected due to compromised immune systems, poor circulation and damaged capillaries. Lastly, pressure ulcers afflict patients who are bedridden or limited in mobility. The mechanisms of chronic wound-healing are poorly understood, although dysfunctional inflammation and macrophage behavior have been strongly implicated (9-11). While the roles of each macrophage population are poorly understood, disruption in the transition from the pro-inflammatory to the anti-inflammatory state has been associated with impaired wound-healing in animal models (9, 12-16).

Another tell-tale sign of chronic-wounds is an imbalance of proteases and inhibitors possibly due to the aforementioned inflammation glitch and/or microbial infection (6). Typically, chronic-

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wounds are associated with elevated matrix metalloproteinases (MMPs) production coupled with attenuation of tissue inhibitors for metalloproteinases (TIMPs) and, therefore, defective ECM modeling (17). Concurrently, significant up-regulation of cytokines such as tumor necrosis factor-α (TNF-α) contributes to delayed healing by hampering TIMP synthesis by fibroblasts (17). Consequently, down-regulation of proteinase production with MMP inhibitor improves wound-healing (18, 19).

Revascularization of the neotissue is a critical phase of wound-healing. Unsurprisingly, vasculopathy is a key cause of impaired healing in chronic-wounds. The mechanisms surrounding this include reduced bioavailability of growth factors and receptors, disruption of matrix proteins, reduced proliferative ability of resident cells and insufficient recruitment of progenitors (20). In particular, abnormal patterns of vascular endothelial growth factor receptors (VEGFR) have been implicated (21).

The pH of chronic-wounds is always alkaline (pH: 7.2-8.9) compared to that of acute wounds which is characteristically neutral or alkaline (pH: 6.5-8.5) (22). Acidosis of the initial wound is essential for proliferation of fibroblasts, oxygenation, collagen formation, angiogenesis and macrophage activity. These key processes are jeopardized by the alkaline environment of chronic-wounds. Bacterial infection and their subsequently released by-products can cause wound pH to become alkaline (23), which can in turn further promote colonization of pathogenic bacteria. As a result, chronic-wounds experience higher occurrences of biofilm formation (24) which presents increased resistance to biocides compared to free floating microbes (25, 26). Biofilms themselves result in the development of chronic-wounds (27-29).

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Approaches in Wound-healing Technologies

Table 1 provides a comprehensive list of wound healing technologies at various stages of development, based on using biologics, biomaterials and cell-based approaches. These approaches are summarized hereon, with specific description of representative products and emerging concepts.

Biologics

Biologics are naturally occurring, single or complex molecules, which are produced by living organisms or contain components of living organisms. They are bioactive and capable of eliciting specific biological responses. Given that the chronic wound environment is known to be deficient in specific bioactives, introducing biologics which play critical roles in wound repair, as a supplement to induce revascularization and re-epithelization is therefore a logical approach to direct wound-healing. While numerous studies have shown promising results, the only approved biologic available commercially is Regranex®, a carboxymethyl cellulose based gel which incorporates platelet-derived growth factor (PDGF), which has been demonstrated to work in foot ulcers (30) but has not been widely adopted in clinical practice. There have also been suggestions that incorporating other growth factors like transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) can promote healing (31).

Given the importance of defective inflammation, several studies have explored immunomodulatory bioactive factors. For example, Mirza et al.(10) showed that the

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administration of antibodies that block signaling of the pro-inflammatory cytokine IL-1b improved wound-healing in diabetic mice, although it should be emphasized that this treatment was administered 3 days after wounding so as not to inhibit the early inflammatory phase of wound-healing. In another study that emphasized the importance of timing in administration of immunomodulatory therapies, Leal et al. (32) administered the neuropeptide substance-P (SP) to diabetic wounds in a murine model. They found that the treatment promoted an acute inflammatory response characterized by increased expression of pro-inflammatory cytokines IL-6 and MCP-1 at day 3 after wounding. However, by day 10, SP treatment reduced the levels of these cytokines, concomitant with a reduction in the relative levels of pro-inflammatory macrophages. These early peaks in inflammation followed by transition to anti-inflammatory behavior ultimately resulted in improved wound-healing.

Another approach is to mimic, promote repair of or replace damaged ECM components. CACIPLIQ20® is a bioengineered product (33) that mimics damaged heparin sulphate glycosaminoglycan, thereby stimulating revascularization (33, 34). It is primarily composed of a poly-glucose-based polymer resistant to endoglycosidase and capable of restoring the structural and functional properties of the ECM. Retrospective studies with significant numbers are not yet available but an individual case of successfully treating a 22-year old male with lower extremity ulcer diagnosed with Stewart-Bluefarb syndrome (SBS) has been recently reported (35).

A prospective product undergoing clinical trials for chronic wound-healing is Epiceram® (36). Currently, the gel is a prescription based controlled-release skin-barrier-repair emulsion therapy for eczema and atopic dermatitis. Containing three key essential lipids – ceramides, conjugated

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linoleic acid (CLA), and cholesterol in a physiologically balanced patented 3:1:1 ratio, Epiceram® claims to restore barrier function of chronic and defectively closed wounds. The same trial is also testing Ceramiseal™ which is a similar concoction of dermally relevant lipids including squalene, ceramide-3, oryzanol, cholesterol, etc. but the exact mode of action is unclear based on the product description. Another drug, deferoxamine, is also undergoing clinical trial aimed to investigate the efficacy of a hypoxia-inducible factor to improve chronic diabetic wound-healing (37). Deferoxamine is currently the only HIF inducer approved for clinical trials having previously shown to work on animal models. A recently developed technique showing great promise involves using platelet-rich plasma (PRP) to deliver a rich source of not only coagulation and associated proteins but also proteases and protease inhibitors, antimicrobial proteins, cytokines and growth factors. A meta data analysis revealed that 65.3 % of DFU patients achieved complete healing after receiving PRP treatment compared to 45.5 % of DFU patients receiving standard wound care (38).The meta data analysis also suggested that PRP source and preparation has an effect on the efficacy.

The biologics space is becoming increasingly active especially with the development of PRP as a viable alternate treatment for chronic wounds. However, the harsh biochemical environment of a chronic wound remains a threat to maintaining the viability of biologics to achieve the desired outcome. There is thus a need to develop standardized clinical protocols and guidelines, as well as stronger clinical evidence to support their use.

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Biomaterials

A biomaterial is typically a material that is derived from natural or synthetic sources and, when processed into a specific template, can be exploited in contact with living systems. Wound healing applications in the form of dressings are the earliest use of biomaterials. Today, wound dressings go beyond passive coverings that simply provide an isolated environment for healing. Here, we summarize the more significant known and emerging biomaterial based applications that actively participate in the enhancement of wound healing.

Bioactive Wound-Dressings

An advanced wound-dressing provides immediate physical protection to the ablated skin, maintains a conducive environment for wound-healing and at the same time incorporates active ingredients or elements that enhances the wound healing process. A plethora of wound-dressings is available, each with its own pros and cons (39).

Inspired by its success in ophthalmological reconstructions, amniotic membranes (AM) have emerged as wound-dressings (40). Derived from human placentae after cesarean delivery, each of which can provide four to five AM tissue fragments 5 cm in diameter, AM supply and cost are favorable. AM is composed of collagen types I, III, IV, V and VI along with ECM components like fibronectin, laminin, glycosaminoglycans and proteoglycans. In addition, AMs also contain a range of active growth factors. A systematic review assessing 6 clinical trials amounting to 331 patients with diabetic leg ulcers reported that wound-healing with AM occurred 2.32 times more often and 32 days faster than other conventional therapies (41) possibly due to the privileged

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immune and anti-inflammation properties that AM possess (42). Though still new, a few commercial products have surfaced including Grafix®, AmnioBand® and EpiFix® (43-45).

Recently, active agents-incorporated bioactive wound-dressings that additionally enhance the wound-healing process are also evolving. Antimicrobials such as silver sulfadiazine are the most common active agents used (46). However, silver incorporated dressings are exclusively for infection-prone wounds as the released silver ions are cytotoxic to healthy cells as well (47). Another commercially available range of antimicrobial dressings are coated with dialkylcarbamoyl chloride (DACC) patented as the Sorbact™ technology. DACC makes the dressing extremely hydrophobic, which in the aqueous wound environment attracts hydrophobic microbes to bind irreversibly with the dressing (48). A recent review by Totty et al described the limited but encouraging data in favor of DACC-coated dressings (48).

Ichthopaste® is another relatively new product made from open wound cloth (cotton) impregnated with zinc oxide and ichthammol, which is a natural product derived through dry distillation of sulfur-rich oil shale, and is known for its anti-inflammatory, bactericidal and fungicidal properties (49). While other newer and better medications exist, ichthammol's excellent tolerance makes it a safe choice for cutaneous disease care, especially when long term care is needed (49). Apart from the examples above, commercially available dressings with specific drugs are not common although there are some promising recent studies (50-54).

Being the most direct and traditional of approaches, some of the most successful wound healing products today are dressings. Yet, the wound-dressing space continues to be an extremely crowded and innovative one. Current research focuses on incorporating multi-level, bioactive functionalities into the dressings.

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This is showcased in novel approaches to incorporate natural ECM elements into wound dressings, along with cytokines and antimicrobials.

Skin Equivalents

Skin equivalents are constructs that typically aim to replace, repair and regenerate specific compartment(s) of the skin - epidermis, dermis or both – and integrate into wounds like autografts. However, these grafts often behave as temporary dressings that slough off as the wound heals, this group of products remains the most advanced tissue engineered constructs in clinical use. Success, however, is heavily dependent on meticulous wound preparation, wound selection and proper application, all of which require good judgement and experience. In this section, we give emphasis to established and more recent skin equivalents that are indicated for chronic wounds, and incorporate biomaterials as supporting templates or bioactive agents.

i. Cultured Epithelial Autografts (CEAs):

Swift re-epithelialization is a primary challenge of wound-healing in order to reinstate the protective capacity at the injured site; hence the development of cultured epithelial autografts (CEAs). CEAs were realized due to the pioneering work of Rheinwald and Green who successfully cultured human keratinocytes *in vitro* (55). Keratinocytes can now be cultured in a variety of serum-free culture media without the need for a feeder layer (56), thus facilitating more ready acceptance by regulatory bodies. A CEA sheet large enough to cover the entire body can be generated within 3-4 weeks from only a 3 cm^2 large biopsy (57). Epicel®, EPIBASE® and EpiDex® are some long-standing products making use of this approach.

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To circumvent the handling difficulties due to the inherent fragility of CEAs, keratinocytes have been cultured on membranous biomaterials that act as mechanical supports. These are either peeled off after grafting or integrated into the wound over time. Natural materials such as collagen-I, hyaluronic acid, fibrin and chitosan (57-64), and synthetic materials such as silicone (MySkin®), polyurethane (Hydrotherm™), Teflon™ and surface carboxylic acid functionalized poly(hydroxyethyl methacrylate) have all been shown to work as a CEA delivering membrane (57).

An interesting variation to overcoming the fragility of CEAs is to aerosolize sub-confluent keratinocytes and spray them onto the wound bed – a strategy that CellSpray® (65) and Tisseel® (66) adopt. This approach significantly reduces the time lag between wounding and treatment since it negates the need for an *in vitro* culture period. However, lacking the epidermal organization of CEAs limits the use of spray-on keratinocytes to use on partial-thickness and graft donor site wounds (31). Another similar-concept product that showed good promise in its phase II trials is patented under the trade name Allox™ (HP802-247) (67). Constituted of cryopreserved allogeneic growth-arrested human keratinocytes and fibroblasts in a fibrin-forming matrix, it can be sprayed on or used along with a secondary dressing. However, phenotypic changes in the cells was cited as the probable cause of failure during recent phase III trials and the product has since been dropped (68).

CEAs are a major breakthrough in skin grafting technology, but limitations remain. Apart from high costs due to the lengthy culture period needed, scarring remains a problem along with an

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unpredictable success rate owing to complicated handling and application procedures, and uncontrollable donor cell quality.

ii. Dermal Substitutes:

The presence of a dermal layer in skin substitutes can enhance wound-healing in terms of speed and functionality (69, 70). Integra™, the first synthetic dermal substitute to be used clinically, consists of crosslinked bovine tendon collagen-I and shark glycosaminoglycan (chondroitin-6-sulfate). In a recently concluded clinical trial, complete DFU closure after 16 weeks of treatment was achieved in 51 % of patients receiving Integra treatment compared to only 32 % of patients receiving the control treatment of moist wound therapy (p = 0.001). The median time to complete DFU closure was 43 days (7.2 % per week) for Integra treated subjects and 78 days (4.8 % per week) for control subjects in wounds that healed (p = 0.012) (71). However, high cost and risks of infection under the overlaying silicone layer are the main drawbacks (72, 73).

Several acellular dermal substitutes were developed after the success of Integra™, including MatriDerm®, which is made up of non-crosslinked bovine dermal collagen I, III, V and elastin (74), and Pelnac, which is made up of porcine collagen (75). While both Integra™ and MatriDerm® have been shown to increase vascularization, experimental results have also revealed that cellular penetration into these meshes can be slow or absent (76, 77). Host cell vaibility is critical to the performance of such templates, and in patients who have undergone pre-operative radiotherapy, host cell concentrations can be critically low which can significantly impair wound-healing. In contrast, templates made from decellularized tissues like Alloderm® (human cadaveric skin allografts) and Permacol™ (porcine xenografts) can provide the natural

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dermal matrix microarchitecture to support host cell penetration, proliferation and subsequent tissue regeneration. The overall ECM architecture of the skin is preserved in these products, complete with vascular channels in the dermis and basement membrane at the epidermal-dermal junction, while the antigenic cellular components are removed (74). Despite several reports of excellent clinical outcomes (78-80), the use of decellularized templates is again held back by high costs and, in the case of Alloderm®, limited supply of human donor skin.

Consequently, a new product called PriMatrix® has gained popularity because it is easier to handle and does not require any orientation specific placement (81-86). PriMatrix® is decellularized fetal calf dermis that is particularly rich in collagen-III. Encouraging reports about the ability of PriMatrix® to definitively close and re-epithelialize complex wounds (82, 83, 86) and diabetic foot ulcers (84, 85) have emerged. Interestingly, PriMatrix® outperformed Apligraf® (see next section) in terms of wound-healing times for both venous leg ulcers and diabetic foot ulcers regardless of wound size (85).

iii. Bi-layered Substitutes:

A natural transition from epithelial and dermal substitutes is the incorporation of the two to create a more physiologically realistic bi-layered skin construct that can effectively replace damaged or lost skin. It was with this principle in mind that Apligraf®, a bi-layered skin substitute once considered the most advanced tissue engineered product, was developed. It possesses a lower dermal layer of collagen I populated with human fibroblasts and an upper stratified epidermis differentiated from a layer of human keratinocytes (85). Apligraf® replicates

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the split thickness skin architecture without appendages. It acts as an active wound covering with immediate replacement of the lost living cells and ECM components. It was the first FDA approved bi-layered, living skin substitute indicated for both venous leg ulcers and diabetic foot ulcers. In a pivotal multicenter, prospective, randomized control clinical trial, after 12 weeks of treatment, complete wound healing was achieved in 63 patients (56 percent) suffering from DFUs in the Apligraf® treated group compared with 36 patients (38 percent) in the control group (p = 0.0026) (87). The wound healing time for patients achieving complete closure was 65 days for the Apligraf® treated group compared to 90 days for the control group. However, the allogenic cells in Apligraf® do not survive beyond a couple of months post-application which renders the graft closer to an advanced cellularized wound-dressing (88).

OrCel® is a bi-layered cellular matrix with co-cultured keratinocytes and fibroblasts derived from human neonatal foreskin embedded in a porous bovine collagen-I sponge. OrCel® is FDA approved for non-infected split-thickness donor site wounds and is pending approval for VLUs and DFUs. Limited data is available for its usage on chronic wounds, although phase 3 clinical trial is ongoing for its efficacy on DFU (89).

iv. New Material Systems:

Numerous biomaterials are being explored for potential usage as skin substitutes and scaffolds (31). Natural materials, especially those that are native to the ECM, are obvious choices. Fibrin, nature's designer matrix material for healing wounds, is commonly used. Others such as collagen, elastin and fibronectin have also been widely explored. While these ECM molecules are advantageous in that they are recognized by cells and elicit desirable cell responses, limitations

in terms of mechanical properties and processability have led to their increasing use as composites with other natural or synthetic materials.

Fibrin and collagen-I have recently been used as a coating on electrospun polylactic acid and poly(lactide-*co*-glycolic acid) nanofibrous membranes (90). In the same light, by combining the self-assembly properties and excellent mechanical stability of silk fibroin with the bioactivity of elastin, Vasconcelos et al. developed a wound-dressing that performed as well as other commercially available dressings in terms of facilitating wound-healing and managing wound exudate (91). Recently, a novel variation of electrospinning termed rotary jet spinning (RJS) was developed which can produce nanofibers of fibronectin driven by high extensional and shear strain rates (92). This fibrillar conformation is shown to accelerate wound closure and improve tissue restoration. RJS was also applied to engineer a nanofibrous dressing made of soy-protein hydrolase and cellulose acetate (93). The resulting nanofibers successfully mimicked ECM properties while providing other benefits like high water retention, enhanced fibroblast activity, and accelerated re-epithelialization.

New versions of old materials have also been developed, such as photocrosslinkable gelatin to improve versatility of fabrication and improve mechanical outcomes (94). Many other innovative combinations of established biomaterials have also been explored for skin regeneration applications (31). Conventional biomaterials which are typically not used in wound healing applications are also being explored, such as bioactive glasses (95).

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Though natural ECM components found in the human skin are obvious choices as building blocks to fabricate skin substitutes, the challenge remains to find a reliable, abundant and cheap source of such an ECM component. Materials of animal origin are therefore often used but not without shortcomings; immunogenicity and risks of inter-species pathogen transfer are concerns. Keratins have thus emerged as exciting options, because they are abundant, easily accessible, renewable and potentially autologous if extracted from human tissue such as hair. Over the last few decades, a wide range of possibilities to use keratins in wound-healing applications have been demonstrated, from cell carriers to acellular templates and drug delivery vehicles (96, 97). In the form of a 3D template, keratin hydrogels have shown promise as a hemostatic agent (98), cell carrier (99) and also as a template that supports skin regeneration after burns (100). Composites (101), photocrosslinkable platforms (102) and bio-inks (103) based on keratin have also been developed, suggesting versatility of the material.

The development of skin equivalents was largely fueled by an acute shortage of transplantable skin, and represented the early, exciting successes in tissue engineering. However, it has become apparent over time that skin equivalents cannot replace autografts. Nonetheless, specific to chronic wounds, skin equivalents could offer an optimal physical and mechanical template via which actives agents such as cells and biologics can be effectively protected and delivered to effect healing.

Cell-Based Technologies

Cell-based technologies refer to those in which whole living cells are being applied. To differentiate from skin equivalents, we define “cell therapies” in this section as cell-only

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treatment which, in wound-healing, is relatively new. To date, there are only about 20 clinical trials, completed or ongoing, investigating the use of cell-based therapies in healing of chronic wounds (104). Cell types explored for this purpose include bone marrow mesenchymal stem cells (BM-MSCs) (105, 106), adipose-derived cells (ADCs) (107), epidermal cells (68), amniotic fluid stem cells (AFSCs) (108), MSCs derived from umbilical cord Wharton jelly (109) as well as macrophages (110). Therapeutic cells are usually delivered undifferentiated within a matrix such as a hydrogel (109, 111, 112), intravenously (113, 114) or topically to wound sites as a cell solution (115) or together with a complementary material (116). The delivery mechanism in such therapies clearly plays an important role in ensuring success (117, 118), as does wound bed preparation (such as debridement) (119).

Mesenchymal Stem Cells

Apart from skin cells, there is notable interest in MSCs in the wound-healing field (120, 121). The potential role of MSCs in wound-healing was first suggested by Neuss et al who reported that human MSCs express hepatocyte growth factor (HGF) and cognate receptor HGFR/c-met (119). In 2007, Wu et al published findings that BM-MSCs accelerated wound closure in mice through re-epithelization and angiogenesis of the wound site (122). These findings were supported by a separate study which showed that circulating MSCs in mice were recruited to wound sites and promoted tissue regeneration through differentiation into keratinocytes and endothelial cells (114), presumably modulated by the high levels of chemokines and cytokines released by BM-MSCs (123, 124).

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We refer readers to other reviews on chronic wound-healing based on MSCs (125, 126), and stem cell therapy in general (127, 128).

Macrophages

Direct administration of allogeneic macrophages to chronic ulcers has been explored in animal models and humans (129, 130), but incomplete understanding of the kinetics of inflammation in different types of ulcers has limited the successful deployment of these strategies. Impaired transition of macrophages from the M1 to M2 phenotype has been implicated in the defective healing of chronic-wounds (9, 11, 15), Jetten el al. (129) explored the administration of M2-polarized macrophages to the wounds of diabetic mice. Surprisingly, these cells impaired wound-healing instead, possibly because they were administered immediately after wound creation, thus suppressing the critical early inflammatory phase required for wound-healing. Indeed, Maruyama et al. (131) showed that early administration of diabetic macrophages treated ex vivo with the pro-inflammatory cytokine IL-1b significantly improved wound-healing in diabetic mice. Improved understanding of the kinetics of macrophage activation in healthy and impaired wound-healing will lead to better design of macrophage-based therapies.

Commercial cell therapy products under clinical trial

Four commercial cell-based products for chronic-wounds have been listed on the Clinical Trials database: CureXCell™, Grafix®, HP802-247 and APZ2. Although approved in Israel, CureXCell™ by Macrocure Lte., an allogeneic white-blood cell therapy, failed to meet its primary end point in its Phase III multicenter clinical trial for DFU (132). In contrast, Grafix® has achieved a degree of clinical success (133, 134); in multicenter Phase III clinical trials, the

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product was able to achieve complete wound closure in 62% of the patients with chronic DFUs within 12 weeks (133). Marketed by Osiris Therapeutics, Inc. as a placental allograft, Grafix® is essentially cryopreserved placental membrane containing MSCs, growth factors and anti-inflammatory cytokines. HP802-247 (also described before) from Smith and Nephew is an allogeneic spray of irradiated keratinocytes and fibroblasts in fibrin to stimulate healing of venous leg ulcers (135).

The use of autologous cells for chronic-wounds is also being explored in clinical trials. Rheacell GMBH is currently conducting a Phase I/IIa clinical trial for its product APZ2 which is profiled as ABCB5 positive MSCs (136). Previously shown to be a marker of limbal stem cells (137), ABCB5 positive MSCs found naturally in skin have also been shown to aid tissue repair (138).

In addition to cell-based products, companies have also offered devices for preparation of autologous cells for chronic-wound healing. Two examples that are under clinical testing are ReGenerCell™ from Avita Medical and Transpose® RT System from InGeneron. ReGenerCell™ is a device to extract skin cell suspensions from patient skin biopsy in 30 minutes. The resulting cocktail, called Regenerative Epithelial Suspension™, is sprayed onto non-healing DFUs and chronic leg ulcers. It is claimed that cells could adhere to the wound bed as early as day 1 post-harvest (139, 140). Transpose® RT System is an automated system for isolating of stromal vascular fraction (SVF) from liposuction aspirates. The process can be completed within 60 minutes. The SVF is injected around the rim of venous stasis wound subcutaneously. A small pilot study concluded in 2017 reported that SVF promote healing in previously non-healing AVLU and VLU in multimorbid patients (141).

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While cell therapy hold great potential in accelerating wound-healing (118, 142), and could hold the key to finally realizing the possibility of regenerating skin appendages along with the epidermis and dermis (143), effects remain variable between patients and require further clinical validation. With autologous therapies, major challenges include long culture periods and cell quality variability. It will be critical to standardize cell isolation and processing conditions in order to achieve consistent clinical efficacy (118, 128). Other significant factors for consideration include delivery mechanisms (117, 118, 144, 145) and choice of therapy combinations.

Additive manufacturing approaches

Conventional scaffolds

Over the last three decades, artificial skin constructs in the form of thin films (146, 147), electrospun scaffolds (148-150), freeze-dried scaffolds (151-153), and hydrogels (154, 155) have been widely utilized. Despite the skin being a relatively thin and flat organ, the use of 3D scaffolds is favorable over 2D scaffolds in terms of improved cellular attachment and proliferation. Furthermore, a 3D architecture has huge impacts on cell morphology (151, 156) and ECM secretion (157, 158).

3D Bioprinting

3D bioprinting provides a highly automated, layer-by-layer manufacturing platform for the deposition of biomaterials, living cells and growth factors (159, 160). The technology is capable of fabricating complex 3D microenvironments that closely resemble the native ECM of skin and depositing multiple cell types and signaling cues essential for autocrine and paracrine signaling.

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Most importantly, it facilitates a high degree of freedom and flexibility in the design of the skin constructs. The critical aspects of skin bioprinting (161) include imaging of wound sites, selecting the appropriate biomaterials and cells, developing pre-defined computer-aided design (CAD) files and finally the bioprinting process. The three main bioprinting strategies for skin tissue engineering are microvalve-based (162, 163), laser-based (164, 165) and extrusion-based (166-168) where skin cells can be printed in the form of cell suspensions (169), cell spheroids (170) or cell-encapsulated hydrogels (171).

A recent *in-vitro* study demonstrated the laser-based bioprinting of fibroblasts (mouse NIH-3T3) and keratinocytes (human HaCaT) embedded in a collagen gel onto a sheet of Matriderm® (decellularized dermal matrix) (172). Further evaluation of the printed skin constructs revealed the presence of cadherins and connexin 43 in the epidermis which are essential for tissue morphogenesis and cohesion. Interestingly, the *in-vivo* transplantation of the printed skin construct in the dorsal skin fold chamber of nude mice showed good graft-take and angiogenesis after 11 days of post-transplantation (173).

A preliminary microvalve-based bioprinting of multi-layered collagen constructs containing keratinocytes and fibroblasts using nebulized aqueous sodium bicarbonate was reported (174). Collagen layers were first printed and allowed to crosslink, followed by the deposition of cells on top of each layer. The construct maintained its overall shape under submerged culture condition for 7 days and high cell viability was reported. Consequently, fluidic channels were fabricated using a sacrificial gelatin template (175). The presence of fluidic channels resulted in significantly higher cell viability (85 % viability) compared to the ones without any channels

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(60 % viability). Furthermore, microvalve-based printing facilitates drop-on-demand printing to emulate native cellular density of different cell types within bioprinted skin constructs (176). Bioprinting of pigmented human skin constructs (177) was also attempted recently using a two-step approach; bioprinting a hierarchical porous collagen-fibroblast dermal matrices by facilitating matrix deposition through molecular crowding (178) followed by pre-defined patterning of primary human keratinocytes and melanocytes in skin-specific arrangements. The resulting constructs were then matured to achieve the 3D pigmented human skin.

A similar study using fibrin-based bio-inks was also carried out (168). More recently, skin-derived ECM was used to 3D-print pre-vascularized skin patches (consisting of skin-derived ECM, endothelial progenitor cells and adipose-derived stem cells) that accelerate wound closure, re-epithelialization and neovascularization (179). In a cell-based approach, alternating layers of fibrin-collagen containing amniotic fluid-derived stem cells (AFSCs) and thrombin (crosslinking agent) were deposited onto full-thickness skin wounds of nu/nu mice, using the microvalve-based bioprinting approach (180). Although the AFSCs only remained transiently in the wound sites but secretion of trophic factors resulted in increased wound closure rates and angiogenesis.

Advancements in additive manufacturing capabilities have spurred interests in developing both transplantable skin and *in vitro* test models. The possibility to customize a bioprinted skin graft based on a specific wound bed topography is intriguing, although tangible improvements in clinical outcomes have yet to be demonstrated. As the spatial resolution of additive manufacturing techniques improves over the coming years, the anticipation would be for

bioprinting to increasingly impact the field especially in terms of achieving more precise arrangement of cells and ECM components to reconstitute skin appendages.

Alternate Innovations

This section covers other recent unconventional methodologies which have been applied as standalone wound-management systems or as enhancements to other solutions. In particular, there has been a growing debate over the environment which the wound requires for speedy healing in the context of exposure to air. The conventional wound-care approach is to allow wounds to breathe in order for them to dry and receive fresh oxygen to heal faster. Although the paradigm of protecting wounds from the environment and managing exudates has not changed, supplying oxygen to the wound has made a comeback as a catalyst for inducing rapid wound closure. In this respect, continuously diffused oxygen (CDO) therapy using an FDA-approved device, TransCu O_2® System, to deliver pure, humidified oxygen to the wound, has shown potential (181). In a controlled clinical trial, it was found that the CDO therapy achieved complete wound closure in twice as many chronic wounds and at a significantly faster rate of closure compared to controls.

An alternative approach of delivering oxygen is to apply a hemoglobin spray, a concept used in Granulox® (182). Once applied, the hemoglobin immediately harvests oxygen from the surrounding air and transports it to the wound itself. This effect can last up to 72 hours and a recently concluded meta-analysis using Cox proportional hazards log-rank regressions demonstrated substantial increase in the chances of healing in various types of wounds including DFUs and VLUs (182).

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Another unconventional approach is Therapeutic Magnetic Resonance (TMR®) which delivers magnetic fields that pulsate only at tissue-specific frequencies, insofar stimulating and accelerating the healing processes. In a clinical trial, TMR® showed remarkable benefits after 2 weeks of application to DFUs in terms of histological outlook, cellular demographics and ECM composition (183). While the exact mechanism remains obscure, the efficacy is assumed to result from the ability of magnetic fields to interfere with biological processes. Other reported approaches which have been less explored or only recently emerged include negative pressure wound therapy (184), contact and non-contact ultrasound therapy (185) and red light therapy (186).

Animal Models

The complexity of skin wounds encountered in the clinic is reflected in an assortment of wound-healing animal models. Chronic-wounds are different from acute wounds, incisional wounds are different from ulcers, and burns are different from everything else. Much also depends on the patient, their age, overall health status, circumstances of their injury or condition, and co-morbidities, which has made the search for predictive animal models of wound-healing elusive. Previous reviews have been published on rat (187), swine (188), burn (189), and wound-healing models more generally (190), as well as human models (191). Introduction of new wound-dressing products into the market requires a relatively straightforward regulatory path, and as a consequence the requirements of a preclinical model can be rather simplistic, often focusing on providing a protective, moist wound environment and using time to re-epithelialization as the main study endpoint. This has led to the misapplication of animal study data, such as using

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excisional wounds as a surrogate for chronic-wounds, or relying on rodent data as a singular predictive tool for humans. While several models are excellent for elucidating the underlying wound-healing mechanism, and others can mimic the morphology of skin and size of human wounds, an animal model that reliably replicates non-healing chronic-wounds in the human population does not exist. Dhall et al. recently developed a genetically-modified mouse model that sometimes displayed non-healing characteristics, which could be driven by oxidative stress and bacterial contamination (192). It remains to be seen if a direct connection between this model and clinical studies can be established. It may be the case that no animal model is capable of reliably duplicating the complexity of a clinical population. Since non-healing wounds are a relatively small sub-population of patients, one potential strategy may be a multifactorial approach, combining age, comorbidities, concomitant bacterial colonization, and other factors (e.g. oxidative stress, genetic modification, etc.), along with down-selection to the most problematic wounds (i.e. slowest healing within a cohort of animals) to generate the most predictive model for testing advanced therapies. Until such a model is developed and validated, and as advanced skin regeneration technologies work their way through early feasibility testing, this critical need remains.

Conclusions and Outlook/Future Perspective

Advancement in tissue engineering and regeneration medicine strategies have been most apparent in the area of wound-healing. Specific to the healing of chronic-wounds, this article summarized significant past achievements and the most recent and exciting developments that are now available or will soon be available. Approaches based on biologics are promising, especially those developed for immunomodulatory therapies. Cell based therapies based on

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various sources of stem cells continue to receive attention, although the availability of these cells will remain a challenge both in terms of quality and quantity. Biologics and cell based approaches may also complement the more traditional tissue engineering strategies based on 3D biomaterials platforms. With the continuous development of new materials, such 3D platforms will not only provide a physical scaffold to support wound-healing but may also participate in modulating the biochemical environment to enhance the process. To top it off, the revolution that additive manufacturing is bringing, manifested in the form of 3D bioprinting, is catalyzing new perspectives to innovate more complex but realistic products and upscale fabrication processes.

However, there are improvements to be made other than the technologies described above. Clinically, better understanding of how patient heterogeneity affects wound-healing outcomes is necessary to translate findings from bench to clinic. The availability of advanced wound-healing diagnostics will greatly improve the ability of healthcare professionals and caregivers to match product types to wound conditions. Future products could come in the form of sensor incorporated dressings, for example, to give immediate feedback on wound-healing progression or infection type. For scientists and engineers, the need for better and more realistic *in-vitro* and *in-vivo* models is urgent. Currently, animal models of chronic-wounds are available, but they do not adequately mimic human pathologies. Realistic *in-vitro* models of chronic wound-healing are lacking, especially those that replicate the complex and varying 3D, immunological and biochemical environment of different chronic wounds. This may mitigate the late clinical stage failure rate confronted by many of the technologies discussed in this review.

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Collectively, there is still immense potential for the field to further advance current strategies of managing chronic-wounds. It is anticipated that more effective products will be developed in the years ahead, which will combine the essential elements of the physical, mechanical, biochemical and biological environment that are necessary to bring about effective healing of chronic-wounds.

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Table 1: Summary of wound healing technologies indicated for chronic wounds - biologics, biomaterials, cell-based technologies & alternate innovations.

	Material	Functional Strategy/Features	Stage of Maturity	Ref.
Biologics	Carboxymethyl cellulose based gel	• Incorporated with PDGF	Regranex®: commercialized but not widely adopted in clinical practice	(30)
	Poly-(carboxymethylglucose sulphate) solution	• Mimics damaged heparin sulphate glycosaminoglycan • Stimulating revascularization	CACIPLIQ20®: recently reached markets	(33, 34)
	Controlled-release skin-barrier-repair emulsion	• Ceramides, conjugated linoleic acid (CLA), and cholesterol in a physiologically balanced patented 3:1:1 ratio	Epiceram®: prescription only; clinical trials completion end of 2018	(36)
	Topical serum	• Proprietary blend of naturally occurring lipids	Ceramiseal™: commercially available; clinical trials completion end of 2018	(36)
	Topical gel	• Deferoxamine	Desferal: undergoing clinical trials	(37)
	Platelet-rich plasma	• Rich source of platelets • Able to enhance wound-healing relevant biologics	Promising field; needs standardized protocols and more clinical data	(38)

	Material	Functional Strategy/Features	Stage of Maturity	Ref.
Bioactive Wound Dressings	Amniotic membranes (AM) derived from human placentae	• AM which contains immune and anti-inflammation characteristics	Grafix®, Amnioband® and Epifix®: commercially available	(41-45)
	Dressing with dialkylcarbamoyl chloride (DACC) coating	• DACC is extremely hydrophobic and binds microbes irreversibly	Sorbact™: commercially available	(48)
	Open cotton cloth impregnated with zinc oxide and ichthammol	• Ichthammol is an anti-inflammatory, bactericide and fungicide.	Ichthopaste®: Relatively new product	(49)
Cultured Epithelial Allografts (CEAs)	Basic CEA	• Cultured keratinocytes derived from biopsies	Epicel®, EPIBASE®, EpiDex®: Long standing products	
	CEA on membranous biomaterials	• Natural: collagen I, hyaluronic acid, fibrin and chitosan	Shown to work	(57-64)
		• Synthetic: silicone (MySkin®), polyurethane (Hydrotherm™), Teflon™	MySkin®, Hydrotherm™, Teflon™: available commercially	
	aerosolize sub-confluent keratinocytes sprayed onto wound bed	• Applied directly on wound bed • Reduces time lapse between wounding and treatment	CellSpray®, Tisseel™: available commercially	(65, 66)
	cryopreserved allogeneic growth-arrested human keratinocytes and fibroblasts in a fibrin-forming matrix	• Sprayed on or used as secondary dressing	Allox™ (HP802-247): failed in Phase III trials	(67, 68)

	Material	Functional Strategy/Features	Stage of Maturity	Ref.
Dermal Substitutes	bovine tendon collagen type I and shark glycosaminoglycan (chondroitin-6-sulfate) cross-linked with glutaraldehyde	• acellular synthetic dermal substitute • may increase vascularization • cellular penetration into these meshes can be slow	Integra™: available commercially	(74, 77)
	non-crosslinked bovine dermal collagen I, III, V and elastin		MatriDerm®: available commercially	(74)
	porcine collagen		Pelnac™: available commercially	(75)
	human cadaveric skin allograft	• decellularized tissues • provide natural dermal matrix microarchitecture with vascularity • For human allograft – limited availability due to need for donor skin.	Alloderm®: available commercial	(78-80)
	porcine xenografts		Permacol™: available commercially	
	decellularized fetal calf dermis with a matrix that is particularly rich in collagen type III	• support re-epithelialization and re-pigmentation • Gaining popularity due to ease of handling	PriMatrix®: available commercially	(81-86)

	Material	Functional Strategy/Features	Stage of Maturity	Ref.
Bilayered Substitutes	• lower dermal layer of collagen I populated with human fibroblasts • upper stratified epidermis differentiated from a layer of human keratinocytes	• replicates the split thickness skin architecture without details like appendages and melanocytes • once considered the most advanced tissue engineered product	Apligraf®: available commercially	(85, 88)
	Bi-layered bovine collagen-I sponge with human derived co-cultures	• Keratinocytes and fibroblasts derived from human neonatal foreskin • Replicates split-thickness skin	OrCel®: pending approval for use on VLUs and DFUs	(89)
New Material Systems	electrospun polylactic acid and poly(lactide-co-glycolic acid) nanofibrous membranes	• fibrin and collagen I along with fibronectin • enhance cell adhesion and spreading	Research phase	(90)
	self-assembly properties and excellent mechanical stability of silk fibroin	• Elastin	Research phase: similar performance as commercial products in terms of wound healing and exudate management	(91)
	Nanofibers electrospun under high extensional and shear strain rates	• Fibronectin • Soy-protein hydrolase and cellulose acetate	Recently published research; fibrillar conformation is shown to accelerate wound closure and improve tissue restoration	(92, 93)

	Material	Functional Strategy/Features	Stage of Maturity	Ref.
	Keratin based 3D templates	• Keratin extracted from human hair • Composites with other agents • Hemostatic agent, cell carrier and supports regeneration	Research phase	(98-100)
Cell (only)-Based Technologies	Mesenchymal stem cells	• hepatocyte growth factor (HGF) and cognate receptor HGFR/c-met • Delivered undifferentiated within a hydrogel matrix, intravenously or topically as solution or composite	Animal studies	(109, 111-119, 122-126)
	Allogenic macrophages	• diabetic macrophages treated ex vivo with the pro-inflammatory cytokine IL1b	Research still in its infancy, pending better understanding of macrophage role in wound healing	(131)
	Allogenic white blood cell		CureXCell™: Failed in Phase III trials; Still approved in Israel	(132)
	Cryopreserved placental membrane containing MSCs	• growth factors and anti-inflammatory cytokines	Grafix®: complete wound closure in 62% of the patients with chronic DFUs in phase III clinical trials	(133, 134)
	ABCB5 positive MSCs	• ABCB5, a marker of limbal stem cells	APZ2: currently conducting a Phase I/IIa clinical trial	(136-138)

	Material	Functional Strategy/Features	Stage of Maturity	Ref.
Alternate Innovations	TransCu O2® System (fuel cell technology) with moist wound therapy	• EO2 Concepts: Continuously Diffused Oxygen (CDO): Continuous delivery of pure, humidified oxygen to wound	Showed promising results in a randomized, balanced, double blind, sham-controlled, parallel group clinical trial evaluation	(181)
	Hemoglobin spray	• Harvests oxygen from surrounding air and transports to wound	Granulox®: commercially available	(182)
	Magnetic resonance	• Therapeutic effect from external magnetic field • Significantly enhances biological processes related to wound-healing	TMR®: Patented technology	(183)

Figure Caption

Figure 1: Overview of current strategies for managing chronic wounds.

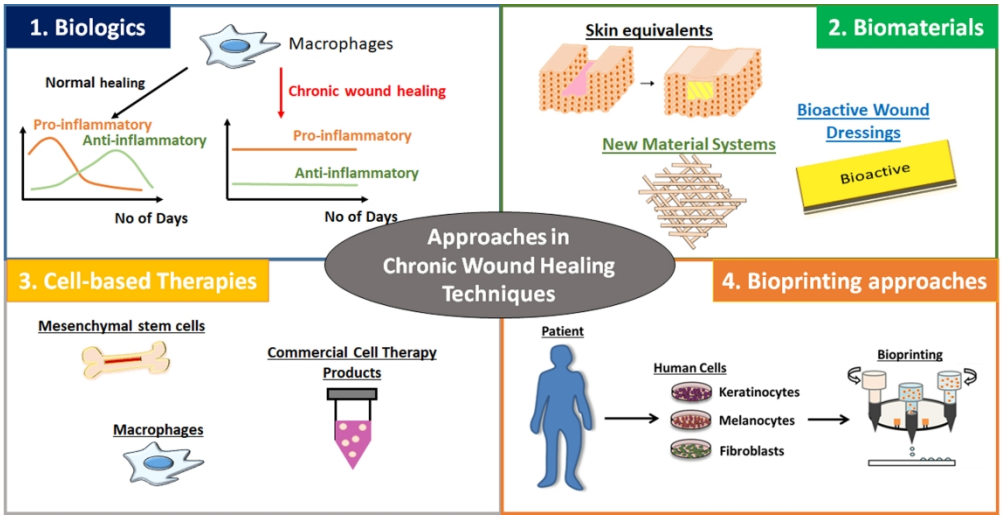

Figure 1: Overview of current strategies for managing chronic wounds.

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